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Sensitivity and Specificity of a Blood and Urine Galactomannan Antigen Assay for Diagnosis of Systemic Aspergillosis in Dogs

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Background: Diagnosis of canine systemic aspergillosis requires fungal culture from a sterile site, or confirmatory histopathology from a nonsterile site. Invasive specimen collection techniques may be necessary.

Objective: To evaluate the sensitivity and specificity of a serum and urine *Aspergillus* galactomannan antigen (GMA) ELISA assay for diagnosis of systemic aspergillosis in dogs.

Design: Multicenter study.

Animals: Thirteen dogs with systemic aspergillosis and 89 dogs with other diseases. Thirty-seven of the 89 dogs had signs that resembled those of systemic aspergillosis and 52 dogs were not suspected to have aspergillosis.

Procedure: The GMA ELISA was performed on serum specimens from all dogs and urine specimens from 67 dogs. Galactomannan indices (GMI) $\geq$ 0.5 were considered positive. Results for dogs in each group were compared.

Results and Conclusions: The sensitivity and specificity of the assay for serum were 92 and 86%, respectively, and for urine were 88 and 92%, respectively. False negatives were seen only in dogs with localized pulmonary aspergillosis. Use of a cutoff GMI of 1.5 increased specificity to 93% for both serum and urine without loss of sensitivity for diagnosis of disseminated infection. High-level false positives (> 1.5) occurred in dogs with other systemic mycoses and those treated with Plasmalyte.

Clinical Relevance: Serum and urine *Aspergillus* GMA ELISA is a noninvasive, sensitive, and specific test for the diagnosis of disseminated aspergillosis in dogs when a cutoff GMI of $\geq$ 1.5 is used.

Key words: Aspergillus; Epidemiology; Fungal; Infectious diseases; Microbiology; Mycology-general.

Aspergillus species are environmentally ubiquitous saprophytic fungi that cause opportunistic infections in veterinary and human patients. In dogs, the most common manifestation of infection is sinonasal aspergillosis, which usually is caused by *Aspergillus fumigatus*.[1] Diagnosis of sinonasal disease is made using a combination of specific antibody detection, imaging of the nasal cavity, rhinoscopic visualization of fungal plaques, and culture of *Aspergillus* spp. from nasal biopsies.[1–3]

Systemic aspergillosis is characterized by disseminated infection that most often involves the intervertebral disks, bones, thoracic lymph nodes, lung, and renal pelvis. *Aspergillus terreus* and *Aspergillus flavus* are isolated from the majority of dogs with systemic disease.[4] Clinical signs are often nonspecific, and include weight loss, anorexia, lethargy, and weakness.[4,5] Definitive diagnosis requires culture of *Aspergillus* spp. from a normally sterile site, or culture of *Aspergillus* spp. from a nonsterile site (such as a bronchoalveolar lavage specimen) together with visualization of fungal hyphae in tissues using light microscopy.

Abbreviations:

ELISA	enzyme-linked immunosorbent assay
GMA	galactomannan antigen
GMI	galactomannan index
GSD	German Shepherd Dog
OD	optical density
TAMU-VMTH	Texas A&M University Veterinary Medical Teaching Hospital
UCD-VMTH	University of California, Davis Veterinary Medical Teaching Hospital

Fungal culture of urine is the least invasive means of diagnosis of systemic aspergillosis, but in 1 study was only positive in 52% of 30 dogs.[4] Collection of specimens for culture from other affected sites, such as the intervertebral disk spaces, is often technically challenging, requires sedation or anesthesia, results in small specimen sizes, and carries substantial risk. Less invasive means to support the diagnosis of systemic aspergillosis clearly are required. Unfortunately, serum *Aspergillus* antibody agar gel immunodiffusion (AGID) testing for *A. fumigatus* antibodies, which has value for diagnosis of nasal aspergillosis,[3] was positive in only 20% of dogs with systemic disease.[4]

An *Aspergillus* galactomannan antigen (GMA) enzyme-linked immunosorbent assay (ELISA)[a] is available for diagnosis of invasive aspergillosis in human patients. This assay detects galactomannan antigen that is released into body fluids by *Aspergillus* spp. In humans, this assay is sensitive and specific for the diagnosis of aspergillosis, with overall sensitivity ranging from 40 to 71% and specificity ranging from 53 to 89%.[6–10] Although this assay appears insensitive for

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diagnosis of nasal aspergillosis, the performance of the assay has not yet been thoroughly evaluated in dogs with systemic aspergillosis.[11,12]

The objective of this study was to determine the sensitivity and specificity of a urine and serum GMA ELISA[a] for diagnosis of systemic aspergillosis in dogs as determined using fungal culture and light microscopic evaluation of tissues as the gold standard. We hypothesized that (1) the GMA assay would be a sensitive and specific test for systemic aspergillosis in dogs when used on urine and serum, and (2) the sensitivity of the assay would be increased when urine was used as a test specimen, because of the common occurrence of *Aspergillus* spp. pyelonephritis in dogs with disseminated disease.

Materials and Methods

Dogs

Dogs examined at 2 university teaching hospitals, the William R. Pritchard University of California, Davis Veterinary Medical Teaching Hospital (UCD-VMTH) and the Texas A&M University VMTH (TAMU-VMTH), were included in the study. In addition, some dogs diagnosed with systemic aspergillosis or other disseminated mycoses at referring institutions were enrolled, provided there was strict adherence to study entrance criteria. Specimens were collected prospectively from dogs seen at UC Davis and private referral practices between January 2009 and July 2011. All sick dogs seen by the UC Davis Small Animal Internal Medicine Service were eligible for inclusion and specimen collection. Although dogs from UCD-VMTH were enrolled prospectively, they were assigned retrospectively to the appropriate group based on ultimate diagnosis. Specimens from Texas A&M had been collected and tested using the GMA assay as part of a diagnostic evaluation for dogs suspected to have aspergillosis before the study was initiated and data were evaluated retrospectively.

Three groups of dogs were defined: dogs with systemic aspergillosis (Asper+), which included dogs with localized pulmonary aspergillosis and disseminated disease; dogs with clinical and diagnostic findings consistent with systemic aspergillosis but that ultimately were diagnosed with a different disease that explained their clinical findings (Asper-like); and, dogs that were not suspected to have systemic aspergillosis and ultimately were diagnosed with another disease that explained their clinical findings (Control). Dogs were excluded from the study if a definitive diagnosis was not made or if they were receiving treatment with antifungal drugs.

All dogs included in the Asper+ group were required to have clinical findings consistent with systemic aspergillosis and culture confirmation of infection. Clinical findings consistent with systemic aspergillosis were defined as diskospondylitis, osteomyelitis, lesions consistent with granulomatous uveitis or chorioretinitis, dilatation and hyperechogenicity of the renal pelvis on abdominal ultrasound examination, hilar lymphadenopathy on thoracic radiographs, and solitary or multiple pulmonary nodules or masses on thoracic radiographs. All dogs in the Asper+ group were diagnosed with systemic aspergillosis by culture of *Aspergillus* spp. either (1) from a normally sterile site, with or without confirmation of *Aspergillus* spp. infection using cytology or histopathology, or (2) from a nonsterile site, with confirmation of *Aspergillus* spp. infection by visualization of fungal hyphae in lesions on cytological or histopathological examination using light microscopy. Dogs were placed in the Asper-like group if they had clinical findings consistent with systemic aspergillosis but an alternate fungal or bacterial organism was isolated from a normally sterile site or the results of histopathology were consistent with another cause for their clinical abnormalities. Dogs were placed in the Control group if they lacked clinical findings consistent with systemic aspergillosis and ultimately were diagnosed with another disease that explained their clinical findings.

In addition, all dogs in the Asper+ and Asper-like groups seen at the UCD-VMTH were evaluated with a thorough history, complete physical examination, CBC, serum biochemistry panel, urinalysis, aerobic bacterial urine culture or fungal urine culture, abdominal ultrasound examination, and thoracic radiographs. The diagnostic evaluation of the control cases varied, but at a minimum consisted of a complete physical examination, CBC, serum biochemistry panel, urinalysis, and either thoracic radiographs or abdominal ultrasonography.

All dogs seen at the TAMU-VMTH and by private referral practitioners were required to have had a thorough history, complete physical examination, CBC, serum biochemistry panel, urinalysis, and imaging performed. The type of imaging (thoracic radiographs, abdominal ultrasound examination, computed tomography, or magnetic resonance imaging of affected tissues) was decided by the attending clinician on the basis of clinicopathological abnormalities. The medical records of these cases were reviewed by 2 board-certified internists (JES, AKC) and a resident (RSG) in order to obtain information on signalment, presenting signs, diagnostic tests performed, and diagnosis and outcome, and to confirm that the dogs adhered to the group assignment criteria for the study. Dogs were assigned to each of the 3 groups based on these results.

Any medications or IV fluids administered at the time of specimen collection were recorded. Efforts were made to enroll control dogs treated with systemic antimicrobial drugs of the penicillin class and IV Plasmalyte[b] solution, because these medications have been associated with false positive GMA test results in human patients.[10] To specifically assess the effect of Plasmalyte on the serum and urine GMA assay results, 2 dogs in the control group that had negative serum and urine GMA assay results subsequently were treated with Plasmalyte 148 and retested after 24 hours of treatment. These dogs were not included in the statistical analysis.

GMA ELISA Assay

A quantity of 3 mL of serum was obtained from each of the dogs in the study. Whenever possible, 5 mL of urine also was obtained for testing either by cystocentesis or sterile catheterization. Serum and urine were refrigerated immediately after collection and shipped on ice within 24 hours to a central laboratory[c] where the GMA ELISA assay was performed. The ELISA assay was performed according to the manufacturer's instructions.[13] Briefly, serum and urine specimens were heat-treated in the presence of EDTA to precipitate proteins that might interfere with the test. The sample and conjugate were added to wells that had been coated by the manufacturer with bound anti-*Aspergillus* galactomannan antibody and incubated at 37°C for 90 minutes. After a wash step to remove unbound material, chromagen was added. This step results in a colorimetric change in the assay, the optical density (OD) of which is proportional to the amount of antigen in the specimen. The OD is determined by using a spectrophotometer. The OD of the test specimen was divided by the OD of the cutoff control, yielding a galactomannan index (GMI). A GMI $\geq$ 0.5 was considered positive for *Aspergillus* antigen in serum, as defined for diagnosis of human systemic aspergillosis.[10] All personnel that performed the assay were blinded to group assignments.

Statistical Analysis

A D'Agostino and Pearson normality test was performed to assess for normality of data. Age, breed, sex, and serum and urine GMIs were compared among the 3 groups of dogs. Among dogs that had both serum and urine specimens collected, serum GMIs were compared to those for urine for dogs with disseminated aspergillosis, and for dogs in the Asper-like and control groups, which were combined into a single group. Categorical data were compared across groups using the chi-squared test and between groups using Fisher's exact test. Comparisons for nonparametric data were performed with the Mann-Whitney test. Normally distributed continuous data were compared using the unpaired *t*-test. All calculations were performed using a statistical software analysis package.[d] *P*-values of $\leq$ .05 were considered significant.

Results

Dogs

A total of 109 dogs had either serum or paired serum and urine specimens submitted for testing and were considered for inclusion in the study. Seven dogs subsequently were excluded from the study because a definitive diagnosis was not obtained. Three dogs were middle-aged, female spayed German Shepherd Dogs (GSD) with multifocal diskospondylitis. Fungal hyphae with morphology consistent with *Aspergillus* spp., which branched at 45° angles, were detected using cytological examination of a disk aspirate in 1 of these 3 dogs and at necropsy in another dog. Disease in the 3rd dog did not respond to antibacterial drug treatment. All 3 dogs had serum GMIs that ranged from 6.39 to 9.84. Urine GMIs were measured in 2 of these 3 dogs and were 4.15 and 6.00. Another dog, a middle-aged Catahoula dog, had diskospondylitis that was refractory to antimicrobial drugs. The serum and urine GMIs for this dog were 0.88 and 3.88, respectively. Disease progressed in the face of antifungal and antibacterial drug treatment, and the dog was lost to follow-up. The remaining 3 of the 7 excluded dogs had negative serum and urine GMI results. None were breeds predisposed to aspergillosis, 2 had intracranial lesions, and 1 had a pulmonary mass lesion. None were treated with antifungal drugs.

Paired urine and serum specimens were available from a total of 67 dogs. Urine was not available from any of the dogs examined at TAMU-VMTH and from 3 UCD-VMTH control group dogs. The origin, signalment, and ELISA assay results for each group of dogs are shown in Table 1. Dogs in the Asper+ and Asper-like groups were younger than dogs in the control group, and dogs in the Asper+ group were 5.8 times more likely to be GSD than dogs in the control group (95% confidence interval, 1.2–27.4; Table 1). Thirteen dogs were included in the Asper+ group. Eleven of these dogs had disseminated aspergillosis, and 2 had localized pulmonary aspergillosis. Necropsy confirmation of the diagnosis was available for 8 dogs, and all had widely disseminated disease.

Thirty-seven dogs were included in the Asper-like group. Fifteen dogs had diskospondylitis, 14 had pulmonary masses or nodules or hilar lymphadenopathy, 3 had non-hilar lymphadenopathy, 3 had chorioretinitis, and 2 had osteomyelitis. Fourteen dogs were diagnosed with bacterial infections, 10 with fungal infections other than aspergillosis, 7 with neoplasia, 4

Table 1. Distribution and characteristics of dogs among Asper+, Asper-like, and control groups and the results of serum and urine GMA ELISA for each group.

	Group			*P*-Value		
Variable	Asper+	Asper-like	Control[b]	Asper+ versus Asper-like	Asper+ versus Control	Asper-like versus Control
Number of dogs[a]	13 (8)	37 (25)	52 (34)	NA	NA	NA
UCD-VMTH	7 (7)	23 (23)	37 (34)			
TAMU-VMTH	5 (0)	12 (0)	15 (0)			
Private referral	1 (1)	2 (2)	0			
Mean age (years) ± SD (median)	4.6 ± 3.2 (4.0)	6.1 ± 3.7 (6.0)	7.8 ± 3.6 (8.5)	.17	.007	.04
Sex						
Male	5	18	26	.75	.54	.67
Female	8	19	25			
Breed						
GSD	4	6	6	.42	.04	.18
Non-GSD	9	31	45			
Mean serum GMI ± SD (median)	7.44 ± 3.32 (8.33)	0.60 ± 1.20 (0.19)	0.51 ± 1.23 (0.21)	<.0001	<.0001	.89
Mean urine GMI ± SD (median)	6.80 ± 3.21 (7.82)	0.54 ± 1.39 (0.10)	0.23 ± 0.51 (0.09)	.0003	.0001	.82

NA, not applicable; GMI, galactomannan index; UCD-VMTH, UC Davis Veterinary Medical Teaching Hospital; TAMU-VMTH, Texas A&M Veterinary Medical Teaching Hospital; SD, standard deviation; GSD, German Shepherd Dog.

[a]All dogs had serum specimens collected; the number of dogs from which urine specimens were obtained is shown in parentheses.

[b]Age and sex known for 51 dogs in the control group.

with noninfectious inflammatory diseases, and 2 with pulmonary edema. Necropsy confirmation of the diagnosis was available for 9 dogs. Fungal diseases in this group included pulmonary coccidioidomycosis (3 dogs), disseminated cryptococcosis (2 dogs), disseminated paecilomycosis (2 dogs), and disseminated geotrichosis, blastomycosis, and penicilliosis (1 dog each). Paired serum and urine samples were available for all of the dogs with mycoses except the dog with blastomycosis.

Fifty-two dogs were included in the control group. Diseases diagnosed in this group included inflammatory brain disease (9 dogs); neoplasia (9 dogs); fungal infections with clinical presentations that did not resemble systemic aspergillosis (8 dogs); diabetes mellitus, nonfungal pneumonia, immune-mediated disease, inflammatory bowel disease, chronic kidney disease, and lower urinary tract disease (3 dogs each); acute kidney injury (2 dogs); and inflammatory nasal disease, chronic bronchitis, prostatic abscess, *Mesocestoides* peritonitis, canine monocytic ehrlichiosis, and inflammatory liver disease (1 dog each). Necropsy confirmation of the diagnosis was available for 4 dogs. The fungal infections were sinonasal aspergillosis (3 dogs), unspeciated fungal rhinitis (2 dogs), colonic zygomycosis, sporotrichosis, and a deep cutaneous nailbed infection caused by an unspeciated mold that did not resemble *Aspergillus* spp. The diagnosis of zygomycosis was based on the histopathologic appearance of the organism in colonic biopsies, negative ELISA serology for anti-*Pythium insidiosum* antibodies,[e] and strong staining of fungal structures in tissue sections with periodic acid-Schiff stain. All dogs with fungal infections had paired serum and urine samples except the 2 dogs with unspeciated fungal rhinitis.

Serum and Urine GMA ELISA Assay Results

In the Asper+ group, all the dogs with disseminated disease had serum GMIs > 5.0 (Fig 1A). The 2 dogs with localized pulmonary aspergillosis had serum GMIs of 0.14 and 0.77. Thus, 1 dog with systemic aspergillosis tested negative using the cutoff of 0.5. Median serum and urine ELISA GMIs for the dogs in the Asper+ group were higher than those for the dogs in the Asper-like and control groups (Fig 1; Table 1). All the 8 dogs with disseminated aspergillosis that had urine assays performed had urine GMIs > 4.0, and the 1 dog with localized pulmonary involvement had a negative urine GMI. This dog had a positive serum GMI (Fig 2). Thus, of 8 dogs that had both serum and urine tested, both specimens were positive in 7 dogs, serum only was positive in 1 dog with localized disease, and urine only was positive in no dog. Eleven of 13 dogs in the Asper+ group had fungal culture of the urine performed. All 11 dogs were serum ELISA-positive, and 7 of 11 had urine cultures that grew *Aspergillus* spp. Urine ELISA and fungal culture of the urine were performed in 7 dogs, all of which had positive urine ELISA and 4 of 7 had positive urine cultures.

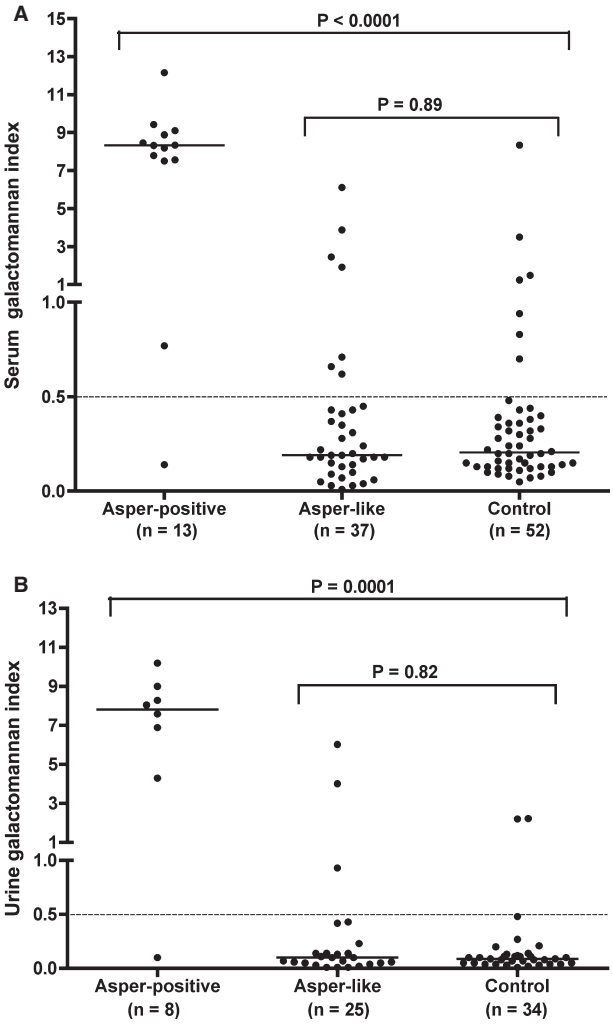

Fig 1. **(A)** Serum *Aspergillus* GMA ELISA optical densities (OD) in 102 dogs. **(B)** Urine *Aspergillus* GMA ELISA optical densities (OD) in 67 dogs. Asper-positive: dogs with culture-confirmed systemic aspergillosis. Asper-like: dogs with findings consistent with systemic aspergillosis but confirmed alternate diagnoses. Control: sick dogs with findings inconsistent with systemic aspergillosis and an alternate diagnosis. The horizontal bar represents the median. The dotted line shows the cutoff OD of 0.5.

Seven of 37 (18.9%) dogs in the Asper-like group had increased serum GMIs, and 3 (12%) of 25 of these dogs had increased urine GMIs. Three dogs had weak positive results when serum was assayed (i.e, GMIs between 0.5 and 1.0) and 4 had serum GMIs > 1.0. The dogs with serum GMIs > 1.0 had disseminated mycoses, including infections with *Paecilomyces* spp., *Geotrichum* spp., *Cryptococcus neoformans* and *Penicillium* spp. (serum GMIs 1.92, 2.46, 3.88, and 6.12, respectively). Of these 4 dogs, the urine GMA ELISA was positive only for the dogs with paecilomycosis and penicilliosis (urine GMIs, 4.01 and 6.02, respectively). One dog with paecilomycosis had negative serum and urine GMIs. This dog was a Doberman mixed-breed dog with cutaneous and local lymph node involvement

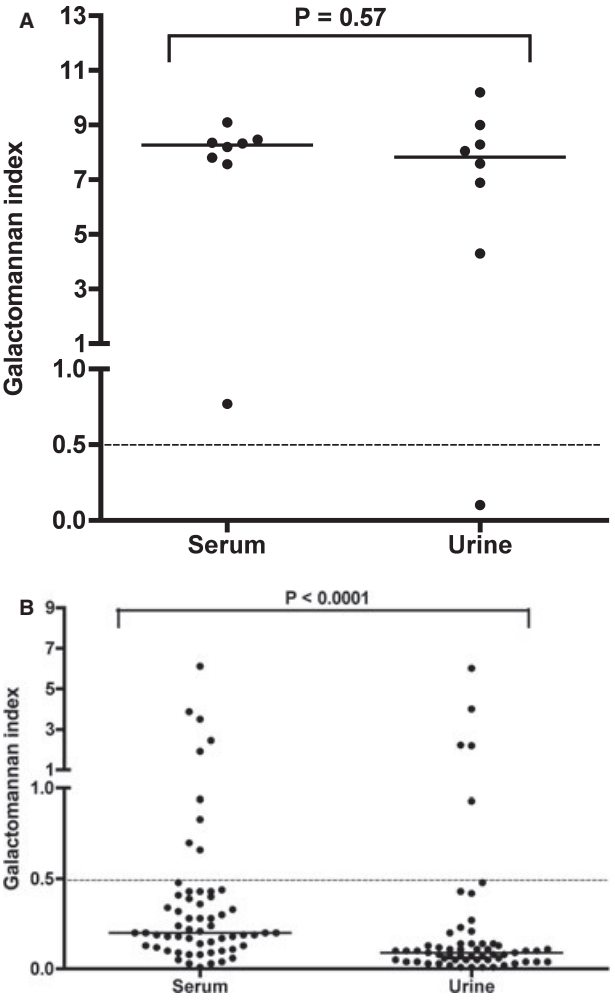

Fig 2. Serum and urine *Aspergillus* GMA ELISA optical densities (OD). **(A)** Dogs with culture-proven systemic aspergillosis (n = 8). **(B)** Dogs without systemic aspergillosis (n = 59), including 25 dogs suspected to have aspergillosis and 34 dogs not suspected to have aspergillosis. The horizontal bar represents the median. The dotted line shows the cutoff OD of 0.5.

that developed after immunosuppressive drug treatment, and recovered fully with antifungal drug administration. In contrast, the dog with paecilomycosis that tested positive on the ELISA assay (a Rhodesian Ridgeback) died 1 month after antigen assays were performed and had widespread disseminated infection at necropsy. The dogs with geotrichosis, cryptococcosis, and penicilliosis were a GSD, American Cocker Spaniel, and Coton De Tulear, respectively. The dogs with weak positive serum GMA test results included a GSD with dirofilariasis and congestive heart failure that was receiving ampicillin, a Shetland Sheepdog with pulmonary osseous neoplasia and a Labrador Retriever with osteosarcoma (serum GMIs 0.62, 0.66, and 0.71, respectively). A urine GMA ELISA assay result was only available for the dog with pulmonary neoplasia and was negative. In addition to the 2 dogs with systemic mycoses, only 1 dog had had a positive urine GMA result, a Collie with bacterial pneumonia (urine GMI 0.93, serum GMI 0.43).

In the control group, 7 (13.7%) of 51 dogs had positive serum GMIs, and 2 (6%) of 34 dogs had positive urine GMIs. Three dogs had weak positive serum assay results and 4 dogs had serum GMIs > 1.0. The 4 dogs with serum GMIs > 1.0 had undifferentiated round cell neoplasia of the brain, an unspeciated intranasal dematiaceous fungal infection, colonic zygomycosis, and diabetes mellitus, respectively (serum GMIs 1.24, 1.49, 3.5, and 8.35, respectively). The dog with colonic zygomycosis was a GSD and had negative urine GMI. Urine GMIs were not available for the other 3 dogs. The dog with diabetes mellitus was being treated with Plasmalyte at the time of specimen collection. Diagnoses in the dogs with weak positive serum assay results were megaesophagus, acute myeloid leukemia, and an unspeciated localized mold infection of the nailbed (serum GMIs 0.70, 0.83, and 0.94, respectively). None of these 3 dogs were breeds predisposed to systemic aspergillosis. The dog with megaesophagus was being treated with ampicillin-sulbactam,[f] and the dog with leukemia with clavulanic acid-amoxicillin[g] at the time of specimen collection. All the 3 dogs with weak positive serum assay results had urine ELISA assay results available, and only the dog with the nailbed mycosis tested positive (urine GMI 2.20). The other dog with a positive urine GMA ELISA assay result was a Boxer with gastric helicobacteriosis and inflammatory bowel disease (urine GMI 2.23).

Thus, of 59 dogs without aspergillosis that had both serum and urine tested, both serum and urine were positive for 3 dogs, all of which had mold infections. Serum only was positive in 6 dogs, 3 of which had fungal infections (cryptococcosis, geotrichosis, and zygomycosis), and urine only was positive in 2 dogs, neither of which had fungal infections.

Effect of Medications on the GMA Assay

In addition to the dog enrolled in the control group that that had a high-level serum false positive result during treatment with Plasmalyte 148, 2 control dogs that had negative GMA test results developed GMIs > 5.0 in both serum and urine after 24 h of IV Plasmalyte 148 treatment.

Eighteen dogs were treated with penicillin derivatives at the time of specimen collection, including clavulanic acid-amoxicillin (PO, 7 dogs), ampicillin (IV, 4 dogs), ampicillin-sulbactam (IV, 4 dogs), procaine penicillin G (IV, 1 dog), procaine penicillin G and ampicillin (IV, 1 dog), and amoxicillin (PO, 1 dog). As described above, weak positive serum ELISA assay results occurred in 3 dogs (GMIs 0.62, 0.70, and 0.83). Urine specimens were available for 14 of these dogs and all were negative.

Comparison of Serum and Urine GMA Results

The sensitivity of the serum and urine GMA assay for diagnosis of systemic aspergillosis using the

Table 2. Calculated specificities of *Aspergillus* GMA ELISA results for diagnosis of systemic aspergillosis in dogs at ELISA cutoff values of 0.5, 1.0, and 1.5.

	Overall Specificity (%)			Specificity after Exclusion of False Positives from Mold Infections and Plasmalyte (%)		
Specimen Type and Group [n]	Cutoff GMI $\geq$ 0.5	Cutoff GMI $\geq$ 1.0	Cutoff GMI $\geq$ 1.5	Cutoff GMI $\geq$ 0.5	Cutoff GMI $\geq$ 1.0	Cutoff GMI $\geq$ 1.5
Serum						
All groups [102]	75/89 (84)	81/89 (91)	83/89 (93)	75/81 (93)	81/82 (99)	83/83 (100)
Asper+ and Asper-like [50]	30/37 (81)	33/37 (89)	33/37 (89)	30/33 (91)	33/33 (100)	33/33 (100)
Asper+ and Control [65]	45/52 (87)	48/52 (92)	50/52 (96)	45/48 (94)	48/49 (98)	50/50 (100)
Urine						
All groups [67]	54/59 (92)	55/59 (93)	55/59 (93)	54/56 (96)	55/56 (98)	55/56 (98)
Asper+ and Asper-like [33]	22/25 (88)	23/25 (92)	23/25 (92)	22/23 (96)	23/23 (100)	23/23 (100)
Asper+ and Control [42]	32/34 (94)	32/34 (94)	32/34 (94)	32/33 (97)	32/33 (97)	32/33 (97)

GMI, Galactomannan Index; n, number of dogs.

"All groups" compares Asper+ cases to combined Asper-like and Control dogs; "Asper+ and Asper-like" compares Asper+ with Asper-like dogs, and "Asper+ and Controls" compares Asper+ and Control dogs.

established cutoff of 0.5 was 12/13 (92%) and 11/13 (88%), respectively. When the cutoff was increased to 1.0 or 1.5, the sensitivity of the serum GMA assay decreased to 11/13 (85%) and the sensitivity of the urine assay remained unchanged. The sensitivities of the assay on both serum and urine for diagnosis of disseminated disease were 100%, regardless of the cutoff used. The overall specificities of the serum and urine galactomannan assays were 84 and 92%, respectively. When the cutoff GMI was increased to 1.5, both dogs with localized pulmonary aspergillosis were classified as negative, with a reduction in the sensitivity of the serum assay for systemic aspergillosis, but not for disseminated disease. However, the specificity increased for both urine and serum assays (Table 2). When dogs with mold infections and those treated with Plasmalyte 148 were excluded, the specificity at the higher cutoff value of 1.5 was 100% for serum and 98% for urine.

Among dogs in the Asper+ group that had paired serum and urine specimens collected, no difference between serum and urine GMIs (Fig 2A) was found. Among dogs in the Asper-like and control groups, serum GMIs were higher than urine GMIs (Fig 2B). Urine GMIs clustered at the bottom of the reference range, whereas serum GMIs were distributed throughout the reference range. Of the 11 dogs that had false positive serum or urine assay results, only 3 had false positive results for both serum and urine specimens. Six dogs only had false positive serum assay results and 2 only had false positive urine assay results.

Discussion

In this study, we demonstrated that the *Aspergillus* GMA ELISA assay is sensitive for the diagnosis of systemic aspergillosis in dogs when applied to either serum or urine specimens, and appears to be specific for diagnosis of systemic mold infections. When compared with the gold standard of culture, the sensitivity of the assay for serum and urine was 93 and 89%, respectively. In a meta-analysis of 27 studies and 4284 confirmed cases of invasive aspergillosis in humans, the sensitivity of the assay was 71%.[9] When dogs with localized pulmonary involvement were excluded in our study, the sensitivity increased to 100% for serum and urine, regardless of the cutoff GMI used. Although a relatively small number of dogs with systemic aspergillosis were studied, the urine and serum of all dogs with disseminated disease had GMIs > 5.0, and serum and urine GMIs for the dogs with systemic aspergillosis were significantly higher than those of the dogs in the Asper-like and control groups. A previous study examined the results of a serum GMA ELISA assay in 6 dogs with presumed systemic aspergillosis with mixed results, but the methods of diagnosis were not described nor were GMIs reported.[12]

In our study, dogs with localized pulmonary aspergillosis had negative or weak positive assay results. Circulating antigen concentrations may be lower in dogs with localized pulmonary disease than in dogs with disseminated infections. In humans with pulmonary aspergillosis, use of the assay on bronchoalveolar lavage fluid has improved sensitivity when compared with use of serum.[7,14] As in human patients, the magnitude of the serum GMI in dogs also may be indicative of disease severity and perhaps predictive of clinical response.[7]

The specificity of the serum GMA assay for diagnosis of systemic aspergillosis in our study approximated that reported in human patients (89%). False positive GMIs with magnitudes similar to those in dogs with disseminated aspergillosis (GMIs > 4) were only seen in dogs treated with Plasmalyte 148 and those with other systemic mycotic infections. Plasmalyte administration also causes false positive GMA assay results in human patients. False positives in patients receiving Plasmalyte occur as a consequence of gluconate, which also is produced by fermentation of glucose by *Penicillium* and *Aspergillus* spp.[10,15] *Penicillium* spp., *Paecilomyces* spp., *Cladosporidium* spp., *Geotrichum* spp., *Histoplasma capsulatum* and *Cryptococcus neoformans* possess antigens that cross-react with antibodies to *Aspergillus* GMA and can produce false

positive GMA ELISA assay results in human patients.[10,16] In the study reported herein, false positives occurred in dogs with disseminated penicilliosis, paecilomycosis, geotrichosis, and cryptococcosis. No dogs with coccidioidomycosis had false positive test results, but disseminated disease was not detected at the time these dogs were tested. Of potential importance, the dogs with paecilomycosis and cryptococcosis that tested positive were breeds predisposed to fungal dissemination,[4,15] whereas those that tested negative were not predisposed breeds.[4,17] Fungal burden may be higher in predisposed breeds. Variable positivity in dogs with cryptococcosis may result from variable expression of the component of the *C. neoformans* capsule believed to cause cross-reactivity.[16] Because only small numbers of dogs with each of the systemic mycoses were evaluated, additional studies that include larger numbers of dogs with each of these diseases are warranted.

Some dogs with localized fungal infections were included in the study, such as dogs with sinonasal aspergillosis. Weak positive results occurred in some of these dogs, but all the dogs with sinonasal aspergillosis had negative assay results. In a study that evaluated the performance of the serum GMA assay for diagnosis of sinonasal aspergillosis in dogs, only 4 of 17 dogs with sinonasal aspergillosis had positive ELISAs, with GMIs that ranged from 0.51 to 0.75.[11] False positive GMA ELISA results were detected in 11 (18%) of 62 dogs without sinonasal aspergillosis, which included healthy dogs and dogs with nasal tumors, lymphoplasmacytic rhinitis, and orthopedic disease. In all dogs, serum GMIs were $\leq$ 1.5.

Various *Penicillium*-derived antibiotics have been documented to cause false positive serum GMA ELISA assay results in human patients, especially piperacillin-tazobactam.[18–20] Only 3 of 18 dogs treated with penicillins in our study had false positive serum assay results, and it was unclear whether the positive test results truly resulted from antimicrobial drug treatment.

A few other dogs had weak positive serum and urine GMIs that could not be explained. Other reported causes of false positive results include contamination of specimens with cotton, cardboard, or even ingestion of soybean protein.[10] The dogs with unexplained false positive results were not breeds predisposed to infection with *Aspergillus* spp.

Although we showed no statistical difference in the sensitivity and specificity of the serum and urine ELISA assays for diagnosis of disseminated aspergillosis in dogs, urine GMIs in the dogs in the Asper-like and control groups were significantly lower than the corresponding serum GMIs, and fewer false positive results were identified in urine specimens. In human patients, serum ELISA is more sensitive and specific than the urine ELISA.[6,7] Additional studies that include urine and serum specimens from large numbers of dogs are necessary to determine if urine is the ideal specimen for submission, but both serum and urine appear to represent adequate specimens for diagnosis. Dogs with false positive serum ELISA assay results did not always have false positive urine ELISA assay results, and vice versa. Although case numbers were small, when false positive results were present in both serum and urine, a non-*Aspergillus* mold infection was present in all cases. When false positive results were present in urine but not serum, nonfungal disease was present. Either a fungal infection or a nonfungal disease was present when serum was positive but urine negative. Submission of both urine and serum may be useful to optimize sensitivity for detection of mold infections.

Because of the profound increases in serum and urine GMI in dogs with disseminated disease, use of cutoff GMIs of 1.0 or 1.5 did not alter sensitivity for diagnosis of disseminated aspergillosis, but improved specificity. Although specificity for the dogs in the study population could have been further improved with the use of even higher cutoff values, we chose not to evaluate them because of the small number of dogs with disseminated aspergillosis included in the study population. The reason for the marked increases in GMIs in affected dogs compared to humans with invasive aspergillosis is unknown. Most human patients have underlying hematological malignancy or are transplant recipients, rather than a genetic predisposition to the disease, which may influence the extent of disease and fungal burdens. Earlier detection in susceptible human populations also may play a role.[10,14]

From a clinical standpoint, the value of separating hyalohyphomycoses such as paecilomycosis from aspergillosis currently is unclear, because there is insufficient evidence in veterinary medicine that treatment recommendations and prognosis differ for these infections. In this study, the sensitivity of the serum and urine GMA assay for diagnosis of systemic mold infections caused by *Aspergillus* or other molds using a cutoff value of 0.5 was 18/19 (95%) and 10/13 (77%), respectively. The specificity of the serum and urine GMA assay for diagnosis of systemic mold infections was 75/84 (89%) and 52/54 (96%), respectively. Given the low number of dogs with systemic mold infections other than aspergillosis in this study, more information is necessary before it can be concluded that the *Aspergillus* galactomannan assay has equivalent sensitivity for detection of *Aspergillus* and non-*Aspergillus* mold infections. Lack of cross-reactivity has been documented for several hyalohyphomycoses in human patients.[21] In addition, prospective studies that compare treatment responses and prognosis among different species of mold infections in dogs are required to determine whether or not differentiation between these agents is of clinical importance. Other mycoses, such as histoplasmosis, with alternative treatment recommendations and prognoses may be associated with cross-reactivity.[22] As such, even in the face of a positive GMA assay together with consistent clinical findings, an effort to identify the fungal organism may be required to ensure appropriate client education and proper treatment.

A weakness of this study was the relatively low numbers of dogs with systemic aspergillosis enrolled. Systemic aspergillosis is a relatively rare disease, and in the largest study to date, only 30 cases were reported over a 17-year period at a large teaching institution.[4] However, the numbers were sufficient to show strong statistical correlations between seropositivity and disseminated disease. To increase case numbers, we retrospectively enrolled dogs from TAMU-VMTH that previously had been tested. Potential limitations of retrospective enrollment included a less consistent clinical and diagnostic approach to each dog, and lack of urine specimens for these dogs. Nevertheless, all the dogs had been evaluated at a large referral hospital and thorough diagnostic evaluations had been performed. Diagnostic evaluations of dogs in the 2 control groups were thorough and allowed for identification of an alternate etiology in every dog that was ultimately grouped in the control or Asper-like groups, and a 2nd diagnosis of systemic aspergillosis was unlikely.

In summary, the results of our study suggest a high serum or urine GMA ELISA assay result (GMI > 1.5) coupled with systemic illness should raise concern for a systemic mycosis. False negatives may be encountered with localized pulmonary involvement, and false positives may occur with Plasmalyte administration and other systemic mycoses. Given the generally poor prognosis associated with disseminated mold infections, this test could serve as a means to identify infection and institute treatment earlier in predisposed breeds, perhaps with better clinical outcomes.[4,23] Decreasing GMIs have been correlated with improved survival in human patients treated for invasive aspergillosis.[24,25] The utility of the GMA ELISA for monitoring treatment in dogs requires additional investigation.

Footnotes

[a] Platelia *Aspergillus*, MiraVista Laboratories, Indianapolis, IN
[b] Plasmalyte 148, Baxter Health, Deerfield, IL
[c] MiraVista Laboratories
[d] GraphPad Prism version 4.0, GraphPad Software, La Jolla, CA
[e] Pythium Lab, Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA
[f] Unasyn, Baxter Health
[g] Clavamox, Pfizer Animal Health, New York, NY

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References

1. Peeters D, Clercx C. Update on canine sinonasal aspergillosis. Vet Clin North Am Small Anim Pract 2007;37:901–916 vi.

2. Johnson LR, Drazenovich TL, Herrera MA, et al. Results of rhinoscopy alone or in conjunction with sinuscopy in dogs with aspergillosis: 46 cases (2001–2004). J Am Vet Med Assoc 2006;228:738–742.

3. Pomrantz JS, Johnson LR, Nelson RW, et al. Comparison of serologic evaluation via agar gel immunodiffusion and fungal culture of tissue for diagnosis of nasal aspergillosis in dogs. J Am Vet Med Assoc 2007;230:1319–1323.

4. Schultz RM, Johnson EG, Wisner ER, et al. Clinicopathologic and diagnostic imaging characteristics of systemic aspergillosis in 30 dogs. J Vet Intern Med 2008;22:851–859.

5. Day MJ, Penhale WJ, Eger CE, et al. Disseminated aspergillosis in dogs. Aust Vet J 1986;63:55–59.

6. Salonen J, Lehtonen OP, Terasjarvi MR, et al. Aspergillus antigen in serum, urine and bronchoalveolar lavage specimens of neutropenic patients in relation to clinical outcome. Scand J Infect Dis 2000;32:485–490.

7. Klont RR, Mennink-Kersten MA, Verweij PE. Utility of Aspergillus antigen detection in specimens other than serum specimens. Clin Infect Dis 2004;39:1467–1474.

8. Scotter JM, Campbell P, Anderson TP, et al. Comparison of PCR-ELISA and galactomannan detection for the diagnosis of invasive aspergillosis. Pathology 2005;37:246–253.

9. Pfeiffer CD, Fine JP, Safdar N. Diagnosis of invasive aspergillosis using a galactomannan assay: A meta-analysis. Clin Infect Dis 2006;42:1417–1427.

10. Wheat LJ, Walsh TJ. Diagnosis of invasive aspergillosis by galactomannan antigenemia detection using an enzyme immunoassay. Eur J Clin Microbiol Infect Dis 2008;27:245–251.

11. Billen F, Peeters D, Peters IR, et al. Comparison of the value of measurement of serum galactomannan and Aspergillus-specific antibodies in the diagnosis of canine sino-nasal aspergillosis. Vet Microbiol 2009;133:358–365.

12. Garcia ME, Caballero J, Cruzado M, et al. The value of the determination of anti-Aspergillus IgG in the serodiagnosis of canine aspergillosis: Comparison with galactomannan detection. J Vet Med B Infect Dis Vet Public Health 2001;48:743–750.

13. Bio-Rad. Platelia™ *Aspergillus* EIA Kit Instructions. Redmond, WA: Bio-Rad; 2008.

14. Hage CA, Knox KS, Davis TE, et al. Antigen detection in bronchoalveolar lavage fluid for diagnosis of fungal pneumonia. Curr Opin Pulm Med 2011;17:167–171.

15. Surmont I, Stockman W. Gluconate-containing intravenous solutions: Another cause of false-positive galactomannan assay reactivity. J Clin Microbiol 2007;45:1373.

16. Dalle F, Charles PE, Blanc K, et al. Cryptococcus neoformans galactoxylomannan contains an epitope(s) that is cross-reactive with Aspergillus galactomannan. J Clin Microbiol 2005;43:2929–2931.

17. Sykes JE, Sturges BK, Cannon MS, et al. Clinical signs, imaging features, neuropathology, and outcome in cats and dogs with central nervous system cryptococcosis from California. J Vet Intern Med 2010;24:1427–1438.

18. Bart-Delabesse E, Basile M, Al Jijakli A, et al. Detection of Aspergillus galactomannan antigenemia to determine biological and clinical implications of beta-lactam treatments. J Clin Microbiol 2005;43:5214–5220.

19. Fortun J, Martin-Davila P, Alvarez ME, et al. False-positive results of Aspergillus galactomannan antigenemia in liver transplant recipients. Transplantation 2009;87:256–260.

20. Zandijk E, Mewis A, Magerman K, et al. False-positive results by the platelia Aspergillus galactomannan antigen test for

patients treated with amoxicillin-clavulanate. Clin Vaccine Immunol 2008;15:1132–1133.

21. Swanink CM, Meis JF, Rijs AJ, et al. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting Aspergillus galactomannan. J Clin Microbiol 1997;35:257–260.

22. Narreddy S, Chandrasekar PH. False-positive Aspergillus galactomannan (GM) assay in histoplasmosis. J Infect 2008;56:80–81.

23. Kelly SE, Shaw SE, Clark WT. Long-term survival of four dogs with disseminated Aspergillus terreus infection treated with itraconazole. Aust Vet J 1995;72:311–313.

24. Miceli MH, Grazziutti ML, Woods G, et al. Strong correlation between serum aspergillus galactomannan index and outcome of aspergillosis in patients with hematological cancer: Clinical and research implications. Clin Infect Dis 2008;46:1412–1422.

25. Maertens J, Buve K, Theunissen K, et al. Galactomannan serves as a surrogate endpoint for outcome of pulmonary invasive aspergillosis in neutropenic hematology patients. Cancer 2009;115:355–362.