Journal of Avian Medicine and Surgery 23(2):125–135, 2009

Galactomannan Assay and Plasma Protein Electrophoresis Findings in Psittacine Birds With Aspergillosis

Carolyn Cray, PhD, Drury Reavill, DVM, Dipl ABVP (Avian), Dipl ACVP, April Romagnano, DVM, PhD, Dipl ABVP (Avian), Fern Van Sant, DVM, Daphne Champagne, BS, Rhoda Stevenson, DVM, Dipl ABVP (Avian), Vanessa Rolfe, DVM, Chris Griffin, DVM, Dipl ABVP (Avian), and Susan Clubb, DVM, Dipl ABVP (Avian)

Abstract: In psittacine birds, the antemortem diagnosis of aspergillosis is usually based on the clinical signalment combined with the results of diagnostic tests such as radiography, routine hematologic and biochemical analysis, and biopsy. For several years, plasma protein electrophoresis has been used as an ancillary diagnostic technique in forming a diagnosis and treatment plan in avian species. More recently, a commercially available assay to measure galactomannan, an *Aspergillus* species antigen, has been described for clinical use in humans, cattle, horses, dogs, and gyr falcons. This report describes several confirmed cases of aspergillosis, with accompanying clinical data, including plasma protein electrophoresis and galactomannan assay results, in addition to results of traditional evaluations by hematology, radiography, and biopsy. In clinical cases in psittacine birds, the galactomannan assay appears useful for detecting circulating *Aspergillus* antibody.

Key words: aspergillosis, plasma protein electrophoresis, EPH, galactomannan, avian, psittacine birds, *Aspergillus* species

Introduction

Aspergillus species is a ubiquitous fungus known to infect various mammals and birds.[1] Fungal infections and granulomas suggest immune suppression as well as support exposure to an overwhelming number of fungal spores.[2] Many avian species are susceptible to infection, but some species, such as goshawks (*Accipiter gentilis*), gyr falcons (*Falco rusticolus*), penguins (subfamily Spheniscinae), quail (*Conturnix* species), and alcid species[3] appear more susceptible to respiratory fungal infections. Because advanced stages of disease may be present before significant clinical signs are observed, early diagnosis and treatment are not always possible. Antemortem diagnostic tests can be as problematic and challenging.[4] Traditional techniques, including routine hematologic testing and biochemical analysis, can be used, but changes may be nonspecific for aspergillosis. Radiographic lesions that are consistent with aspergillosis may be observed but need to be confirmed. Endoscopy is an excellent diagnostic tool but may not be available or advisable if the patient is very ill. Other test avenues have been described, including polymerase chain reaction (PCR) testing for *Aspergillus* species.[5] The use of serodiagnostic testing was described in several avian species, although positive titers for *Aspergillus* antibody and antigen were reported in normal birds.[6–14] Plasma protein electrophoresis (EPH) has been proposed as an accessory tool to serodiagnostic tests.[15–17]

Recently, a commercial assay to measure galactomannan, a major antigen of *Aspergillus*

From the Division of Comparative Pathology, Department of Pathology, University of Miami Miller School of Medicine, PO Box 016960 R-46, Miami, FL 33101, USA (Cray, Champagne, Clubb); Zoo/Exotic Pathology Service, 2825 KOVR Drive, West Sacramento, CA 95605, USA (Reavill); Animal Health Clinic, 5500 Military Trail no. 40, Jupiter, FL 33458, USA (Romagnano); For The Birds, 1136 S. Deanza Boulevard, Suite B, San Jose, CA 95129, USA (Van Sant); Exotic Bird Hospital, 10550-12 Old St. Augustine Road, Jacksonville, FL 32257, USA (Stevenson); Bird and Exotic Hospital, 6147 Lake Worth Road, Greenacres, FL 33463, USA (Rolfe); Griffin Avian and Exotic Veterinary Hospital, 2100 Lane Street, Kannapolis, NC 28083, USA (Griffin); and Rain Forest Clinic for Birds, PO Box 508, Loxahatchee, FL 33470, USA (Clubb).

species, has become available. Galactomannan represents a family of galactofuranose antigens that are secreted during different growth phases of *Aspergillus*.[18] Although it is believed that some degree of angioinvasion is needed, the kinetics and mode of galactomannan release to the circulation are poorly understood.[18] In humans, the galactomannan enzyme-linked immunosorbent assay (ELISA) has been reported to have variable sensitivity and specificity, reaching upward of 100%, and is often dependent on whether patients are receiving antifungal treatment and whether they have a proven or probable diagnosis.[19] In studies that used a rabbit model of infection, the test yielded a sensitivity of 84% and specificity of 90%.[19] Galactomannan concentrations were found to correlate with fungal burden in experimentally and naturally infected cows with systemic infection.[20] Variable galactomannan reactivity was described in dogs with suspected aspergillosis and in horses with guttural pouch mycosis.[21,22] In gyr falcons with confirmed aspergillosis, a poor sensitivity was found, with a 95% specificity.[23] In psittacine birds with suspected infection, an assay with a high positive cutoff level had a low sensitivity, although birds with clinical signs were two times more likely to have positive test results.[24,25] In a study of several avian species with confirmed aspergillosis, circulating galactomannan concentrations were 2.6-fold higher in infected birds than in normal birds when testing used the manufacturer's recommended assay cutoff level.[11] In the current report, the galactomannan and EPH results of 12 psittacine birds are summarized, 11 of which were confirmed cases of aspergillosis. These results were reviewed with results of radiography, routine hematologic testing, PCR testing, and histologic examination.

Materials and Methods

Patients

All samples were submitted to the Avian and Wildlife Laboratory, University of Miami, by practitioners engaged in diagnostic investigations of individually owned pet birds. The species described in this report are the following: palm cockatoo (*Probosciger aterrimus*; n = 1), African grey parrot (*Psittacus erithacus*; n = 3), Moluccan cockatoo (*Cacatua moluccensis*; n = 1), Cape parrot (*Poicephalus robustus*; n = 1), yellow-naped Amazon parrot (*Amazona ochrocephala*; n = 1), quaker parrot (*Myiopsitta monachus*; n = 1), blue and gold macaw (*Ara ararauna*; n = 2), eclectus parrot (*Eclectus roratus*; n = 1), and harlequin macaw (*A ararauna* × *Ara chloroptera*; n = 1).

Plasma protein electrophoresis

Plasma protein electrophoresis by using lithium heparinized plasma samples was performed as previously described.[15,26] All samples were analyzed within 24 hours of submission to the laboratory. Samples were free from hemolysis and lipemia, and were refrigerated until analyzed. All species-specific reference intervals were established in the laboratory (University of Miami) by using plasma samples held for 24 hours.

Galactomannan ELISA

The Platelia ELISA kit (Bio-Rad Laboratories, Hercules, CA, USA) was used for the galactomannan ELISA. The procedural changes were limited to the use of plasma instead of serum and the use of 60-µl rather than 300-µl samples for sample pretreatment. Changes were validated by a technical study that included comparing paired serum and plasma samples and spiking additional samples with galactomannan. The assay results were derived by calculating an index from positive, negative, and cutoff control samples. Specifically, the index relates to the relative reaction of the test sample versus the cutoff control sample. The index carries no unit designation. By the manufacturer's specifications, any sample with an index greater than or equal to 0.5 was considered positive.

Histopathology

All tissue samples were obtained by biopsy or at necropsy and were preserved in 10% neutral buffered formalin solution before routine processing. Paraffin-embedded tissues were sectioned at approximately 5 µm, mounted on glass slides, and stained with hematoxylin and eosin and, in some cases, additional stains. Cases 1 and 6 were stained with acid-fast and case 6 with periodic acid–Schiff stains. Cases 4 and 10 were stained with Gomori methenamine silver stain. Case 8 was stained with a Fite stain.

PCR testing

RNA was isolated by adapting described methods.[27,28] Briefly, 3 sections, each 50-µm thick, of paraffin-embedded tissue were placed in lysis buffer and incubated at room temperature for 15 minutes, followed by treatment with proteinase K for 1 hour at 60°C. After heating to 100°C,

RNA was isolated from the solution by using a traditional phenol-chloroform method. Complementary DNA was transcribed through a reverse transcription (RT)-PCR by using the Access RT-PCR system (Promega Corp, Madison, WI, USA). The PCR reaction was conducted by using previously described methods and genus-specific primers.[29] For the PCR, reaction mixtures consisted of GoTaq Colorless Master Mix (Promega), 50 pmol each of primer (Asp-1 5′-CGGCCCTTAAATAGCCCGGTC-3′ and Asp-2 5′-ACCCCCCTGAGCCAGTCCG-3′) (Sigma, St Louis, MO, USA), template DNA, and nuclease-free water. Amplification was performed in a PerkinElmer GenAmp 2400 thermocycler (PerkinElmer, Waltham, MA, USA) under the following conditions: initial denaturation for 5 minutes at 94°C followed by 30 cycles of denaturation for 45 seconds at 94°C, annealing for 60 seconds at 60°C, and elongation for 45 seconds at 72°C. Negative and positive controls by using a reaction mixture without a DNA template were included in each reaction. These included stock controls as well as controls prepared from paraffin-embedded lung tissue of normal mice and mice infected with *Aspergillus fumigatus*. The primer pairs detect *Aspergillus* species as well as *Paecilomyces variotti*. To distinguish between them, PCR products were digested with the StyI restriction enzyme, run on a polyacrylamide gel, stained with ethidium bromide, and visualized by using ultraviolet light as previously described.[29] Samples positive for *Aspergillus* species produced 2 fragments at 150 bp and 200 bp.

Other analyses

Results of complete blood cell (CBC) counts were reported by the submitting practitioner, as were results from radiography and other diagnostic techniques. Reference intervals noted in the text represent those generated and partially published from the University of Miami Avian and Wildlife Laboratory.[15]

Results

Clinical cases with histologic confirmation of fungal infection are reviewed in Table 1. In addition, brief summaries are presented below.

Case 1: A 9-month-old male Congo African grey parrot was presented with a 2-day duration of difficulty in breathing and lethargy and died shortly after admission. The white blood cell (WBC) count was slightly low, at 4500 cells/μl (reference interval, 5000–11 000 cells/μl. The beta-globulin fraction was high, at 1.46 g/dl (reference interval, 0.35–0.64 g/dl). The galactomannan assay result was strongly positive. At necropsy, a third of the lung tissue contained a caseated mass. On histologic examination, a mycotic pneumonia and airsacculitis were identified. The lung lesions contained fungal hyphae that were septate and branching, and hyphal structures, as well as inflammatory cells, infiltrated across the vessel walls within the lung sections, consistent with fungal vasculitis. Mature sarcocysts were identified within skeletal fibers. Tissues were positive for *Aspergillus* species by PCR testing, and culture of the lung mass revealed *A fumigatus*.

Case 2: A 5-month-old female Congo African grey parrot was presented with open-mouth breathing. On physical examination, the bird was underweight and gasped after handling. A blood sample was collected, and the bird was treated with oxygen, nebulized with terbinafine (1 mg/ml), and administered ketoconazole (30 mg/kg PO q12h). The parrot died during the placement of an air sac tube. Results of hematologic testing revealed a high WBC count (34 600 cells/μl) with heterophilia. Results of the galactomannan assay were positive, whereas EPH results showed a decrease in the albumin/globulin (A : G) ratio to 0.83 (reference interval, 1.3–2.7) and an increase in beta-globulin concentration to 1.62 g/dl (reference interval, 0.35–0.64 g/dl). At necropsy, the trachea was congested, with an exudative focal plug near the syrinx and congestion in the lungs. On histologic examination, the trachea was effaced by a focally extensive area of mixed inflammation to the level of the tracheal rings, and a fungal granuloma composed of fungal hyphae with internal septations and branching was present. The result of PCR testing of a tissue sample was positive for *Aspergillus* species.

Case 3: A 20-year-old female Congo African grey parrot was presented with difficulty in breathing. On clinical examination, the bird was wheezing, with heavy upper-respiratory sounds. Radiographs demonstrated an area of decreased opacity above the heart and distal to the trachea. A rhinolith was present in the right naris, and *Aspergillus* species was cultured from this site. Results of a CBC count and the EPH were within reference intervals, and the galactomannan assay result was positive. The bird was examined 3 additional times over the next 2 months. On the next 2 visits, the bird was presented with seizures of unknown origin. On the last visit, a visible white fungus was observed in the right naris. At

Table 1. Summary of 12 clinical cases of psittacine birds diagnosed with fungal infection that were tested by galactomannan assay.

Case	Species	Age/sex	Time point, d	Galactomannan index (≥0.5 = positive)	A : G ratio[a]	Beta globulins, g/dl[a]	WBC ($\times 10^3$ cells/μl)[a]	*Aspergillus* confirmed	Location of fungal lesions
1	African grey parrot	9 mo/M	0	5.3	0.38 (1.3–2.7)	1.46 (0.35–0.64)	4.5 (5.0–11.0)	Culture, PCR[b]	Lung and air sacs, also sarcocystosis
2	African grey parrot	5 mo/F	0	0.5	0.83 (1.3–2.7)	1.62 (0.35–0.64)	34.6 (5.0–11.0)	PCR	Trachea
3	African grey parrot	20 y/F	0	1.8	WNL	WNL	WNL	Culture	Sinus; possible respiratory involvement
4	Yellow-naped Amazon parrot	Adult/M	0	0.9	WNL	WNL	WNL	PCR	Air sacs
5	Cape parrot	2 y/UNK	0	6.7	0.39 (1.8–2.5)	1.27 (0.49–0.75)	14.9 (4.5–11.5)	Histology, PCR	Lung and air sacs
6	Moluccan cockatoo	15 y/M	0	0.2	WNL	WNL	WNL	PCR	Trachea
			23	0.3	WNL	WNL	ND		
			60	0.2	WNL	WNL	ND		
7	Palm cockatoo	13 y/M	0	0.4	0.84 (1.3–3.6)	1.19 (0.34–0.75)	24.0 (5.0–11.0)	PCR	Trachea
			30	2.7	WNL	WNL	10.1		
			120	1.9	WNL	WNL	16.0		
8	Eclectus parrot	8 y/F	0	3.5	0.40 (1.6–3.2)	0.87 (0.35–0.65)	18.0 (4.0–10.0)	PCR	Crop and surrounding tissue, also possible mycobacteriosis
			53	0.6	0.64	2.57	32.0		
9	Blue and gold macaw	27 y/M	0	0.5	0.68 (1.2–2.6)	1.68 (0.38–0.54)	WNL	Culture, PCR	Nasal
			120	0.1	0.69	1.54	WNL		
			210	0.2	0.68	1.85	WNL		
			270	0.9	0.49	2.11	WNL		
			300	0.4	0.50	1.80	WNL		
			420	0.3	0.57	1.81	WNL		
10	Blue and gold macaw	7 y/F	0	1.6	WNL	WNL	WNL	PCR	Granuloma in coelomic cavity
			96	1.4	WNL	WNL	WNL		
11	Harlequin macaw	10 y/M	0	0.7	0.86 (1.2–2.6)	1.33 (0.38–0.54)	WNL	Unconfirmed	Trachea and air sacs
12	Quaker parrot	1 y/M	14	0.9	0.54 (1.5–2.8)	1.66 (0.33–0.68)	WNL	PCR	Lung and air sacs

Abbreviations: A : G indicates albumin/globulin ratio; WBC, white blood cell; WNL, results within reference interval; PCR, polymerase chain reaction; UNK, unknown; ND, not done.

[a] Reference intervals for values are given in parentheses.

[b] Positive results for *Aspergillus* DNA by PCR testing of fixed tissues.

this time, the EPH results were within reference intervals, except for a mild increase in the beta-globulin fraction (0.73 g/dl; reference interval, 0.35–0.64 g/dl). This same sample tested positive for galactomannan. The bird was treated with ketoconazole (10 mg/kg PO q12h) but was lost to follow-up.

Case 4: An adult male yellow-naped Amazon parrot was presented severely lethargic. Results of radiographs demonstrated a large soft-tissue mass in the caudal abdomen and a barium gastrointestinal contrast study suggested hepatomegaly. The CBC count and EPH results were within reference intervals, whereas the galactomannan assay result was positive. An air sac biopsy was obtained by endoscopy, and histologic results revealed necrotizing airsacculitis, with a mixed-cell population of macrophages, multinucleate cells, and occasional degenerate heterophils. No definitive fungal hyphal structures were identified on hematoxylin and eosin or Gomori methenamine silver stained sections. A tissue sample submitted for PCR testing was positive for *Aspergillus* species. The bird was treated with ketoconazole (30 mg/kg PO q12h) and itraconazole (10 mg/kg PO q12h) for approximately 6 months, as well as given supportive therapy and was asymptomatic 3 years later.

Case 5: A 2-year-old cape parrot presented with severe lethargy. On clinical examination, the bird was severely emaciated, with an overgrown maxilla. Radiographic results demonstrated an increased opacity throughout the abdomen with the obliteration of organ placement. Hematologic test results showed a borderline low hematocrit (HCT) (reference interval, 43–50) (33%) and mild leukocytosis (14 900 cells/μl; reference interval, 4500–11 500 cells/μl), with heterophilia. Results of the EPH revealed that the A:G ratio was decreased to 0.39 (reference interval, 1.8–2.5), with a moderate increase in beta-globulin concentration (1.27 g/dl; reference interval, 0.49–0.75 g/dl). The galactomannan test result was strongly positive. Treatment with ketoconazole (30 mg/kg PO q12h) and supportive therapy was not successful, and the bird died 5 days after initial presentation. At necropsy, the lungs and air sacs were congested and full of caseous material. The histologic diagnosis was fungal pneumonia and coelomitis characterized by fragmented fungal structures admixed with cell debris and surrounded by a mantle of multinucleate cells, macrophages, and numerous degenerate heterophils. The air sac and lung lesions supported intact hyphal structures with parallel cell walls,

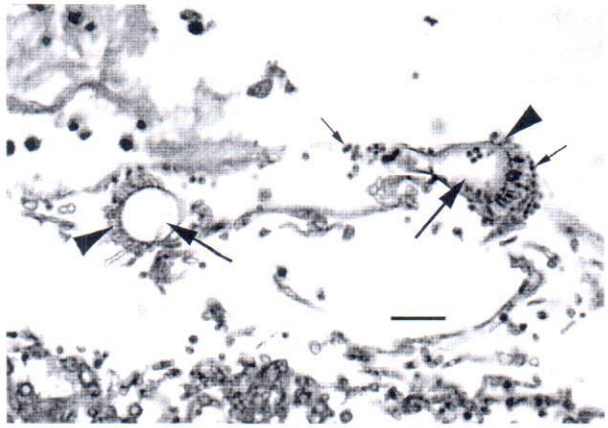

Figure 1. Photomicrograph showing the fruiting bodies of the fungus *Aspergillus fumigatus* forming in the air sac lumen of a cape parrot. The large arrows indicate the vesicles. The sterigmata (arrowheads) and fragmented chains of conidia (small arrows) are visible (hematoxylin and eosin, bar = 20 μm).

internal septations, and branching. In one area, brown fruiting bodies were present, characterized by a vesicle covered with a layer of sterigmata supporting chains of conidia (Fig 1). These structures, as well as PCR results, confirmed the diagnosis of aspergillosis.

Case 6: A 15-year-old male Moluccan cockatoo was referred for mild-to-moderate chronic intermittent dyspnea of 8–9 months duration. The history included treatment for aspergillosis within the previous 5 years. On physical examination, moderate dyspnea with exertion was observed. Radiographs demonstrated small and rare mineralized foci within the lung fields but not in the air sacs. The CBC count and EPH results were within reference intervals, and the galactomannan test result was negative. Tracheal fungal and bacterial cultures showed no growth. Treatment included oxygen supplementation, terbinafine (10 mg/kg PO q12h), and subcutaneous fluids. Twenty-three days later, the bird was re-presented with an acute exacerbation of dyspnea. Tracheal endoscopic examination revealed an intraluminal stricture that encompassed 80%–90% of the lumen approximately 4.0 cm caudal to the laryngeal mound. A tracheal resection and anastomosis was performed to remove the stricture. The histologic diagnosis was heterophilic to pyogranulomatous necrotizing tracheitis, with partial tracheal occlusion, intralesional fungal hyphae, and tracheal epithelial hyperplasia, with loss of cilia. The fungal hyphal structures were septate with rare branching and forming thick mats admixed with inflammatory cells. The

hyphal structures had a pleomorphic appearance with a variation in cross-sectional size of the hyphae. Results of PCR testing of the tissue were positive for *Aspergillus* species. Therapy continued for another 3 months, including F-10 nebulization (0.4%, 15 minutes, q12h; Health and Hygiene, Ltd, Sunninghill, South Africa). At days 23 and 60, the EPH results showed a mild decrease in albumin concentration. The galactomannan assay was negative at both time points. The bird responded well and was asymptomatic 8 months later.

Case 7: A 13-year-old male, captive-bred, palm cockatoo was presented with severe dyspnea after a 1-day duration of wheezing. Radiographs demonstrated opacities in the left air sac. Endoscopic examination revealed a caseous plug above the syrinx and abnormalities of the air sacs. The WBC count was high (24 000 cells/μl; reference interval, 5000–11 000 cells/μl), with a mild heterophilia. Results of the EPH were abnormal, with an A : G ratio of 0.84 (reference interval, 1.3–3.6) and high beta-globulin concentration (1.19 g/dl; reference interval 0.34–0.75 g/dl). The galactomannan assay result was negative. The bird was hospitalized, and an air sac tube was placed. A 10% solution of amphotericin was administered intratracheally, and itraconazole (10 mg/kg PO q12h) therapy was begun. Three days later, the tracheal mass was removed by endoscopy and examined by histology. The entire mass was a fungal granuloma, with mats of hyphae that formed a radial pattern and was surrounded by degenerate heterophils; focal areas of hemorrhage and macrophages were identified. The fungal hyphae had parallel cell walls, rare internal septations, and infrequent branching (Fig 2). Subsequent PCR testing of the tissue confirmed the presence of *Aspergillus* species. The bird improved and was discharged 12 days after admission with continuing itraconazole therapy. On recheck examination 1 month later, radiographs demonstrated that the air sac lesions had improved but were not completely resolved. Results of the CBC count and EPH test were within reference intervals, but the galactomannan assay was strongly positive. Itraconazole therapy was continued. On a subsequent recheck at 4 months after presentation, air sac lesions were still visible on radiographs. The WBC count was high (16 000 cells/μl), with mild heterophilia. The EPH results were within reference intervals, and the galactomannan assay was positive. Itraconazole therapy was continued until a 7-month recheck, when the radiographs appeared normal. The owner reported that the bird was active and vocalizations were normal.

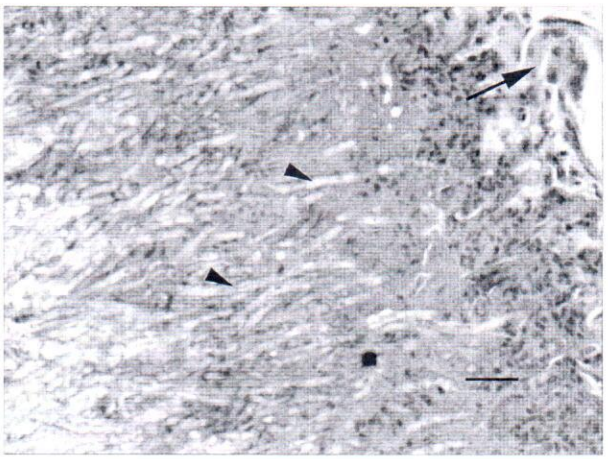

Figure 2. Photomicrograph of a tracheal mass in a palm cockatoo showing a typical fungal granuloma with a mat of fungal hyphae radiating outward (arrowheads). Multinucleate inflammatory cells are forming a capsule (arrows) (hematoxylin and eosin, bar = 20 μm).

Case 8: An 8-year-old female eclectus parrot with a chronic history of recurrent illnesses was presented with feather picking. The WBC count was high, at 18 000 cells/μl (reference interval, 4000–10 000 cells/μl). Results of the EPH revealed a marked increase in gamma-globulin concentration (3.31 g/dl; reference interval, 0.22–0.65 g/dl) and mild increase in beta-globulin concentration (0.87 g/dl; reference interval, 0.35–0.65 g/dl), which resulted in a decreased A : G ratio to 0.40 (reference interval, 1.6–3.2). The galactomannan assay result was strongly positive. Therapy with oral itraconazole (10 mg/kg q12h) and nebulization with clotrimazole (10 mg/ml in polyethylene glycol; 10 min/d for 1 week, then 3 times per week) was initiated. Two months later, the bird was presented with a large hard mass that extended from the base of the skull to the right shoulder. On radiographic examination, there was a healed clavicular fracture, with evidence of pneumonia and a cranial thoracic mass lesion. The galactomannan test result was again positive, the WBC count was high, and the EPH results reflected a marked beta-globulin increase. Despite attempts at treatment and debridement of the mass, the bird died. On necropsy, the histologic diagnosis was a severe chronic ingluvitis and cellulitis that supported numerous branching septate fungal hyphae throughout the lesion and admixed with the inflammatory cells. Additional lesions of pneumonia and a bacterial enteritis positive by Fite's stain were also identified. These lesions suggested

systemic mycobacterial infection; however, further characterization was not performed. On PCR testing of the ingluvies and surrounding tissues, results were positive for *Aspergillus* species.

Case 9: A 27-year-old male blue and gold macaw was presented with a soft swelling on the right side of the rhinotheca. Radiograph results were suggestive of a focal granulomatous lesion. Results of the WBC count were within reference intervals, and the galactomannan assay result was positive. The EPH results showed marked increases in beta- and gamma-globulin concentrations, with the A : G ratio decreased to 0.68 (reference interval, 1.2–2.6). A biopsy of the mass was done, and histologic results showed densely packed, radiating mats of fungal hyphae, with irregular internal septations and rare branching. Culture results of this lesion revealed *Aspergillus* species, and PCR test results of formalin-fixed tissue were positive. The bird was treated with clotrimazole nebulization (10 mg/ml in polyethylene glycol, 10 min, once daily for a week, then 3 times per week) and itraconazole (10 mg/kg PO q12h). The bird was rechecked 4 months later, and radiographs and endoscopic examinations demonstrated clinical improvement. No growth for *Aspergillus* was obtained by routine culture, and hematologic results remained unremarkable, although the EPH results continued to have changes similar to the first submission. Results of a follow-up galactomannan test were negative. The patient improved but maintained a high beta-globulin fraction. Nine months after initial presentation, the assay result for galactomannan was positive. Treatment with itraconazole was resumed, and follow-up testing showed negative galactomannan results but continued hyperbetaglobulinemia as well as mild-to-moderate increases in gamma globulins. Itraconazole therapy was continued.

Case 10: A 7-year-old female blue and gold macaw was presented with sneezing, panting, and pododermatitis. The bird was examined over 4 successive visits, which spanned 7 months. Results of the CBC count and EPH test were within reference intervals, and the galactomannan assay was positive at the initial and last assessed time points. Endoscopic examination demonstrated a white plaque between the ovary and kidney. The histologic diagnosis was a chronic granuloma comprised primarily of macrophages. No microorganisms were identified on hematoxylin and eosin and Gomori methenamine silver staining, but PCR test results were positive for *Aspergillus* species. The patient was treated with ketoconazole (30 mg/kg PO q12h) and itraconazole (10 mg/kg PO q12h) for approximately 6 months. The bird was clinically normal after treatment and later lost to follow-up.

Case 11: A 10-year-old male harlequin macaw was presented with wheezing. Tracheal endoscopy revealed excessive mucus but no visible mass. Results of the CBC count were within reference intervals. The A : G ratio was decreased to 0.86 (reference interval, 1.2–2.6) because of a moderate increase in beta-globulin fraction to 1.33 g/dl (reference interval, 0.38–0.54). The galactomannan assay result was positive. The bird was placed on terbinafine (15 mg/kg PO q12h) and enrofloxacin (15 mg/kg PO q24h; Baytril, Bayer Healthcare, Shawnee Mission, KS, USA). The patient's condition worsened 3 days later, and the bird died, despite emergency treatment. On histologic examination, multifocal areas of necrosis were found throughout the air sacs, and fungal granulomas were observed in the trachea and air sacs. The fungal hyphae lacked significant internal septations, and branching was not identified. The bird was concurrently identified with thyroid hyperplasia. Results of PCR testing of the tissues were negative for *Aspergillus* species.

Case 12: A 1-year-old male quaker (monk) parrot was presented for a recheck of a respiratory infection. The bird had been treated with enrofloxacin prescribed by another veterinarian. On clinical examination, the bird was found to have an irregular heartbeat, and, on radiographs, an enlarged heart and spleen. Initial test results, including a CBC count and total protein concentration, were unremarkable. Two weeks later, the bird began wheezing and was lethargic. The EPH results showed marked increases in the alpha-2-, beta-, and gamma-globulin fractions, with an A : G ratio of 0.54 (reference interval, 1.5–2.8). The galactomannan assay result was positive. The bird died 2 days later, and, at necropsy, the liver, heart, and spleen were enlarged, and multiple focal areas of congestion were present in the lungs. On histologic examination, multifocal, moderate, fungal granulomatous pneumonia was present, and fungal granulomas were found in the air sacs. The fungal granulomas were characterized by fungal hyphae supporting internal septations and branching. A vasculitis was present in the heart and into the great vessels, but no fungal hyphal structures were identified. Results of PCR testing of the tissues were positive for *Aspergillus* species.

Discussion

In the present study, clinical data from 12 cases of fungal infection in psittacine bird species were

reviewed. Aspergillosis was confirmed by PCR testing, fungal culture, and histologic diagnosis in 11 of these 12 cases. In antemortem diagnosis of aspergillosis, these cases demonstrated a use for protein electrophoresis and the galactomannan ELISA, a newly implemented test for the quantitation of circulating *Aspergillus* antigen.

On histologic examination, the inflammatory response of the fungal lesions was similar in all cases and was characterized by a mixed inflammatory cell population. The cell population typically consisted of macrophages, lymphocytes, plasma cells, and heterophils that were both viable and degenerate, as well as, in more chronic cases, multinucleate cells. Fungal hyphae with septations and branching were identified in all but 3 cases. In cases 4 and 10, definitive fungal hyphae were not identified. In case 11, the hyphal structures lacked internal septations, and branching was not recognized. In case 5, the characteristic fruiting bodies of *Aspergillus* species were present within the lumen of the submitted air sac lesions. In 2 cases, 1 and 12, lesions of a vasculitis with fungal hyphae were present within the lumen of blood vessels. Vascular invasion and dissemination are recognized as a feature of some fungal infections and have been described with *Aspergillus* in a passerine bird and *Absidia* in psittacine birds.[30–32]

Aspergillus species, although a common etiologic cause of fungal infections in psittacine birds, is not the only fungal organism that can produce similar clinical signs and lesions.[2,32] Other species of fungus that produce a similar disease to aspergillosis have been described in psittacine birds.[31–35] Definitive classification of the fungal organisms identified on routine histologic sections should be supported by ancillary tests, such as culture, immunohistochemistry, or serologic analysis, unless the characteristic sporulating structures (fruiting body) can be identified.[36–38] PCR-based identification of fungal agents in paraffin-embedded tissue has also been used to successfully resolve the nonspecificity of histologic examination.[39,40] Not all branching and septate hyphae are *Aspergillus* species; less-common organisms, such as *Fusarium* and *Penicillium*, will also produce septate and branching hyphal structures in tissue.[33,37,38] Even those fungal organisms classically identified by irregular hyphal cell walls and lacking internal septations can be morphologically difficult to correctly identify and differentiate from septate and branching fungi in lesions with severe inflammation or by the steric orientation of the fungal elements.[36] This may be the situation in case 11, in which a definitive diagnosis could not be made by histologic examination. Case 5 had the most definitive histologic lesion for an aspergillosis infection, as characterized by the presence of the fruiting bodies present in the lumen of the air sac. These structures were consistent with *A fumigatus*.[30]

In this study, 10 of 12 birds were galactomannan test positive on initial presentation, with indices that ranged from 0.5 to 6.7, where 0.5 is considered the cutoff value for a positive test result. In another study, 27% of samples from presumptively normal birds (versus 67% of confirmed *Aspergillus*-infected birds) were observed to have detectable levels of galactomannan.[11] These data indicate that the current recommended positive cutoff value of 0.5 may allow for potential false-positive results. In humans, these types of results have been linked to environmental exposure to *Aspergillus* antigens by air or food and also possible cross reaction with similar antigens on other fungal species.[18,41,42] Because the assay has a detection limit of 1 ng/ml, weakly positive results may lack specificity.[18,41,42] The low galactomannan concentrations observed in case 11 may be related to these issues. Although this case was diagnosed as a fungal tracheitis and airsacculitis, a definitive diagnosis of aspergillosis was not made by culture or PCR. On histologic examination, the fungal hyphae lacked the characteristic septations and branching expected of *Aspergillus* species. This could be a function of the severe inflammation, as well as the sections available for histologic review. In addition, the positive galactomannan test results may reflect cross reactivity.[18] Another possibility is that the bird may have had a dual infection and that the PCR and culture sampling was not applied to the areas where the *Aspergillus* organisms were present. Dual infections, although uncommon, were reported in psittacine birds and mammals.[32,43]

In other studies, birds with confirmed infection had more than 2.6-fold higher levels of galactomannan and a significantly higher seroprevalence than presumed non–*Aspergillus*–infected clinically normal birds.[11,24,25] These data indicate the promising value of galactomannan testing in aspergillosis serodiagnostics. Although refinements may be possible to optimize assay cutoff levels for different species, veterinarians must understand the potential for false-positive and false-negative test results.[18,41] Since the assay was implemented for use with human samples, the cutoff index has

been reduced from 1.5 to 0.5, with differences in sensitivity and specificity.[42] In addition, clinicians will often perform serial testing to reproduce reported positive or negative results.[42] Lastly, serology is not used as a single test; diagnosis of aspergillosis in humans is formed by using a panel of tests, including serology, hematology, biochemical analysis, culture, PCR testing, and radiography, as well as clinical signs.

The cases in this study reflect the varying types of infection, including the classic respiratory presentation. Location of infection may be especially important in the overall test sensitivity. In the current study, case 6 may reflect this issue, with the apparent infection limited to the tracheal lesion. The impact of location on test results has been proposed with galactomannan testing in humans, as has the differences in the microenvironment at the site of infection.[18] Areas of necrosis that are not nutrient or oxygen rich may decrease the amount of released galactomannan. Also, because galactomannan antigens are large, a degree of angioinvasion has been proposed as necessary for the antigen to be able to reach the circulation. The presence of circulating antigen will also certainly affect the degree of immune reactivity and antibody production. The latter may complex with galactomannan and reduce test sensitivity. In addition, antigenemia would be considered to be variable under any infectious process and site of infection. This has been observed in humans who had daily serial galactomannan tests and in dogs in which periodic testing was performed.[18,21]

EPH remains valuable in determining the resolution of underlying inflammatory or infectious conditions.[15–17,26] However, because inflammatory pathways are similar in different diseases, EPH results are rarely diagnostic of a particular disease. In the current case review, 8 of 12 birds showed moderate to marked changes in EPH results on initial presentation, with increases primarily in the beta-globulin fraction. Because plasma samples are routinely used with avian species, the beta-globulin fraction may reflect fibrinogen as well as other acute-phase reactants.[15] The increases in the globulins observed in the current study are consistent with previous findings regarding the value of EPH in aspergillosis diagnostic testing.[8,11,12,17]

Only 6 of the patients in the current study were still alive at last contact. In 5 cases, more than one test point was available. In case 7, the EPH values normalized with treatment, although the galactomannan result remained positive (but decreased). Lesions were still found by radiography, and therapy was continued. In case 6, normal EPH and negative galactomannan results were present before and during treatment. In case 10, normal EPH and positive galactomannan results persisted over 2 visits and after surgery done to remove a granuloma. In case 9, although an abnormal EPH result persisted, the galactomannan test result went from positive to negative during treatment over a several month period, during which the clinical condition improved. Additional confirmed clinical cases need to be collected to understand the use of the galactomannan assay as a prognostic indicator in avian species. In a review of cases of suspected aspergillosis, decreasing galactomannan levels were observed during treatment and with clinical improvement (C. C., unpublished data, August 2007). Decreases in antigen values would be expected with the elimination of infection. In humans, galactomannan values decrease with successful treatment with a variety of antifungal drugs.[18,42] Normalized EPH results are an expectation of the reduction of the inflammatory process. The prognostic value of EPH has previously been reviewed.[16,26]

In the diagnosis of avian aspergillosis, Jones and Orosz[4] stress a multidimensional approach to diagnosis, including hematologic testing, biochemical analysis, radiology, biopsy, EPH, and microbiologic and serologic testing. In this case series, 4 of 8 patients demonstrated radiographic changes that the submitting practitioners reported were indicative of aspergillosis. Radiographs were also used to monitor disease progression in many cases. Only 4 of 11 patients demonstrated leukocytosis. Mild heterophilia was observed in 3 cases, and monocytosis was not reported in any case. Routine biochemical analyses were not consistently available for evaluation as part of this study group. Overall, a strong clinical suspicion of aspergillosis was reported in most cases, and most birds had both EPH changes and a positive galactomannan status. However, the sample size of cases with complete clinical information in this study is small, and some information may be interpreted as anecdotal and included only opportunistic time points of galactomannan testing. A controlled study that uses experimentally infected birds may better reflect the kinetics of galactomannan expression and relative fungal burden and tissue pathology. Because our study was of birds that presented with established infection, future studies may also better address differences between acute and chronic infection. The diagnostic value of a

galactomannan assay as a screening test versus a diagnostic test is not known. Clearly, although no single technique or test can be solely diagnostic, the combined use of methodologies appears to aid in the diagnosis as long as practitioners understand the limitations of both serologic and routine diagnostic testing for aspergillosis. Further research is needed to address the value of single versus combination testing that uses EPH and galactomannan assays as well as the application of other tests such as PCR and other potential biomarkers and antigens.

Acknowledgments: We thank Ashley Hydrick, Marilyn Rodriguez, and Toshiba Watson for their contributions to data management and sample analysis.

References

1. Tell LA. Aspergillosis in mammals and birds: impact on veterinary medicine. *Med Mycol.* 2005;43(suppl):S71–S73.
2. Reavill DR. Fungal and bacterial diseases. In: Rosskopf WJ, Woerpel RW, eds. *Diseases of Cage and Aviary Birds.* 3rd ed. Baltimore, MD: Williams & Wilkins; 1996:586–612.
3. Redig PT. Avian aspergillosis. In: Fowler ME, ed. *Zoo and Wild Animal Medicine Current Therapy 3.* Philadelphia, PA: WB Saunders; 1997:323–331.
4. Jones MR, Orosz SE. The diagnosis of aspergillosis in birds. *Sem Avian Exotic Pet Med.* 2000;9:52–58.
5. Dahlhausen B, Abbott R, Van Overloop P. Rapid detection of pathogenic *Aspergillus* species in avian samples by real-time PCR assay: a preliminary report. *Proc Annu Conf Assoc Avian Vet.* 2004: 37–40.
6. Buxton I, Sommer CV. Serodiagnosis of *Aspergillus fumigatus* antibody in migratory ducks. *Avian Dis.* 1980;24:446–454.
7. Brown PA, Redig PT. *Aspergillus* ELISA: a tool for detection and management. *Proc Annu Conf Assoc Avian Vet.* 1994:295–297.
8. Reidardson TH, McBain JF. Diagnosis and treatment of aspergillosis in temperate penguins. *Erkrankungen der Zootiere.* 1992;34:155–158.
9. Graczyk TK, Cockrem JF. *Aspergillus* spp. seropositivity in New Zealand penguins. *Mycopathologia.* 1995;131(3):179–184.
10. German AC, Shankland GS, Edwards J, Flach EJ. Development of an indirect ELISA for the detection of serum antibodies to *Aspergillus fumigatus* in captive penguins. *Vet Rec.* 2002; 150:513–518.
11. Cray C, Watson T, Rodriguez M, Arheart K. Application of galactomannan analysis and protein electrophoresis in the diagnosis of aspergillosis in avian species. *J Zoo Wild Med.* 2009;40:64–70.
12. Cray C, Zielezienski-Roberts K. Application of *Aspergillus* antigen assay in the diagnosis of aspergillosis. *Proc Annu Conf Assoc Avian Vet.* 1997:219–221.
13. Zielezienski-Roberts K, Cray C. Update on aspergillosis testing. *Proc Annu Conf Assoc Avian Vet.* 1998:219–221.
14. Graczyk TK, Cranfield MR, Klein PN. Value of antigen and antibody detection, and blood evaluation parameters in diagnosis of avian invasive aspergillosis. *Mycopathologia.* 1998;140:121–127.
15. Cray C, Rodriguez M, Zaias J. Protein electrophoresis of psittacine plasma. *Vet Clin Pathol.* 2007;36:64–72.
16. Werner LL, Reavill DR. The diagnostic utility of serum protein electrophoresis. *Vet Clin North Am Exotic Anim Pract.* 1999;2:651–662.
17. Ivey ES. Serologic and plasma protein electrophoretic findings in 7 psittacine birds with aspergillosis. *J Avian Med Surg.* 2000;14:103–106.
18. Mennink-Kersten MA, Donnelly JP, Verweij PE. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. *Lancet Infect Dis.* 2004;4:349–357.
19. Marr KA, Balajee SA, McLaughlin L, et al. Detection of galactomannan antigenemia by enzyme immunoassay for the diagnosis of invasive aspergillosis: variables that affect performance. *J Infect Dis.* 2004;190:641–649.
20. Jensen HE, Stynen D, Sarfati J, Lapge JP. Detection of galactomannan and the 18 kDa antigen from *Aspergillus fumigatus* in serum and urine from cattle with systemic aspergillosis. *Zentralbl Veterinarmed B.* 1993;40:397–408.
21. 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.* 2001;48:743–750.
22. Guillot J, Sarfati J, de Barros M, et al. Comparative study of serological tests for the diagnosis of equine aspergillosis. *Vet Rec.* 1999;145:348–349.
23. Arca-Ruibal B, Wernery U, Zachariah R, et al. Assessment of a commercial sandwich ELISA in the diagnosis of aspergillosis in falcons. *Vet Rec.* 2006;158(13):442–444.
24. Le Loc'h G, Arne P, Bourgerol C, et al. Detection of circulating serum galactomannan for the diagnosis of avian aspergillosis. *Proc 16th Annu Cong Intl Soc Hum Anim Mycol.* 2006:P–0020.
25. Le Loc'h G, Deville M, Risi E, et al. Evaluation of the serological test Platelia Aspergillus for the diagnosis of aspergillosis. *Proc Eur Assoc Avian Vet.* 2005:260–266.
26. Cray C, Tatum LM. Applications of protein electrophoresis in avian diagnostics. *J Avian Med Surg.* 1998;12:4–10.
27. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res.* 1999; 27:e12i–iii.

28. Morgan K, Lam L, Kalsheker N. A rapid and efficient method for DNA extraction from paraffin wax embedded tissue for PCR amplification. *Clin Mol Pathol.* 1996;49(3):M179–M180.
29. Melchers WJG, Verweij PE, van den Hurk P, et al. General primer-mediated PCR for detection of *Aspergillus* species. *J Clin Microbiol.* 1994;32:1710–1717.
30. Young EA, Cornish TE, Little SE. Concomitant mycotic and verminous pneumonia in a blue jay from Georgia. *J Wildl Dis.* 1998;34:625–628.
31. Dawson CO, Wheeldon EB, McNeil PE. Air sac and renal mucormycosis in an African gray parrot (*Psittacus erithacus*). *Avian Dis.* 1976;20:593–600.
32. Carrasco L, Bautista MJ, de las Mulas JM, Jensen HE. Application of enzyme-immunohistochemistry for the diagnosis of aspergillosis, candidiasis, and zygomycosis in three lovebirds. *Avian Dis.* 1993;37:923–927.
33. Bengoa A, Briones V, Lopez MB, Paya MJ. Beak infection by *Penicillium cyclopium* in a macaw (*Ara ararauna*). *Avian Dis.* 1994;38:922–927.
34. Clark FD, Jones LP, Panigrahy B. Mycetoma in a grand eclectus (*Eclectus roratus roratus*) parrot. *Avian Dis.* 1986;30:441–443.
35. Baross SS, Londero AT. Cardiac zygomycoses in an Australian parakeet. *Sabouraudia.* 1972;10:189–190.
36. Jensen HE, Aalbaek B, Lind P, Krogh HV. Immunohistochemical diagnosis of systemic bovine zygomycosis by murine monoclonal antibodies. *Vet Pathol.* 1996;33:176–183.
37. Kaufman L. Laboratory methods for the diagnosis and confirmation of systemic mycoses. *Clin Infect Dis.* 1992;14(suppl 1):S23–S29.
38. Richardson MD, Warnock DW. Laboratory diagnosis of fungal infection. In: Richardson MD, Warnock DW, eds. *Fungal Infection Diagnosis and Management.* 3rd ed. Malden, MA: Blackwell Publishing Ltd; 2003:22–23.
39. Bialek R, Konrad F, Kern J, et al. PCR based identification and discrimination of agents of mucormycosis and aspergillosis in paraffin wax embedded tissue. *J Clin Pathol.* 2005;58:1180–1184.
40. Paterson PJ, Seaton S, McLaughlin J, Kibbler CC. Development of molecular methods for the identification of *Aspergillus* and emerging moulds in paraffin wax embedded tissue sections. *Mol Pathol.* 2003;56:368–370.
41. Verweij PE, Mennink-Kersten MA. Issues with galactomannan testing. *Med Mycol.* 2006;44(suppl):S179–S183.
42. Verdaguer V, Walsh TJ, Hope W, Cortez KJ. Galactomannan antigen detection in the diagnosis of invasive aspergillosis. *Expert Rev Mol Diagn.* 2007;7:21–32.
43. Jensen HE, Halbaek B, Lind P, et al. Development of murine monoclonal antibodies for the immunohistochemical diagnosis of systemic bovine aspergillosis. *J Vet Diagn Invest.* 1996;8:68–75.