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APPLICATION OF GALACTOMANNAN ANALYSIS AND PROTEIN ELECTROPHORESIS IN THE DIAGNOSIS OF ASPERGILLOSIS IN AVIAN SPECIES

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Abstract: Previous studies support the possible application of galactomannan, a major antigen of *Aspergillus* sp., to aspergillosis diagnosis in avian and other animal species. An assay is commercially available for use with human serum and bronchoalveolar lavage fluid samples. In the current study, galactomannan results from plasma samples were compared between birds with histologically confirmed aspergillosis and those that were clinically normal presumptively non-*Aspergillus* infected birds per submitting practitioners' responses to a questionnaire. It was observed that infected birds demonstrated a 2.6-fold increase in galactomannan over birds without evidence of aspergillosis. With the use of a galactomannan index of 0.5 as a cutoff, the sensitivity and specificity of the test were found to be 67% and 73%, respectively. In addition, plasma samples were analyzed for abnormalities in protein electrophoretic patterns. Infected birds had a higher incidence of increased beta and/or gamma globulin concentrations. Test sensitivity and specificity were 73% and 70%, respectively. If the 2 tests were used as a panel, then the sensitivity was 89% and specificity was 48%. These data indicate that both galactomannan and protein electrophoresis may be valuable tools in the diagnosis of avian aspergillosis.

Key words: Aspergillosis, *Aspergillus*, enzyme-linked immunosorbent assay (ELISA), protein electrophoresis, galactomannan, serodiagnostics.

INTRODUCTION

Aspergillosis remains difficult to diagnose in avian species. Traditional techniques, including routine hematologic and biochemical analysis, culture, and radiography, have value but lack the ability to reliably provide a definitive diagnosis.[18] Endoscopy is a good antemortem tool but is not always practical in ill or debilitated patients. Practitioners are thus often dependent on basic testing and clinical signalment to form a diagnosis and initiate treatment.

Serodiagnostic testing for aspergillosis was first described in ducks and pigeons.[6,24] In 1994, the first application of an ELISA in the diagnosis of infection of caged birds and raptors was described.[5] Other studies documented a high incidence of antibodies in captive and wild penguins.[11,12,27] Antibody testing in conjunction with the detection of circulating *Aspergillus* antigen and changes in protein electrophoresis (EPH) was also described.[9,30] The value of antigen testing was also reported in a model of experimental infection in Pekin ducks.[13] EPH was reported to measure proteins associated with acute inflammation and stimulation of humoral immunity in avian species.[7,8,29] Although not solely diagnostic of aspergillosis, the positive application of abnormal EPH in confirmed cases in psittacine birds was reported.[16] The utility of EPH in the diagnosis of aspergillosis in penguins, a species reportedly with a high antibody reactivity, even in the absence of disease, was also demonstrated.[27]

Galactomannan is a polysaccharide soluble antigen released from the cell wall during the logarithmic growth phase of *Aspergillus* hyphae and can be found in the circulation.[25] A commercial enzyme-linked immunosorbent assay (ELISA) assay to measure this *Aspergillus* antigen has been widely applied in human medicine.[2,23,25] This assay has also been used in studies of aspergillosis in dogs, cows, and horses, as well as with various laboratory animal models of infection.[1,4,10,14,17,20,23] A recent publication profiled its application to the diagnosis of infection in falcons with a high specificity but low sensitivity.[3] In addition, other investigators reported a higher incidence of positive galactomannan results in psittacine birds with suspected infection, although there were many false-positive results.[21,22] The purpose of the current study was to examine galactomannan and protein electrophoretic changes in birds with and without confirmed aspergillosis.

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MATERIALS AND METHODS

Study samples

Lithium heparinized plasma samples were submitted to the University of Miami Avian and Wildlife Laboratory from 2004 through 2007 for analysis by an *Aspergillus* serodiagnostic panel. All samples were analyzed via electrophoretic techniques within 24 hours of arrival at the laboratory. Galactomannan analysis was conducted within 5 days. All samples were refrigerated at 4°C until analyzed.

All samples came from captive birds. Submitting practitioners were sent patient questionnaires to collect information, such as whether there was clinical confirmation of infection (by histology), clinical signs, accessory diagnostic testing (i.e., radiography, hematology, chemistry, culture), and if the patient responded to treatment. Two groups were defined for further statistical analysis: presumptive nonaspergillosis (n = 72) and confirmed aspergillosis (histologic confirmation, n = 56). Birds were categorized as presumptive nonaspergillosis birds on the basis of the practitioner's report, and other routine blood work, and included those birds reported to be clinically normal during the study period. This group did not include birds with other confirmed diseases (i.e., chlamydophilosis, etc.) The histologic confirmed group included birds confirmed at necropsy (n = 53) and biopsy (n = 3). The presumptive nonaspergillosis group was primarily composed of psittacine species (n = 55) and various birds from other orders (n = 17; Falconiformes, Anseriformes, and Galliformes). The psittacine group included the following: African grey parrot (n = 13), Amazon parrot (n = 9), caique (n = 3), cockatiel (n = 1), cockatoo (n = 8), conure (n = 4), eclectus parrot (n = 3), lovebird (n = 1), macaw (n = 10), pionus (n = 1), Quaker parrot (n = 1), and Senegal parrot (n = 1). The confirmed group included penguins (n = 3), osprey (n = 1), duck species (n = 5), peafowl (n = 1), nene goose (n = 1), and various psittacine birds (n = 45). The latter included the following: African grey parrots (n = 10), Amazon parrot (n = 6), caique (n = 1), Cape parrot (n = 1), cockatiel (n = 1), cockatoo (n = 5), conure (n = 1), Eclectus parrots (n = 6), Jardine's parrot (n = 1), macaws (n = 10), Meyer's parrot (n = 1), parrotlet (n = 1), and a Quaker parrot (n = 1). Exact age and sex information was not obtained in many cases and did not form a base for further analysis in this study.

Galactomannan assay

The Platelia ELISA kit (Bio-Rad, Hercules, California 94547, USA) was used for galactomannan quantitation. The procedure was modified by using 60 ul of plasma instead of 300 ul of serum. These changes were validated by an in-house study, which included the comparison of paired serum and plasma samples and spiking additional samples with galactomannan to test the sample volume variables. Other investigators also successfully altered the pretreatment volume while maintaining the proper sample to pretreatment solution ratio.[20,22] The assay results include optical density results of positive, negative, and cutoff control samples provided in the kit. Results of test samples are recorded as an index relative to the reading of 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.

Plasma EPH

Plasma EPH was performed as previously described.[7,8,29] The Paragon system and SPEP-II gels (Beckman, Fullerton, California 92834, USA) were used. Fraction quantitation was performed by using a Beckman densitometer. Results were categorized as normal or abnormal based on in-laboratory species-specific reference intervals, many of which were previously published.[7] The A:G ratio was calculated following its traditional presentation: (prealbumin + albumin)/(alpha 1 + alpha 2 + beta + gamma globulins).[7] Abnormal EPH was subcategorized based on at least a 20% increase in absolute concentration of beta globulins, gamma globulins, beta and gamma globulins, or alpha globulin. For example, the beta globulin fraction range in the African grey parrot ranges from 3.5 to 6.1 g/L. An abnormal beta globulin fraction was considered to be anything greater than 7.32 g/L. Results were examined as percent increases in each fraction in contrast to grams per liter values to account for the differing normal reference intervals of all the species as well as species composition of the 2 analysis groups.

Statistical analysis

A t test for independent samples was used to determine if the assay indices were significantly different between the clinical groups, and chi-square tests were used to determine if the proportion of abnormal findings were significantly different. Standard formulas were used to calculate the sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy of galactomannan and EPH. All calculations were conducted by using Statistical Analysis System software version 9.1 (SAS Institute, Inc., Cary, North Carolina 27513, USA).

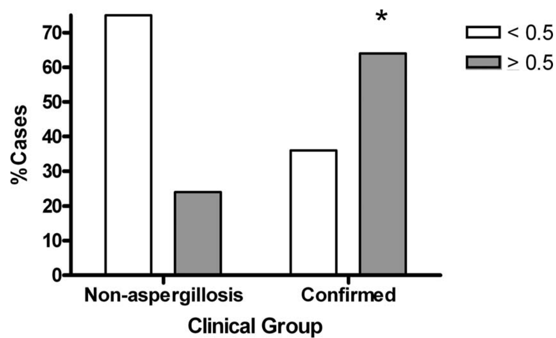

Figure 1. Galactomannan reactivity on the Platelia assay is graphed as the percentage of cases in the presumptive nonaspergillosis and confirmed groups are shown by the calculated index of reactivity. Significant differences are shown with *.

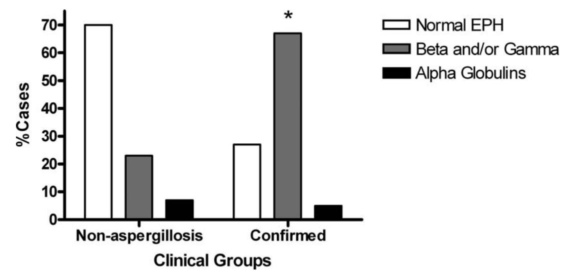

Figure 2. The percentage of cases with normal EPH versus those with greater than 20% increases in beta and/ or gamma globulins and alpha globulins are shown by clinical group. Significant differences are shown with *.

RESULTS

Galactomannan analysis

The majority (73%) of nonaspergillosis birds were negative (index < 0.5) by using the galactomannan assay versus 33% of the confirmed group (Fig. 1). Specifically, the average galactomannan index of the nonaspergillosis group was 0.64 ± 0.19 (mean $\pm$ standard error) and the confirmed group averaged 1.68 ± 0.23 ($P = 0.001$). Test sensitivity was found to be 67%, and the specificity was 73%. The diagnostic accuracy was 70% (Table 1). When the cutoff index was raised to 1.0, the test sensitivity was found to be 39% and the specificity was 83%. The diagnostic accuracy was 65%.

Plasma EPH

By using species-specific reference intervals, 30% of the EPH results were found to be abnormal in the presumptive nonaspergillosis bird group (Fig. 2). This is in contrast to the confirmed group, which was 72% abnormal ($P < 0.001$). Of the confirmed group, 40% of cases demonstrated increases in beta globulins, 9% of the cases demonstrated increases in gamma globulins, 18% of cases had increases in both beta and gamma globulins, and 5% of cases had increases in alpha globulins. The test sensitivity was 73%, and specificity was 70%. The diagnostic accuracy was 71% (Table 1).

Combined analysis

Galactomannan assay and plasma EPH results were examined in combination. The combined test sensitivity was 89% and specificity was 48%. The diagnostic accuracy was 65% (Table 1).

DISCUSSION

The diagnosis of aspergillosis is problematic in avian species and humans alike.[18,23,24] Acute and chronic clinical manifestations, site of infection, ongoing therapies, and immunity to fungal elements all complicate the diagnostic picture. In avian medicine, routine hematology and clinical chemistry, radiography, culture, and biopsy are all traditional antemortem techniques but lack reliability and specificity.[18] EPH was reported to have some value as an ancillary diagnostic test, although it too lacks specificity.[7,8,18,29,30] Galactomannan testing has been applied quite widely for the diagnosis of aspergillosis to human patients and to a lesser degree in animal species.[2,3,10,14,15,21–26] The present study examines galactomannan and plasma EPH results from presumptive non-*Aspergillus* infected birds and those with confirmed infection.

Table 1. Sensitivity and specificity calculations for galactomannan and protein electrophoresis.

Result	% Sensitivity	% Specificity	% Negative predictive value	% Positive predictive value	% Accuracy
Galactomannan index ≥0.5	67	73	76	63	70
Abnormal protein electrophoresis (increase in any globulin fraction)	73	70	76	66	71
Galactomannan–protein electrophoresis combination	89	48	86	54	65

Galactomannan analysis

Galactomannan measurement has been applied to the diagnosis of human fungal infection. Patients undergoing chemotherapy, transplantation, or treatment of autoimmune disorders, e.g., chronic pulmonary disease, may have profound immune suppression.[2] Opportunistic infection with *Aspergillus* sp., among other infectious agents, results in high mortality. By conducting a meta-analysis of 27 studies by using galactomannan testing, an overall sensitivity of 71% and specificity of 89% in humans with hematologic malignancy was reported.[26] Accessory testing, including radiography, routine blood tests, and cultures, aids in diagnosis of disease.

In veterinary medicine, galactomannan testing has been examined in horses, cows, dogs, rabbits, and other laboratory animals.[10,14,17] In contrast to its implementation in human medicine, galactomannan testing has been implemented in veterinary medicine, usually in the absence of a high magnitude of immune suppression as observed in patients with hematologic malignancies. In horses, galactomannan testing was found not to be a sensitive indicator of guttural pouch aspergillosis, which is generally considered to be a superficial infection.[14] However, in a single case of invasive aspergillosis, the galactomannan test was positive.[14] Both invasive and nasal aspergillosis has been described in dogs. No false-positive results were found, but the utility of galactomannan appeared limited in confirmed cases of nasal infection.[10] Positive results were observed in one case of systemic aspergillosis and a case of bronchopneumonia.[10] Jensen et al[17] reported that the detection of galactomannan in cows was sensitive in cases of systemic aspergillosis and that the magnitude of the galactomannan value may be related to the fungal burden. Galactomannan testing has been successfully used in a wide number of laboratory animal models of the human disease, including those that used mice, guinea pigs, and rabbits.[1,4,20,23] In each model, infection was introduced after a immune suppression regimen and was generally limited to the respiratory tract.

Aspergillosis is a common fungal infection in birds and can affect the lower- and upper-respiratory tract. Infection can also become invasive and is generally believed to be a result of environmental contamination and stressors, perhaps leading to reduced immunity.[18] Studies that used galactomannan testing in avian species have been limited to just a few reports.[3,21,22] Based on extensive data from clinical examination, including endoscopy, 142 normal and 50 infected falcons (some suspect, some confirmed by histology) were examined by using the galactomannan assay, with a resultant 12% sensitivity and 95% specificity.[3] This study was conducted with a high cutoff value (2 times higher than the manufacturer's current recommendations). The use of the assay on multiple avian species with suspected aspergillosis was reported to have a specificity of 86% but a sensitivity of 30% also with the higher cutoff value.[21,22] Notably, in the current clinical case study (which is also dominated by psittacine bird samples), an average of 2.6-fold increased indices was found in the confirmed group versus the presumptive nonaspergillosis group. By using the percentage of positive cases, the confirmed group was also found to have a much higher incidence of positive samples. Overall, in the current study, the sensitivity was found to be 67%, and the specificity was 73% when using the current manufacturer's recommended threshold. A diagnostic accuracy of 70% was found. The notable difference in sensitivity compared with the previously published avian studies is likely related to the use of only cases confirmed by histology and the use of the current manufacturer's recommendation of a 0.5 index for a cutoff level versus the 1.0 index used by others. To this point, when the current results were analyzed with the higher 1.0 cutoff index, the specificity increased to 83%, while the sensitivity decreased to 39%.

It is important to note that most humans with positive galactomannan results have a minimal immune response to *Aspergillus* because of the nature of their treatment regimen. That is, chemotherapy to treat hematologic malignancy and immune suppressive drug treatments after stem-cell transplantation often limit immune responses to opportunistic pathogens. However, in those human patients with the anti-*Aspergillus* antibody, diagnostic sensitivity of galactomannan analysis is reduced.[15] This is believed to be related to the antibody binding galactomannan and clearing it from circulation so that it is not present in the sample. In the current study, some birds were found to have very high levels of galactomannan (index > 3.0). Le Loc'h hypothesized that once infection becomes more advanced and the immune system is rendered ineffectual, the circulating antigen concentration can greatly increase.[22] It is known that *Aspergillus* toxins, including gliotoxin, have very immunosuppressive properties in humans and turkeys.[19,28] In animal models of aspergillosis, the addition of exogenous mycotoxin was found to worsen the disease pathogenesis.[19] It is hypothesized that mycotoxins are an important virulence factor of *Aspergillus* and work to depress anti-*Aspergillus* immune respons-

es. In the infection of avian species, this may translate to weakened production of anti-*Aspergillus* antibodies during chronic infection. To this point, this study documents the absence or very low levels of anti-*Aspergillus* antibody in birds with confirmed aspergillosis (Cray, unpub. data).

Experimental rabbit and cow models of aspergillosis demonstrated that galactomannan concentrations were correlative to the fungal burden in the tissues.[17,23] In addition, the site of infection has been proposed to potentially alter the amount of circulating galactomannan in humans.[14,25] This idea is consistent with mostly negative galactomannan results in horses in which infection was limited to the guttural pouch versus higher consistent levels in cows with systemic aspergillosis.[14,17] In human clinical pathology, it is now recommended to perform 2 or 3 sequential tests to confirm negative results.[2] This recommendation stems from differences caused by circulating antibody and the site of infection, which manifest in variable circulating galactomannan concentration throughout the course of infection.[25]

In the current study, it was observed that the mean galactomannan index of the presumptive nonaspergillosis group fell slightly above the recommended cutoff of 0.5. This may indicate misclassification by the submitting practitioner based on clinical data collected during the study period. However, patients were reported to be clinically normal and had normal hematologic and biochemical analyses. Samples from some of the birds did have abnormal plasma EPH results, possibly indicating underlying inflammation or infection; but these were not consistently observed in samples with higher galactomannan results. The manufacturer of the galactomannan assay reports no false positives when using samples from patients with viral infection, cancer, nonviral cirrhosis, and autoimmune disease (Bio-Rad galactomannan assay technical insert). Similar studies have not yet been fully conducted in avian species. In addition, these positive results in presumptive nonaspergillosis birds may reflect colonization but no overt disease, exposure by food or environment, or the presence of an unknown cross-reactive antigen; all of which have also been hypothesized to be variables in human sample analysis.[25] These results may reflect the need for different screening threshold concentrations for different avian species.

Overall, the findings of the current study indicate the potential for galactomannan testing in a diagnostic panel for aspergillosis. Moderate sensitivity and specificity levels are reached by using the manufacturer's recommended 0.5 index cutoff level, and specificity could be improved with the use of a more restricted interpretation of positive results greater than an index of 1.0. Because galactomannan results in the histologic confirmed group averaged an index of 1.68, this also supports the premise that higher galactomannan results are associated with infection. In contrast to humans under treatment for a hematologic malignancy who have little ability to mount an immune response, it should be recognized that birds may not present with the same lack of response. The presence of concurrent anti-*Aspergillus* antibody titers in these birds was not addressed in the current study; but, in other studies, we observed that antibody titers alone are not sensitive diagnostic tests for the diagnosis of aspergillosis (Cray, unpub. data). Cross-reactive antigens and false positives from sample contamination are a valid source of false positives in other human testing and should be expected in avian species.[25] Positive galactomannan results in clinically normal birds should be followed by additional testing, including possible recheck of galactomannan results. Likewise, if a negative galactomannan result is obtained from a suspect bird, retesting should be considered if other routine testing continues to support the possibility of infection.

Plasma EPH

EPH can detect underlying inflammatory or infectious conditions in avian species.[7,8,29] However, because inflammatory pathways are similar in different disease states, the test is rarely solely diagnostic of a particular disease. In the current study, it is notable that the presumptive nonaspergillosis group contained clinically normal patients with abnormal EPH. It was previously observed that approximately 30% of psittacine samples with normal hematologic and chemistry results do exhibit abnormal EPH and hypothesize that EPH may offer a different sensitivity to inflammation and infection than traditional clinical pathology testing (Cray, unpub. data). In the current study, this may reflect a misclassification by the submitting practitioner indicative of possible aspergillosis or other underlying disease or infection. However, as with the galactomannan assay, a higher percentage of abnormal EPH results was found in the confirmed group versus the presumptive nonaspergillosis group. This was inclusive of changes in beta, gamma, and alpha fractions. Only 23% of the presumptive nonaspergillosis group had beta and/or gamma globulin increases versus 67% in the confirmed group. These data are consistent with a small case study that indicated the presence of beta globulin increases in 3 of 7 cases of aspergillosis in psittacine birds and a

larger study in penguins.[16,27] The 73% sensitivity of electrophoresis was thus not surprising, but the specificity was higher than expected. This is likely because of the use of reportedly clinically normal birds in the current study. Clearly, other infectious and inflammatory processes present in birds will also result in changes in the globulins; thus, these changes are not solely diagnostic of aspergillosis. As previously proposed, EPH can be used as an accessory diagnostic and prognostic tool to complement galactomannan testing as well as other tools in clinical evaluation of birds with possible aspergillosis.[8,9,18,29]

Combined analysis

When results of the galactomannan test and plasma EPH were examined in combination, the sensitivity improved to 89%, although the specificity decreased to 48%. These changes are reflected in a decreased positive predictive value and increased negative predictive value. Because the assays are 2 different tests, they have independent false-positive rates that do not occur in the same study cases. The combination of these 2 independent errors results in a decrease in specificity rather than an enhancement when used in combination. Alternatively, the combination of the tests does serve to better correctly diagnose true negative cases.

CONCLUSIONS

Overall, this study demonstrates the utility of both galactomannan analysis and plasma EPH in the diagnosis of avian aspergillosis. Both tests afford moderate levels of sensitivity and specificity, and an increased sensitivity when used in combination. Routine hematologic and biochemical testing, clinical examination, radiography, culture, and endoscopy are still important tools for diagnosis of disease. All test results should be considered in conjunction with these latter traditional techniques. Positive galactomannan results over an index of 1.0 can be viewed as more likely to be a true positive, but, factors, such as site of infection, chronicity of infection, and the presence of anti-*Aspergillus* antibody may affect the amount of galactomannan in circulation. In human pathology, these and other factors are recognized and have resulted in a test plan that often includes a minimum of 3 sequential tests before a diagnosis is made. Because such a level of testing is not always reasonable in many avian patients, galactomannan results can instead be viewed in conjunction with plasma EPH results and the other traditional techniques.

The current study included birds with focal and generalized infection, although all were chronic cases. As the database of clinical presentation, specific infection sites, species, concurrent treatments, presence of anti-*Aspergillus* antibody, and prognostic use grows, the galactomannan and EPH tools will need to be reassessed for further application to the aspergillosis diagnostic dilemma.

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