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ADAPTATION OF A COMMERCIALLY AVAILABLE WESTERN BLOT KIT FOR THE DETECTION OF ANTIBODY TO *ASPERGILLUS* IN PENGUINS IN FRANCE AND THE UNITED STATES

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Abstract: Antemortem serodiagnosis of aspergillosis remains challenging in Sphenisciformes. Protein electrophoresis, serology (antibody, antigen) by ELISA, and gliotoxin detection provide variable diagnostic value. In the present study, a commercially available Western blot (WB) validated for use in humans and dolphins was adapted for use with penguin samples. Using the same method and reagents, samples were analyzed from multiple institutions in the United States and one facility in France. This was inclusive of normal juvenile African penguins (*Spheniscus demersus*, n = 10) and various species of penguins in the United States with confirmed infection (n = 9) as well as 52 samples from Humboldt penguins (*Spheniscus humboldti*) in France. Cumulative WB scores (based on reactivity to different antigens) were found to be significantly higher in the group of penguins with confirmed infection ($p < 0.0001$). Significant differences were also observed between the clinically normal penguins in the two populations, with higher scores in the United States (median score 1.0, 95%CI [0–5], min 0, max 11) compared to France (median score 0,95%CI [0–0], min 0, max 5). The utilization of the WB as a diagnostic tool is inconclusive due to the use of samples from varying institutions, environmental background, age, and stages of infection. However, this tool may provide an overview of antigen reactivity in penguins infected with *Aspergillus* to help design a more robust serology assay and further understand the humoral immune response during infection.

INTRODUCTION

Aspergillosis is a major cause of mortality in Sphenisciformes.[1,20] Culture and identification of *Aspergillus* spp. from associated lesions is considered the gold standard diagnostic method.[1] Alternative diagnostic procedures include hematology, blood chemistry, diagnostic imaging, and endoscopy.[1] However, these procedures have a limited and variable diagnostic value. Antemortem serodiagnosis of aspergillosis in penguins remains challenging. With the use of an ELISA made with sonicated *Aspergillus fumigatus* cultures, penguins were found to be reactive even in the absence of clinical signs.[3] This was a similar finding to the report of an ELISA made with a wide range of metabolic antigens implemented for use in Humboldt penguins (*Spheniscus humboldti*).[8] While the detection of circulating *Aspergillus* galactomannan was reported to have utility in some cases of aspergillosis, this assay was not sensitive in penguins.[4,7] In contrast, the combination of adjunct testing including 3-hydroxybutyrate levels and plasma protein electrophoresis provides high specificity and negative predictive value.[7] More recently, an assay to measure gliotoxin, one of the major toxins of *A. fumigatus*, was found to be highly specific although the utility of this test for diagnosis has not been fully determined.[18] Similarly, a rapid immunochromatographic test targeting an *Aspergillus* antigen detected 75% of cases in the positive group when combining plasma and glottis swab samples, but larger prospective studies and comparison with other assays are needed to assess its performance.[13]

Western blotting (WB) is a diagnostic technique used to measure antibody reactivity to specific antigens through the use of a membrane for immunodetection.[10,11] A commercially available WB kit for the detection of IgG antibody to *Aspergillus* has been described with high specificity and sensitivity for use in non-immunocompromised human patients.[14] This kit was adapted for use in bottlenose dolphins (*Tursiops truncatus*) and found to have high specificity.[6] The goal of the current preliminary study was to adapt and validate this assay for the detection of IgG to *Aspergillus* antigens in penguins.

METHODS

Samples – United States (US)

The Maryland Zoo in Baltimore (MZIB) hosts the largest breeding colony of African penguins

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(*Spheniscus demersus*) in North America. The exhibit includes a 625 m^3 round freshwater pool surrounding a central island with multiple land areas and a large indoor holding area. The pool is managed with sand filtration and ozone disinfection. The colony of approximately 100 penguins has indoor and outdoor access year-round. The indoor building has a high-performance air-handling system with pleated filters with minimum efficiency reporting value of 8 or 12 rating, which are changed monthly. MZIB samples all penguins during the first 2 yr at the facility weekly from April to November as part of a routine avian malaria monitoring program. In the 2021 season this was 10 juvenile birds (0–2.5 years). The birds were all clinically normal throughout the testing period. Blood samples were drawn from the right jugular vein into lithium heparin and serum separator tubes. Tubes were centrifuged, plasma or serum was removed and kept frozen at –80C. Samples (n = 22) were shipped to the University of Miami (Miami, FL 33136, USA) for analysis.

In addition to the MZIB routine penguin samples, 16 samples from nine different penguins with aspergillosis (confirmed by histopathology at time of necropsy or culture/endoscopy) were obtained from various facilities and analyzed at the University of Miami. They included one African penguin, one gentoo penguin (*Pygoscelis papua*), four Humboldt penguins, and three little blue penguins (*Eudyptula minor*).

Samples – France

ZooParc de Beauval (ZPB) hosts a colony of approximately 150 Humboldt penguins in an outdoor semi-naturalistic enclosure with a 600 m^3 chlorinated pool, artificial beach, and 400 m^2 wooded and rocky nesting area. Aspergillosis is a significant cause of death in the colony, with 22.5% of necropsied penguins showing laboratory evidence of the disease.[2]

During the 2017–2021 period, 52 blood samples were opportunistically collected by venipuncture of the venous sinus at the dorsal base of the tail, or medial metatarsal vein, from 46 different penguins. After centrifugation, serum or heparinized plasma was retrieved, and frozen at –20° C until the time of analysis. Forty-five samples were collected from penguins considered unaffected by aspergillosis at the time of sampling based on clinical examination, including normal auscultation and absence of respiratory signs, and absence of confirmed or suspected aspergillosis in the 2 months following sample collection. Five samples were excluded from the analyses due to uncertain aspergillosis status at the time of sampling. Two samples from two different individuals were considered positive at the time of sampling based on compatible clinical signs and postmortem confirmation by both histopathology and culture within 7 d of sample collection. Age of penguins at the time of sampling ranged between 1 month and 23 yr, with 38 samples (73%) from adult birds (≥3 yr old). Stored frozen samples were sent to LDBio Lab (LDBio Diagnostics, 69009 Lyon, France) on three occasions, in October and December 2020, and September 2021.

Western blot

Samples were tested with the *Aspergillus* Western Blot IgG kit (LDBio Diagnostics, 690009 Lyon, France) using the manufacturer's recommendations. For detection of penguin antibody, a goat anti-chicken IgG (polyclonal) conjugate was used (Rockland Immunochemicals, Inc., Limerick, PA 19468, USA). The immunoblot reactivity was compared to a human positive control provided by the manufacturer. The intensity of four bands (30, 22, 18–20, and 16 kDa) that have been shown to be specific for *Aspergillus* antibody in a previous publication[14] was rated and compared to this control. Western blots for the US and French penguin populations were performed and scored by two different operators with considerable experience in using the kit and following recommendations from the manufacturer. The sum of the results of the four bands was calculated with a maximum of 16 (0 to 4 scoring for each band). In dolphins, a cumulative score of 6/16 is considered a positive result,[6] while in humans, the simultaneous presence of two well defined bands indicates a positive result.[14]

Statistics

The United States data was found to be normally distributed using the D'Agostino-Pearson test. The French data from the normal group was non-normal in distribution; the small sample size of the confirmed group did not allow for such assessment. Descriptive statistics and T-test or Mann Whitney test were calculated using MedCalc version 20.022 (8400 Ostend, Belgium) and GraphPad Prism 6.07 (LaJolla, CA 92037, USA).

RESULTS

Varying intensity of reactivity to 30, 22, 18–20, and 16 kDa antigens were observed in the samples from penguins (Fig. 1). In addition to these 4 antigens, some samples demonstrated reactivity with

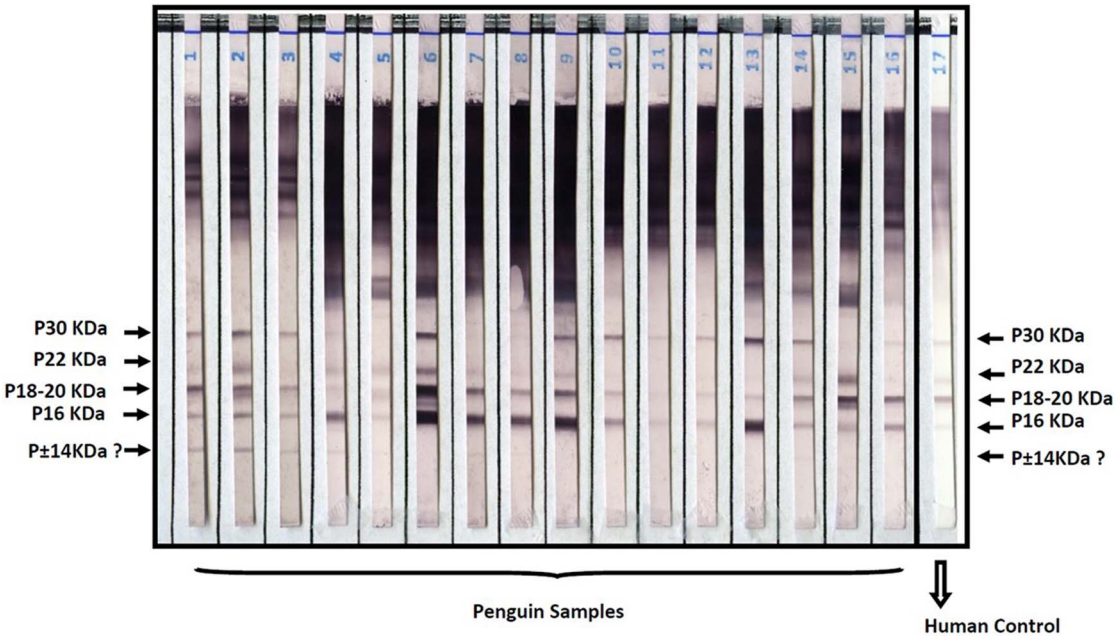

Figure 1. Examples of anti-*Aspergillus* immunoblot testing in penguin samples. Lane 17 represents the human positive control sample. **As examples for reactivity scoring, lane 11 was scored as 5 and lane 6 was scored as 16.

a 14 kDa band and reactivity with antigens greater than 30 kDa.

In the analysis of the samples from the facilities in the United States, the majority of the juvenile penguins showed reactivity under a cumulative score of 6. A significant difference was observed between the mean reactivity of this group and the group of samples from penguins with confirmed infection ($p < 0.0001$). Approximately 45% of the juvenile penguins showed reactivity to 2 or more bands versus 93% of the samples from confirmed penguins (Table 1).

Unaffected penguins in France (median score 0, 95%CI [0–0], min 0, max 5) had significantly lower scores ($p < 0.001$) compared to unaffected penguins in the US (median score 1.0, 95% CI [0–5], min 0, max 11). They also had lower median scores than positive penguins in the same institution, but the low number of the latter ($n = 2$) precluded any statistical comparison (Table 1).

Table 1. Summary of data from penguin samples tested with the *Aspergillus* Western Blot IgG kit in the United States and France. The United States data was normal in distribution. The France data Normal group was non-normal in distribution; the distribution of the Confirmed group could not be assessed given the small sample size.

Group/Clinical status	Number with minimum 2 visible bands	Number with cumulative score > 6	Mean (95% CI) cumulative score	Median (95% CI) cumulative score	Min–Max cumulative score
United States					
Juveniles from MZIB – Normal	10/22	5/22	3.0 (1.3–4.6)[a]	1.0 (0.0–5.0)	0–11
Adults from multiple institutions – Confirmed	16/16	15/16	10.8 (9.2–12.5)[b]	10.5 (9.6–12.4)	5–16
France					
Normal	4/45	0/45	0.3 (0.0–0.6)	0.0 (0.0–0.0)[c]	0–5
Confirmed	2/2	2/2	10.5 (0–29.5)	10.5 (9.0–12.0)[d]	9–12
Uncertain	1/5	0/5			

[a, b] $p < 0.0001$ [c,d] $p = 0.0011$.

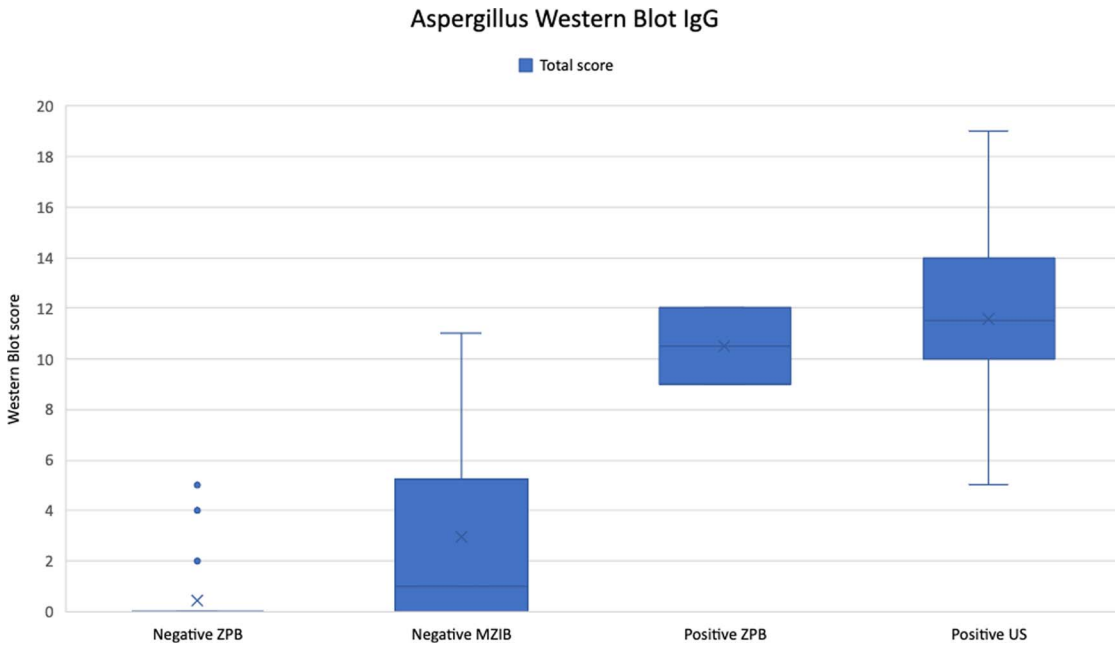

Figure 2. Box plot representation of Western Blot scores from the United States and France penguin samples. Results are grouped by clinical status, i.e. penguins considered positive or negative for aspergillosis. **ZPB, ZooParc de Beauval (France); MZIB, The Maryland Zoo in Baltimore.

Regardless of the country and institution of origin, unaffected penguins (median score 0, 95% CI [0–5], min 0, max 11) had lower scores than penguins with confirmed aspergillosis (median score 10.5, 95% CI [9–12], min 5, max 19, Fig. 2).

Two cases from the United States were examined through repeated measures. In case 1, a juvenile African penguin was found to be gliotoxin positive with a markedly abnormal electrophoretogram, including low A/G ratio (0.14), decreased albumin and moderate to marked increases in alpha 2, beta, and gamma globulins. At this timepoint (time 0), the cumulative immunoblot score was 11/16. On day 7, the same score was observed although the inflammatory changes in the electrophoresis had begun to resolve. On day 23, the cumulative score was 6. Throughout this period, this penguin was clinically normal and a previous immunoblot (day –111) showed no reactivity to any of the antigens. The penguin remained clinically normal for the following 16 months. In case 2, an approximately 1-yr old African penguin was started on antifungal treatment after the onset of clinical signs approximately 2 mon prior to the first testing by immunoblot. At this time, there were variable episodes with periods of no clinical signs, and the immunoblot score was 11/16. One month later the bird continued to have some respiratory signs and weight loss, and the score was 8/16. Three months after initial testing, while still on treatment and displaying some improvement, the score was 5/16. Clinical signs worsened 5 mon after initial testing, at which point the sample was gliotoxin positive and the score was 14/16. The penguin was euthanized 1 mon later, and aspergillosis was confirmed by postmortem culture and histopathology.

Highest WB scores (12 and 9) in the penguins from France were obtained for two animals (cases 3 and 4, respectively) that died of confirmed aspergillosis 1 and 3 d after sampling, respectively. Case 4 had also been tested 42 mon prior to death, with a WB score of 0.

DISCUSSION

This pilot study has documented the reactivity of penguins to various *Aspergillus* antigens in a commercially available WB with confirmed moderate to excellent specificity and sensitivity in humans and dolphins.[6,14] While the sample size of the study precluded the definition of a cutoff value for the cumulative reactivity scoring system, the use of the system defined in dolphins showed significant differences between results of clinically normal penguins versus those with confirmed or presumed aspergillosis. The specificity and sensitivity of the

assay would be presumed to be lower if the human system of the identification of two or more bands with reactivity had been implemented. Differences in species reactivity to the assay, and subsequently suggested cutoff values, could reflect various levels of exposure to *Aspergillus*, with animals being likely more exposed, when compared to humans. Similarly, great variability in cutoff values was reported for ELISA tests in human medicine, based on the control population.[15,16]

The present study included some penguin samples that reacted with other antigens (14 kDa, >30 kDa) that were not reported in the original report of this commercial assay or in the study of the application of this assay in bottlenose dolphins.[6,14] Bands above 30 kDa are often hidden due to reactions to nonspecific antigens. Interestingly, IgE isotype antibody reactivity to these novel antigens has been observed in humans with allergic bronchopulmonary aspergillosis, allowing their use to discriminate between allergic bronchopulmonary aspergillosis and IgE sensitization.[17] This disease is known for its severity in patients with asthma, cystic fibrosis, and chronic obstructive pulmonary disease, and treatment is often a combination of immune suppression and antifungal medications.[12] The avian IgG, better noted as IgY, is believed to be the precursor to mammalian IgG and IgE.[21] Among its effector functions is mediating anaphylactic reactions, so perhaps this broader reactivity on the immunoblot is reflective of this dual role of IgY.[9] It is important to note that the immune response of penguins to aspergillosis is distinct from that of mammals.[5]

Penguins from ZPB (France) showed lower scores when compared to penguins from US institutions. All but three of the ZPB negative samples showed a score of 0, while higher reactivity was noted in negative penguins from MZIB. These differences could be due to different environmental exposures in different institutions. Interestingly, environmental studies on *Aspergillus* contamination had already been conducted both in ZPB and MZIB. Environmental exposure was found to be higher in late summer and lower in winter and spring in both institutions, but contamination burdens between institutions cannot be compared due to different methodologies.[2,19] Therefore, differences in exposure to *Aspergillus* spores between French and US institutions could account for differences in reactivity patterns, both in negative and positive individuals. While no cutoff value to differentiate positive and negative individuals could be proposed based on our results, institution-specific cutoffs may be required. Also, the penguin populations studied between the two countries differed, both in terms of age and species: the ZPB population consisted mostly of adult Humboldt penguins, versus juvenile African penguins in MZIB, and different species were represented in the positive group from the US. Differences in species sensitivity to environmental exposure and/or species-specific reactivity patterns could explain the differences observed between the populations of this study. Additionally, there may be differences related to individual penguins based on immunogenetics, previous exposure and treatment, and current treatment protocols. To this latter point, preliminary data indicates that repeated measures of positive penguins may be reflective of clinical condition. In tandem with other measures (i.e., protein electrophoresis), additional studies may indicate that this new serological method could provide prognostic information.

While more extensive analysis of the application of the assay for clinical diagnosis cannot be made at this time, the study did include several samples that were notable. First, a juvenile penguin that remained clinically normal during infection showed seroconversion and decreasing reactivity (Case 1). It is proposed that this was the penguin's first exposure to this agent and a robust immune response was generated, negating any long-term issues or the need for treatment. In contrast, a similarly aged penguin showed clinical signs consistent with aspergillosis and received antifungal treatment for approximately 9 mon (Case 2). While clinical signs waxed and waned, the antibody reactivity did decrease to a low of 5/16 although rebounded to 14/16 approximately 1 mon prior to euthanasia. Consistently high reactivity was observed in other cases in which repeated measures were available. Case 4, from the French institution, is a good example of marked reactivity just prior to death due to confirmed aspergillosis in an individual that previously tested negative (with a score of 0) at the time of normal clinical status. Unfortunately, no other samples were available from this individual, and potential reactivity at a lower level during preclinical stages of the disease could not be evaluated.

There are several limitations to this study. First, the sample size and differences in populations preclude a thorough statistical analysis of the data. Second, there was no consistent procedure to evaluate the status of the animals for aspergillosis, which is a common limitation to studies on aspergillosis in penguins.[3,4,7,13,18] Most unaffected penguins were considered so based on clinical evaluation alone. In

some cases, other testing options (diagnostic imaging, including CT scan, or plasma protein electrophoresis, for example) were carried out. Positive penguins were considered positive based on postmortem evaluation, including but not limited to culture of the fungus from compatible lesions. Also, there was no consistency in timing of sample collection prior to death and necropsy in positive animals, as individuals were opportunistically sampled. Freezing temperature of the stored samples was different in the US and in France, and the assays were performed and read by different operators, which may have impacted the results. Lastly, evaluation of environmental fungal contamination was performed in some instances, but this was not necessarily concurrent with the timepoints that the penguins were sampled or showed clinical disease. The potential effect of high environmental exposure on WB reactivity pattern remains speculative at this point.

The completion of this preliminary study serves as proof of concept that the immunoblot methodology may have application in the diagnosis of aspergillosis in penguins. While labor intensive and expensive, the true value of this type of assay may be to better understand the humoral immune response of penguins to this fungal infection. It may be proposed that with further study of a wider group of samples, one or more immunodominant antigens may be identified. These antigens may then be the focus of ELISA or other immunoassays with lower expense and higher throughput to assess specificity and sensitivity in diagnosis and prognosis.

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