APPLICATION OF A NOVEL ASPERGILLUS LATERAL-FLOW DEVICE IN THE DIAGNOSIS OF ASPERGILLOSIS IN CAPTIVE GENTOO PENGUINS (PYGOSCELIS PAPUA PAPUA)

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APPLICATION OF A NOVEL *ASPERGILLUS* LATERAL-FLOW DEVICE IN THE DIAGNOSIS OF ASPERGILLOSIS IN CAPTIVE GENTOO PENGUINS (*PYGOSCELIS PAPUA PAPUA*)

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Abstract: Aspergillosis is the primary fungal disease affecting captive penguins globally. Its diagnosis remains challenging, and currently no tests are both sensitive and specific for the detection of early infection. The present study evaluated a recently developed *Aspergillus* lateral-flow device (AspLFD) for the detection of *Aspergillus* spp. antigen in plasma and glottis mucus from captive penguins. In a pilot retrospective study, banked frozen plasma samples from captive penguins were reviewed: samples from 11 gentoo penguins (*Pygoscelis papua papua*) and 4 king penguins (*Aptenodytes patagonicus*) fulfilled the inclusion criteria and were used in the analysis. Positive plasma AspLFD test results were found in 80% (four of five) of the aspergillosis-positive cases tested. All of the aspergillosis-negative cases tested negative (10 of 10) on the AspLFD test. In a cohort prospective study, paired plasma and glottis swab samples were opportunistically and nonrandomly collected from captive gentoo penguins. In total, 26 penguins were tested. In the negative control group, AspLFD test was negative on plasma and swab in 100% of birds (14 of 14). In the aspergillosis-positive group, AspLFD test was positive on plasma samples from 33% (4 of 12) of birds, on swab samples from 50% (6 of 12) of birds, and on either plasma or swab samples from 75% (9 of 12) of birds. The AspLFD is currently used for the diagnosis of aspergillosis in humans and also shows promise for use in penguins. Larger prospective studies are recommended.

INTRODUCTION

Aspergillosis is the primary fungal disease affecting penguins in captivity and is a significant threat to captive populations worldwide.[1,12,18,24] In penguins, early clinical signs of aspergillosis are frequently nonspecific, including anorexia, weight loss, self-isolation, and death.[11,28] Respiratory signs, such as open-beak breathing, coughing, and aphonia, are not always present.[3,11,28]

The diagnosis of *Aspergillus* spp. fungal infections remains challenging, and currently no tests are both highly sensitive and specific for the detection of early infection.[14,23] An antigen-based test for galactomannan (GM) is commercially available and widely used for the diagnosis of aspergillosis in birds.[8] In a survey conducted in numerous avian species, the test had a sensitivity of 67% and a specificity of 73% (with a cutoff index of 0.5).[8] Positive results in clinically normal individuals may occur due to test cross-reactivity or environmental GM exposure.[4,16] In addition, antifungal therapies may reduce GM test sensitivity, and the presence of antibody may react with circulating GM so that it is not detected.[4,29] The dichotomy between *Aspergillus* spp. antibodies and circulating GM is of particular importance in penguins, where high circulating antibody levels have been demonstrated, regardless of infection status.[4,7]

Recently, an *Aspergillus* lateral-flow device (AspLFD) has been made commercially available by OLM Diagnostics (Newcastle upon Tyne, NE4 5TF, United Kingdom).[26] The AspLFD test is a rapid immunochromatographic test for the qualitative detection of an extracellular glycoprotein antigen secreted during the active growth of the fungus.[26] The *Aspergillus*-specific point-of-care assay can be performed easily and allows quick results, which may allow timely initiation of antifungal therapy.[10] Several studies in humans, including multicenter studies and a meta-analysis, have suggested the usefulness of the AspLFD in diagnosing aspergillosis in serum and bronchoalveolar lavage (BAL) fluids.[10,17,31] To the authors' knowledge, the AspLFD has not been assessed in penguins.

In human medicine, BAL fluid samples are used to diagnose aspergillosis, in some cases, with better results than with serum samples.[25] In birds, standard respiratory sample collection techniques include tracheal and air sac lavages, both of which typically require anesthesia.[21] In penguins with aspergillosis, excessive mucus presence at the glottis has been reported.[3,30] Unlike tracheal and

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air sac lavages, collection of the mucus from the glottis can be performed in the conscious bird.[21] Thus, further evaluation of this noninvasively collected sample is warranted.

The present study evaluated the application of the AspLFD as an assay for aspergillosis detection in penguins. The hypotheses were that AspLFD test has the potential to detect *Aspergillus* spp. antigen in penguins and that it has a better performance than in plasma tested alone in diagnosing aspergillosis in gentoo penguins (*Pygoscelis papua papua*) if tested using both plasma and mucus from the glottis from the same penguin.

MATERIALS AND METHODS

Ethical approval

This study received ethical and welfare approval by the Royal Zoological Society of Scotland (RZSS) internal scientific working group. Plasma samples were available as surplus from blood collections performed during routine annual health examinations or diagnostic medical investigations; no blood was collected for the purpose of this study. For the prospective study, glottis swabs were collected at the same time as the aforementioned blood collections. Because of the noninvasive nature of the swab technique, no additional approval was determined to be required.

Retrospective pilot study

To evaluate the ability of the AspLFD test to detect *Aspergillus* spp. antigens in penguins, plasma samples banked at RZSS Edinburgh Zoo were reviewed. Plasma samples had been stored at –40°C and obtained from birds during routine or diagnostic medical examinations. The inclusion criteria were as follows: 1) sample from deceased penguin species; 2) available information on species, sex, age, and date of the blood collection; 3) available postmortem report; and 4) minimum volume of 0.3 ml. The birds were classified as belonging to a confirmed-aspergillosis group or to a negative control group, according to the gross postmortem and histopathological results. Samples from 11 gentoo penguins and 4 king penguins (*Aptenodytes patagonicus*) fulfilled the criteria and were used in the analysis.

Prospective study

After determining the test's efficacy through the analysis of retrospective samples, a cohort prospective study was conducted. Blood and swabs of mucus from the glottis (hereafter swab) were opportunistically and nonrandomly collected from the captive population of gentoo penguins at RZSS Edinburgh Zoo between November 2019 and October 2020. Sample collections occurred during routine annual health examinations or during diagnostic investigations of birds with clinical disease. For clinically diseased birds, samples were collected on the day that the clinical signs were first reported. For clinically normal birds, samples were collected in February 2020, during routine annual health examinations, and included vaccination, physical examination, and blood collection for routine analysis. At the same time, a swab was collected and banked.

Case details including signalment, clinical signs, ancillary testing (including plasma protein electrophoresis [PPE], hematology, biochemistry, and diagnostic imaging), response to antifungal treatment, and postmortem report (if applicable) were collected. The birds were categorized as control group or Aspergillosis group according to the following inclusion criteria.

Control group inclusion criteria: This group included clinically normal birds with no signs of disease and no history of antifungal treatment administered in the past 2 yr or deceased birds that died for reasons other than aspergillosis (postmortem confirmed noninfected). For antemortem cases, selected birds were followed for 12 mon after sampling and excluded if any signs of disease compatible with aspergillosis were observed.

Aspergillosis group inclusion criteria: This group included birds that 1) had begun to show clinical signs compatible with aspergillosis (such as anorexia, weight loss, cough, dyspnea, aphonia, increased mucus at the glottis, and sunken interclavicular air sac), 2) had not been diagnosed with a condition causing analogous signs, and 3) responded to antifungal treatment.

Once included in the aspergillosis group, the birds were classified as proven, probable, or possible cases of aspergillosis, to facilitate the interpretation of the robustness of the results. These classification were adapted from criteria used in human medicine and according to the European Organization for Research and Treatment of Cancer and the Mycoses Study Group 2008 guidelines.[20] This approach was adopted due to the absence of a reference standard for the diagnosis of aspergillosis; a similar method has been applied in other human and veterinary

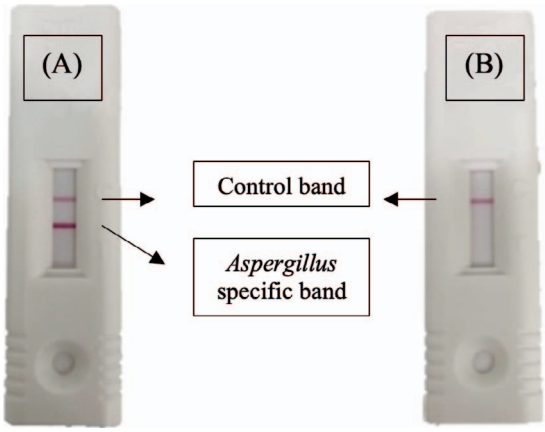

Figure 1. Example of a positive (**A**) and negative (**B**) AspLFD result in gentoo penguins (*Pygoscelis papua papua*).

medicine studies of aspergillosis.[7,9,10,25,31] Proven cases were based on histopathological evidence; probable cases had positive fungal cultures, corroborative imaging investigations, or both; and possible cases had responded to antifungal treatment, but had neither positive fungal cultures, histopathology results, nor imaging findings available. Other ancillary diagnostics, including hematology and PPE, were performed in most cases, but not used for case definition due to their nonspecific nature.

For sample collection, gentoo penguins were manually restrained for blood sample collection from the medial metatarsal vein by using an 1-ml syringe and a 25-ga needle. The blood was immediately transferred into lithium heparin blood tubes and gently agitated. Blood samples were centrifugated for 5 min at 3,000 *g*, and plasma was extracted.

Immediately following blood sampling, one swab (APTACA sterile transport swab, Tip rayon, Shaft plastic, 303/SG, APTACA Spa, Brescia, 10-25125, Italy) was introduced just beyond the glottis opening of the penguin and rotated one or two times (<2 s in duration). The swab was then transferred into an Eppendorf tube with 1 ml of 0.9% sodium chloride (NaCl) solution. Both plasma and the Eppendorf tube containing the swab were stored at –40°C until the analysis. The maximum time interval between storage and analysis was <6 mon.

AspLFD test

For the plasma samples, the AspLFD (OLM Diagnostics) was performed as follows: 250 µl of plasma was mixed with 110 µl of sample buffer. The solution was incubated for 8 min in a water bath at 100°C and then centrifuged for 5 min at 14,000 *g*. Next, 70 µl of the supernatant was transferred into the sample port of the cassette. The device was held at room temperature, and the results were read at 30 min. Results were determined as positive or negative as indicated in Figure 1.

For the swabs (previously placed in an Eppendorf tube with 1 ml of 0.9% NaCl solution), no pretreatment was required. After mixing the sample thoroughly, 70 µl of the NaCl solution was applied into the sample port of the cassette and the results were read at 30 min.

RESULTS

Retrospective pilot study

In total, 15 plasma samples from 15 birds were included and analyzed: 11 gentoo penguins and 4 king penguins, collected over an 11-yr period between 2008 and 2019. The confirmed-aspergillosis group contained five birds: four with acute invasive infections and one with chronic disease. The negative control group contained 10 birds.

Positive plasma AspLFD test results were found in four of the five (80%) confirmed-aspergillosis cases. One case of chronic aspergillosis produced a negative result on the AspLFD test. Ten of the ten negative control cases (100%) tested negative on the AspLFD test.

Prospective study

In total, 52 samples from 26 gentoo penguins were tested: 26 plasma samples and 26 swabs. Twelve birds were included in the Aspergillosis group and 14 in the control group. Table 1 presents signalment, clinical signs, tracheal swab culture and radiographic results, medication received at the time of sample collection, postmortem report (if applicable), fungal disease classification, and AspLFD results of the birds belonging to the aspergillosis group.

Diagnostic performance of AspLFD in the aspergillosis group (total and subgroups of proven, probable, and possible cases) and control group is presented in Table 2. In the control group, AspLFD was negative in all plasma (14 of 14) and swab (14 of 14) samples. In the aspergillosis group, AspLFD was positive in 33% (4 of 12) of plasma samples, 50% (6 of 12) of swab samples, and 75% (9 of 12) of birds (by plasma, sample, swab sample, or both).

Table 1. Details of the aspergillosis gentoo penguin (*Pygoscelis papua papua*) group.[a]

Case	Age	Sex	Clinical sign(s)	Tracheal swab culture results	Radiographic results	Medication at sampling time	PM result	Fungal disease classification	AspLFD results	
									Plasma	Swab
1	24 yr 0 mon	M	Lethargy, inappetence, SIA, SMG, wheeze (lung fields)	No growth	NA	None	NA	Possible	+	–
2	2 yr 6 mon	F	Anorexia	NA	Normal	None	NA	Possible	–	+
3	0 yr 2 mon	F	Loss of voice, weight loss, SMG, crackles (lung fields)	NA	NA	None	Asp	Proven	+	+
4	14 yr 1 mon	M	Lethargy, inappetence, SIA	No growth	NA	None	NA	Possible	–	–
5	13 yr 3 mon	F	Weight loss, SIA	NA	NA	None	NA	Possible	–	+
6	0 yr 1 mon	M	Weight loss, SMG, dyspnea	NA	NA	Itraconazole	Asp	Proven	+	–
7	13 yr 3 mon	M	Coughing	NA	NA	None	NA	Possible	–	–
8	12 yr 3 mon	M	Loss of voice, inappetence, SIA, dyspnea	NA	Thickened air sac membranes	None	NA	Probable	–	+
9	12 yr 3 mon	F	SIA, SMG	NA	NA	None	NA	Possible	+	–
10	11 yr 7 mon	M	Lethargy, inappetence, coughing, SIA, SMG	*Aspergillus* spp.	NA	Voriconazole	NA	Probable	–	+
11	29 yr 5 mon	F	Lethargy, SMG	No growth	Thickened air sac membranes	None	NA	Probable	–	–
12	16 yr 5 mon	M	Lethargy, anorexia, hoarse vocalizations	No growth	Thickened air sac membranes; hyperinflation of abdominal air sacs	None	NA	Probable	–	+

[a] PM, postmortem; M, male; SIA, sunken interclavicular air sac; SMG, stringing mucus around glottis; NA, not applicable; F, Female; +, positive; –, negative; Asp, aspergillosis.

Table 2. Diagnostic performance of AspLFD for diagnosis of proven + probable + possible aspergillosis in gentoo penguin (*Pygoscelis papua papua*) in plasma, swab, and plasma or swab.

Criteria for positivity	*n*	Positive on plasma (%)	Positive on swab (%)	Positive on plasma or swab (%)
Aspergillosis group	12	33	50	75
Proven cases	2	100	50	100
Probable cases	4	0	75	67
Possible cases	6	33	33	67
Control group	14	0	0	0

DISCUSSION

To the best of the authors' knowledge, there are no published studies analyzing the performance of the AspLFD test in avian medicine. Results from the retrospective pilot study, using banked plasma samples suggested that the AspLFD test may be valuable for the diagnosis of aspergillosis in penguins. However, those results were biased toward the most severely affected cases, that is, those that did not respond to antifungal treatment and died. As disease progresses via angioinvasion and dissemination, the chances of detecting circulating antigen may increase. In the confirmed-aspergillosis group, one false-negative AspLFD result was observed. The false-negative result was from a penguin that died from an intestinal foreign body, but was found to have two small chronic thick-walled granulomas in the right and left cranial thoracic air sac at the postmortem examination. It is theorized that the walled-off nature of the lesion may have prevented dissemination of fungal antigens.

The target of AspLFD is an antigenic mannoprotein released exclusively during the active growth of *Aspergillus* hyphae.[26,27] A meta-analysis of human studies showed that the AspLFD test had a sensitivity of diagnosing invasive aspergillosis of 86% in BAL samples and 68% in serum.[19] However, the performance of AspLFD has not been extensively validated in chronic pulmonary aspergillosis (CPA) and further studies are needed. In a retrospective study by Salzer et al., AspLFD showed a poor performance for diagnosing CPA in humans by using BAL, with a sensitivity below 10%.[22] Takazono et al. (2019), using a larger sample size, reported sensitivities of 62 and 66.7% in serum and BAL, respectively.[25]

In the prospective study, plasma AspLFD detected fewer positive cases than a meta-analysis of human studies (33% compared with 68%).[19] Compared with GM testing (the most commonly used antigen test in birds), plasma AspLFD results compared favorably to a large study in falcons (12% detection of positive cases when using a 1.0 index cutoff level), but were lower than detection rates reported in a study of multiple avian species when using a 0.5 index cutoff level (67%).[2,8]

Optimal performance in this study was achieved by combining the results of AspLFD in plasma and swab. Considering a positive case as any bird testing positive on either the plasma or swab sample, the test detected 75% of cases in the aspergillosis group, which is superior to studies of GM testing in avian medicine.[2,8,15] However, further studies with a larger sample size are needed to determine the preferred sample for AspLFD testing in penguins. Additional cases in the proven category would also increase the robustness of findings, although pratically this would require a more invasive diagnostic approach.

In human medicine, BAL has been widely used for the diagnosis of aspergillosis.[25,31] The present study showed that the AspLFD test was able to detect *Aspergillus* spp. antigens from the mucus of the glottis or trachea in gentoo penguins collected with a swab. Anecdotally, at the RZSS Edinburgh Zoo, the interclavicular air sac has been the location in the respiratory tract most frequently affected by aspergillosis lesions in gentoo penguins and the close anatomical location of the interclavicular air sac and trachea may have contributed to a better performance of AspLFD from swab and plasma samples compared with plasma alone. In addition, the test interference caused by circulating antibodies, which is seen in GM blood testing and may theoretically also occur with plasma AspLFD testing, would be avoided by using glottis or tracheal mucus samples. This could be of particular importance in penguins where high circulating antibody levels are common.[4,7]

An additional encouraging finding of this study was the absence of positive results in the control group. This suggests that each time a positive result is obtained, additional diagnostic testing is warranted and initiation of treatment should be considered.

An experimental study in guinea pigs (*Cavia porcellus*) showed that the sensitivity of AspLFD testing of serum samples was decreased as a result of antifungal agent exposure; however, BAL samples remained positive.[29] In the present study, Cases 6 and 10 were on antifungal treatment at the time of sample collection: one case had a positive result on plasma and the other case had a positive result on swab. According to the aforementioned study, it is possible that Case 10 had a negative plasma AspLFD test due to exposure to antifungal therapy; however, more studies are needed to confirm the effect of treatment on test performance in penguins.

In the present study, the majority of clinically abnormal penguins (aspergillosis group) presented with a depressed albumin-globulin (A-G) ratio and increased α-2-, β-, and γ-globulins on the PPE result. A previous retrospective study in African penguins (*Spheniscus demersus*) also showed a significantly decreased A-G ratio and increased

α-2-, β-, and γ-globulins in *Aspergillus*-diseased birds versus clinically normal penguins ($P < 0.05$).[9] In avian medicine, an increased β-globulin fraction, with compatible clinical data, supports the diagnosis of aspergillosis.[5,8,13] Although PPE is generally considered to have high sensitivity, normal PPE results can also be obtained in clinically or subclinically infected birds.[6] In Case 6, a 1-mon-old chick, the electrophoretic result was normal, despite the bird dying a few days later with a widespread aspergillosis infection. In this case, the plasma AspLFD result was positive. Because the AspLFD test is an antigen-based test, it does not rely on a competant immune response for detection of a positive case, which may offer an advantage in some cases.

This study has several limitations. First, the retrospective design used frozen plasma samples, stored at –40°C for a long period, which may have influenced the performance of the test. No published studies are available regarding the long-term stability of the mannoprotein antigen detected by the AspLFD, and it is possible that the chemical structure had degraded over time. The prospective design was implemented to remove this bias. A second limitation was the small sample size of the patient cohort and the lack of proven cases of aspergillosis. The absence of a reference standard for the diagnosis of aspergillosis is a major limitation for prospective cohort studies. Moreover, if only penguins with postmortem examination are included, a selection bias for the most severe cases of aspergillosis is inevitable; in clinical practice it is important to diagnose cases of aspergillosis early, to allow prompt initiation of therapy. Several studies in human and veterinary medicine have therefore used similar inclusion criteria to classify aspergillosis cases.[7,9,10,25,31]

The pretreatment step required for the plasma sample before using the AspLFD may provide an additional limitation for the test because it requires an equipped laboratory (to heat and centrifuge the sample). Nevertheless, the use of the AspLFD with the swab proved to be a fast (30 min), easy-to-perform point-of-care test.

CONCLUSION

The hypothesis that the AspLFD could be used to detect of *Aspergillus* spp. in penguins was confirmed. However, further studies are recommended to confirm the second hypothesis that the AspLFD test has a better performance for diagnosing aspergillosis in gentoo penguins if tested on both plasma and swab rather than plasma alone. The AspLFD test is currently used in the diagnosis of aspergillosis in human medicine and also shows promise in captive penguins. This is the first study of the AspLFD test in penguins and further larger prospective studies including comparison of AspLFD with GM and the levels of anti-*Aspergillus* antibody might improve understanding of the performance of this test.

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