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RH: HYDROXYBUTYRATE IN PENGUIN ASPERGILLOSIS

**APPLICATION OF 3-HYDROXYBUTYRATE MEASUREMENT AND PLASMA
PROTEIN ELECTROPHORESIS IN THE DIAGNOSIS OF ASPERGILLOSIS IN
5 AFRICAN PENGUINS (*SPHENISCUS DEMERSUS*)**

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25 **Abstract:** New alternative laboratory means are needed to improve the options for *ante-*
mortem diagnosis of avian aspergillosis. In this study, 3-hydroxybutyrate was measured in
plasma samples collected from a cohort of African penguins (*Spheniscus demersus*) maintained
under human care. Results were interpreted in combination with those of protein
electrophoresis and compared to anti-*Aspergillus* antibody and galactomannan antigen
30 detection. Overall, 3-hydroxybutyrate levels were found significantly increased in *Aspergillus-*
diseased cases *versus* control penguin groups ($P=0.002$). Mean absolute concentration of β -
globulins was increased $>20\%$ in samples from infected birds, and α_2 -globulins were also
found to be significantly increased *vs.* control groups ($P=0.001$). Consequently, the
combination of increased 3-hydroxybutyrate and increased α_2 -globulins did have a higher
35 specificity than both markers alone (98.4% *vs.* 78.6% and 92.2%, respectively; $P<0.05$).
Elevated 3-hydroxybutyrate levels combined with increases in α_2 -globulins or with any
electrophoresis abnormalities reflected a high negative predictive value of 79.9% or 86.7%,
respectively, *vs.* 64.7% when 3-hydroxybutyrate was considered alone ($P<0.05$). In contrast,
anti-*Aspergillus* antibody and galactomannan antigen did not distinguish between infected cases
40 and controls ($P>0.05$). This study demonstrates that basic testing in tandem with the new
biomarker 3-hydroxybutyrate may provide reliable evidence for the diagnosis of aspergillosis in
penguins.

Key words: *Aspergillus*, aspergillosis, electrophoresis, penguin, hydroxybutyrate, diagnostic
45 assay.

INTRODUCTION

Aspergillosis is a fungal airborne infection caused by ubiquitous molds of the *Aspergillus* genus. Aspergillosis can lead to fatal outcome both in diseased humans and animals, especially when the diagnosis is delayed and the antifungal therapy is inadequate.^{19,23} Some birds are known to be particularly susceptible to aspergillosis, including African penguins (*Spheniscus demersus*) which are maintained under human care in aquaria or during rehabilitation.⁹ Unfortunately, avian aspergillosis remains difficult to diagnose as current laboratory diagnostics do not have consistent high sensitivity and specificity. In penguins, samples are often found negative for galactomannan antigen detection,⁸ while most have high levels of anti-*Aspergillus* antibody reactivity regardless of clinical status.⁷ Biopsy sampling for fungal culture or histopathology can be difficult to perform in ill or debilitated patients. Furthermore, some medical imaging, *e.g.* celioscopy, is also not readily available in every facility, while radiography is often unreliable and rarely yields findings for aspergillosis in penguins.⁷

Veterinary practitioners currently depend on plasma protein electrophoresis (EPH) to address the diagnosis of aspergillosis and initiate preemptive antifungal treatment.^{3,8} Quantitation of serum proteins has been shown to provide a reflection of acute phase responses through the demonstration of change in albumin, a negative acute phase protein, and four globulin fractions which are often increased with different disease processes.¹² The diagnostic and prognostic application of data obtained from commercial semi-automated methods of protein fractionation has been well described in veterinary medicine.^{12,17} Thus, plasma electrophoresis has become more widely utilized in avian medicine over the last 20 years.^{2,4,5,20,25}

Proteomics and metabolomics are innovative tools that enable the *ex vivo* study of pathologic processes. Their use in research has previously defined possible new biomarkers of infection.^{10,11} Recently, a significant increase of some lipoproteins, fatty acids and ketone bodies including 3-hydroxybutyrate,²⁴ was highlighted in falcons with *Aspergillus fumigatus*

versus healthy birds,²¹ through proton nuclear magnetic resonance spectroscopy and robust multivariate analysis. Elevation of 3-hydroxybutyrate is believed to indicate behavioral and/or metabolic adjustments directed to spare energy and body protein during infection.⁸ It indirectly
75 reflects the need for activating fat mobilization.¹ In experimental studies, it was demonstrated to increase in some vertebrate species especially in birds,^{1,22} but comprehensive clinical trials are lacking. In the present study, the diagnostic value of 3-hydroxybutyrate measurement in a cohort of blood samples from African penguins that were undergoing aspergillosis was assessed with comparison and in combination with EPH.

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MATERIALS AND METHODS

Case definition. The definitive diagnosis of “proven” or “probable” fungal infection had been assigned according to the classification derived from the definition utilized in human medicine.⁹ “Proven” cases were based on histopathologic evidence ($n=7$) and “probable” cases had
85 positive fungal cultures in conjunction with clinical signalment and corroborative imaging investigations ($n=15$). Penguins with clinical signs of aspergillosis, but with neither positive fungal cultures / histopathology evidences nor imaging findings available, were considered “possible” diagnosis ($n=25$), if they responded to antifungal treatment.⁷ The control groups included penguins undergoing varied non-*Aspergillus* inflammatory diseases and clinically-
90 normal birds.

The ages of the clinical groups were as follows: *Aspergillus*-diseased, 8.5 +/- 3.9 years; inflammatory controls, 23.8 +/- 4.0 years; clinically normal controls, 8.4 +/- 3.8 years. The percentages of male patients in each group were 69.6%, 56.3%, and 51.6%, respectively.

Diagnostic testing. Blood samples were drawn from penguin flipper vein, then heparinized in
95 lithium tubes and centrifuged. If free from hemolysis and lipemia,³⁻⁶ plasma specimens were

refrigerated until analysis within 24 hours of submission to the laboratory.¹⁰ Otherwise, they were frozen at -80°C.

Detection of anti-*Aspergillus* antibody was conducted *via* a previously described ELISA technique using bulk *Aspergillus* ID antigens made of pooled mycelial-phase culture filtrates of
100 *Aspergillus* species (IMMY, Norman, OK, 73071, U.S.A.).⁷ Galactomannan antigen was measured in blood according to the manufacturers' recommendations (Bio-Rad Clinical Diagnostics, Hercules, CA, 94547, U.S.A.).^{3,7,8,13}

EPH results were obtained from the SPIFE 3000[®] electrophoresis analyzer (Helena Labs, Beaumont, TX, 77707, U.S.A.).⁵ They were subcategorized as abnormal on the basis of at least
105 a 10%-change (in absolute concentration) compared to the reference intervals of at least one protein fraction.

3-hydroxybutyrate measurement analysis was conducted using the RX Daytona+[®] instrument with Randox[®] reagents (Randox Laboratories, Kearneysville, WV, 25430, U.S.A.).

Statistical analysis. Methods validation was conducted according to prior recommendations.¹⁵
110 Statistical analysis was completed by using XLStat *Premium* v.18.07[®] software (Addinsoft, Paris, France). The *P*-values were obtained by the non-parametric Kruskal-Wallis test used herein for comparing three independent groups of different sample sizes. The guidelines of American Society for Veterinary Clinical Pathology for publication of clinical pathology data were followed.¹⁴ Reference intervals (RI) were calculated using MedCalc v.16.4.3 (MedCalc
115 software, 8400 Ostend, Belgium) according to parametric double-sided method for normal distribution with 90% confidence intervals.

RESULTS

Clinical group definition Characteristics of included penguins are listed in Table 1. Overall,
 120 172 blood samples were collected from naturally-infected cases ($n_1=47$) and from control
 penguins with inflammatory disease ($n_2=29$, inclusive of chronic arthritis, mild viral bronchitis,
 malaria, chronic anemia, and metastatic adenocarcinoma) or clinically-normal status ($n_3=96$, as
 reported by submitting institutions).

Clinical suspicion of aspergillosis was very strong in most diseased cases: 67.6% showed
 125 respiratory signs (*e.g.* dyspnea, wheezing, gasping, stridor, open-mouth breathing, coughing,
 changes in vocalizations), 11.8% neurological signs (*e.g.* ataxia, opisthophonos, torticollis, limb
 paresis, blindness, behavioural changes), and 76.5% general signs (*e.g.* lethargy, anorexia,
 weight loss, lack of appetite, sternal recumbency, regurgitation).

Antibody and antigen detection. The level of reactivity for anti-*Aspergillus* antibody was not
 130 different between the *Aspergillus*-diseased cases and the control groups ($P=0.76$): 92.3%
 69.2%, and 79.2%, samples expressed positive reactivity with an index ≥ 1.4 among
Aspergillus-diseased, non-*Aspergillus* inflammatory control and clinically-normal penguins,
 respectively.

There were also no significant differences between the penguin groups for the mean measure
 135 of galactomannan antigen ($P=0.14$, Table 1). For example, 23.1% *Aspergillus*-diseased birds
 had an antigen an index ≥ 0.7 , whereas 37.5% non-*Aspergillus* inflammatory controls had index
 ≥ 0.7 .

Plasma protein electrophoresis. The albumin/globulin (A/G) ratio, prealbumin concentration,
 and $\alpha 1$ -globulins were found to be significantly lower in the *Aspergillus*-diseased cases *vs.*
 140 clinically-normal penguins ($P<0.005$). In contrast, $\alpha 2$, β , and γ -globulins were significantly
 increased in *Aspergillus*-diseased birds *vs.* clinically-normal penguins ($P<0.005$) (Table 1).
 Notably, respective mean concentrations for the *Aspergillus*-diseased cases were not different

from those for the non-*Aspergillus* inflammatory controls, except for α 2-globulins ($1.13 \text{ g/dL} \pm 0.27$ vs. $0.86 \text{ g/dL} \pm 0.20$, $P < 0.05$). A typical EPH pattern for a clinically normal penguin and a
145 penguin with aspergillosis is shown in Figure 1.

Overall, significant changes were observed for A/G ratio in 66.7% and 58.6%, for pre-albumin in 25.6% and 36.4%, for albumin in 58.5% and 34.6%, for α 1-globulins in 7.3% and 13.6%, for α 2-globulins in 45.2% and 18.2%, for β -globulins in 57.1% and 48.1%, and for γ -globulins in 36.6% and 32.1% of samples from *Aspergillus*-diseased cases and non-*Aspergillus*
150 inflammatory controls, respectively. β -globulins reflected the largest increases in *Aspergillus*-diseased penguins (+20.4% compared to upper normal limit). EPH specificity to *Aspergillus*-disease was determined at 57.3% [CI 49.4 to 64.6] and 74.5% [CI 66.9 to 80.5], when ≥ 1 and ≥ 2 protein fraction(s) showed $>10\%$ significant change from normal ranges, respectively. However, elevated α 2-globulins demonstrated high specificity to aspergillosis at 92.2% [CI
155 86.0 to 95.3], when positive cutoff was set at 1.0 g/dL (but sensitivity was only 45.2% [CI 33.6 to 57.7]), and the positive (PPV) and negative predictive values (NVP) were at 70.4% [CI 55.9 to 84.8%] and 80.5% [CI 74.5 to 86.5%], respectively.

3-hydroxybutyrate measurement. The mean concentration of 3-hydroxybutyrate in penguin plasma was $1.33 \text{ mmol/L} \pm 0.59$ in *Aspergillus*-diseased cases (Table 1). It was significantly
160 higher than that observed in the two control groups, *i.e.* $0.94 \text{ mmol/L} \pm 0.13$ and $0.77 \text{ mmol/L} \pm 0.22$ for the clinically-normal and the non-*Aspergillus* inflammatory penguins, respectively ($P=0.002$) (Figure 2).

Receiving operator characteristic (ROC) curve established the lower positive cutoff at 0.60 mmol/L, but the specificity was not satisfactory (53.6%, [CI 35.8 to 70.4]), in spite of high
165 sensitivity (91.2%, [CI 77.8 to 95.9]). Thus, the upper positive cutoff was set at 0.94 mmol/L (sensitivity: 64.7%, [CI 50.3 to 76.0]; specificity: 78.6%, [CI 71.2 to 84.0]) (Table 2).

Diagnostic performance of combined tests. With the lower cutoff of 3-hydroxybutyrate (0.60 mmol/L) and at least one significant change >10% for any protein fraction in EPH, sensitivity and specificity of the combined test were assessed at 64.5% [CI 49.4 to 76.3] and 73.5% [CI 65.3 to 78.8], respectively. The NPV was high, *e.g.* 86.7%, [CI 80.1 to 92.9]. When the upper cutoff of 0.94 mmol/L was used with at least one significant change >10% for any protein fraction in EPH, the sensitivity and specificity were at 38.7% [CI 26.4 to 53.6] and 86.9% [CI 79.7 to 91.2]. The upper 3-hydroxybutyrate cutoff combined with α 2-globulins absolute concentration (with positive threshold at 1.0 g/dl), provided excellent overall specificity, *i.e.* 98.4% [CI 94.4 to 99.4], while NPV and PPV were both elevated at 79.9 % [CI 72.9 to 85.2] and 77.8 % [CI 55.0 to 1.00], respectively.

DISCUSSION

As with other animal species and humans, diagnosis of aspergillosis in birds remains difficult.⁴⁻⁶ *Ante-mortem* diagnostics display poor performance. As demonstrated in this study, serological assays are often not able to distinguish between controls and *Aspergillus*-diseased cases. In the current assay, the mean indices for anti-*Aspergillus* antibody detection were similar to those observed in 108 other penguins in a prior study.⁷

In contrast to psittacine electrophoretograms where β -globulins are the dominant globulin fraction in clinically-normal birds, the electrophoretogram of the penguin demonstrates a significant level of protein in the α 2-, as well as in β -globulins, and is a reflection of an increase in a myriad of positive acute phase proteins (Figure 1).^{4,12,17} The significant change in α 2-globulins in *Aspergillus*-diseased penguins is interesting and may reflect an increase in haptoglobin which may reflect a chronic inflammatory process (C. Cray, personal observation). Changes in γ -globulins would be consistent with the stimulation of humoral immunity. The α 1-

fraction remains poorly defined regardless of clinical condition. A prealbumin fraction is present as a shoulder migrating to the left of the albumin fraction. The clinical significance of prealbumin in birds is not been defined; however, prealbumin has been observed to decrease in falcons with naturally-acquired and experimentally-induced aspergillosis.^{18,26} In clinically-
195 abnormal penguins, a moderate decrease in percent albumin is often present; this is supportive of its designation as a negative acute phase protein. Importantly, EPH has been demonstrated to be the only valid method (*vs.* bromocresol green methods) for albumin quantitation.⁶

Plasma changes in 3-hydroxybutyrate concentration were hypothesized to have diagnostic relevance upon observation of increased concentration in experimentally-infected falcons.²¹ Its
200 elevation was assumed to reflect the need for energy activating during the infection.¹

In the current study, it was statistically determined in a cohort of *Aspergillus*-diseased penguins, with an elevated mean measurement at 1.33 mmol/L \pm 0.59. Notably, a special caution should be raised to the wide confidence interval of the results, suggesting important inter-individual variations, and to the age difference with penguins undergoing non-aspergillosis inflammatory
205 (the potential effects that age could play in the interpretation of results have not been clearly demonstrated). Anyway, in nine penguins with history of resolved *Aspergillus*-infection (diagnosis established 68 days \pm 14 earlier; 100.0% recovered when treated with antifungal drugs), the mean concentration for 3-hydroxybutyrate was low (0.86 mmol/L \pm 0.10). In contrast, a high value of 6.00 mmol/L was reported in a severely infected penguin three days
210 prior its death. Overall, concentrations >1.90 mmol/L were observed in penguins at the apparent peak of the *Aspergillus*-disease with significant clinical signs including hyper inflated cervical air sacs, coughing, wheezing and stridor, and strongly suggestive radiographic findings. These findings suggest a possible prognostic value of 3-hydroxybutyrate concentration, as levels return to normal when penguins recover in contrast to very elevated levels when clinical signs
215 of aspergillosis are severe. However, 3-hydroxybutyrate levels have been also studied in finches

adapting to winter conditions compared to levels measured in the summer: it was found to increase in fasting birds as well as in birds adapted to winter conditions, though stayed relatively static in fed birds in both summer and winter.² This suggests that the variations of 3-hydroxybutyrate may be also strongly linked to fasting and overall nutritional status/energy balance. So in order to improve its diagnostic performance, its measurement in blood perhaps requires concomitant additional analysis like comparison in tandem with EPH results. Indeed, combined tests are assumed to be theoretically able to overcome specificity limitations. Because EPH and 3-hydroxybutyrate assessment are two distinct tests, they have independent false-positive and false-negative rates that are unlikely to occur in the same study cases. The results showed that if both EPH fractions and 3-hydroxybutyrate levels were normal, the probability appeared high that the penguin was not infected with *Aspergillus* with NPV calculated at 86.7%. Furthermore, α 2-globulins demonstrated high specificity to aspergillosis at 92.2%, and it is the reason why its combination with the upper 3-hydroxybutyrate cutoff offered very elevated specificity at 98.4%.

In conclusion, this brief report underscores the potential utility of 3-hydroxybutyrate measurement for the initial diagnosis of aspergillosis in penguins especially when combined with routine testing like EPH. Further studies are warranted in order to confirm the diagnostic and prognostic benefits of 3-hydroxybutyrate in other penguin and avian species. For instance, it may be relevant to compare its measurements in subjects with well-controlled food intake to assert that its increases were not only due to these birds not eating because of severe infection.

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FIGURE LEGENDS

Figure 1: Typical electrophoretograms of a clinically normal (A) and *Aspergillus*-diseased African penguin (B). The fractions, from left to right, are prealbumin, albumin, α 1-, α 2-, β -, and γ -globulins.

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Figure 2: Boxplots showing distribution of 3-hydroxybutyrate measurement in samples collected from *Aspergillus*-diseased penguins (dark grey, $n=47$) and from control penguins undergoing miscellaneous non-*Aspergillus* inflammatory processes (light grey, $n=29$).