Applicability of the Platelia EIA® *Aspergillus* test for the diagnosis of aspergillosis in penguins


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(With 2 figures)

Abstract

Even today, an effective diagnostic test for aspergillosis in penguins is unknown, being the gold standard post-mortem examinations. The fungal antigen galactomannan (GM) has been used as a biomarker of disease in humans and is detected by the Platelia *Aspergillus* EIA (BioRad)*, a commercial kit based on the sandwich ELISA technique. It is standardized for use in neutropenic patients, however studies have demonstrated its usefulness also possible for birds. The aim of our study was to evaluate the effectiveness of Platelia *Aspergillus* EIA* test (BioRad-US) in the diagnosis of aspergillosis in Magellanic penguins, determining sensitivity, specificity, and positive and negative predictive values for different cut-off points. Were included in the study, blood serum samples (n = 29) Magellanic penguins in captivity that died by aspergillosis. Detection of GM was performed following manufacturer’s instructions and the GM index was obtained by dividing the average value of OD of the duplicate of the clinical sample by duplicate OD of the average value of the cut-off sample provided by the kit. Through information database results were obtained for the presence of anti-*Aspergillus fumigatus* antibodies detected by agar gel immunodiffusion (AGID) for all serum samples. Results were analyzed using chi-square test and Kruskal-Wallis from SPSS 20.0, IBM®. ROC curve was obtained and from this, rates of sensitivity, specificity, positive and negative predictive values were also calculated based on four different cutoff points (0.5, 1.0, 1.5 and 2.0). The serum GM index did not differ between animals of the case and control group (pKW=0.097). In determining the ROC curve for serum GM detection the value of area under the curve was 0.635. From the values determined by the coordinate of the curve, four different cut points (0.5, 1.0, 1.5 and 2.0) were analyzed, resulting in sensitivity rates ranging from 86.2 to 34.5% % and specificity between 87% and 26.1%. By comparing the serum GM index in group case as the presence or absence of antibodies detected by AGID was found p=0.503. The detection of GM the Platelia *Aspergillus* EIA* test seems is not be useful for the diagnosis of aspergillosis in naturally infected penguins.

Keywords: galactomannan, immunoenzimatic test, mycosis, birds.

Aplicabilidade do Platelia EIA® *Aspergillus* como teste diagnostico da aspergilose em pinguins

Resumo

Ainda hoje, um teste diagnóstico eficaz para aspergilose em pinguins não é conhecido, sendo o padrão-ouro os exames post-mortem. O antígeno fúngico galactomannana (GM) tem sido utilizado como biomarcador da doença em humanos, sendo detectado pelo Platelia *Aspergillus* EIA (BioRad)*, um kit comercial que se baseia na técnica ELISA sanduíche. É padronizado para utilização em pacientes neutropênicos, no entanto estudos tem demonstrado sua possível utilidade também para aves.O objetivo de nosso estudo foi avaliar a eficácia do teste Platelia *Aspergillus* EIA® (BioRad-US) no
diagnóstico da aspergilose em pinguins-de-Magalhães, determinando sensibilidade, especificidade e valores preditivos positivos e negativos em diferentes pontos de corte. Foram incluídas no estudo, amostras de soro sangüíneo (n=29) de pinguins-de-Magalhães em cativeiro que vieram a óbito por aspergilose. A detecção de GM foi realizada seguindo instruções do fabricante e o índice de GM foi obtido dividindo o valor da média da DO da duplicata da amostra clínica pelo valor da média da DO da duplicata da amostra de cut-off fornecida pelo kit. Através de informações em banco de dados foram obtidos resultados sobre a presença de anticorpos anti-Aspergillus fumigatus, detectada por Imunodifusão em gel de ágar (IDGA) em todas as amostras séricas. Os resultados foram analisados utilizando-se teste de qui-quadrado e Kruskal-Wallis a partir do programa estatístico SPSS 20.0, IBM®. Curva ROC foi obtida e a partir dela, taxas de sensibilidade, especificidade, valores preditivo positivo e negativo foram igualmente calculados considerando quatro diferentes pontos de corte (0.5, 1.0, 1.5 e 2.0). O índice de GM sérica não diferiu entre os animais do grupo caso e controle ($\chi^2 = 0.097$). Na determinação da curva ROC para detecção de GM sérica o valor da área sobre a curva foi de 0.635. A partir dos valores determinados pelas coordenadas da curva, quatro diferentes pontos de corte (0.5, 1.0, 1.5 e 2.0) foram analisados, resultando em taxas de sensibilidade variando de 86.2% a 34.5%, e de especificidade entre 87% e 26.1%. Ao comparar o índice de GM sérica nos animais do grupo caso quanto a presença ou não de anticorpos detectados pela IDGA foi encontrado $p=0.503$. A detecção de GM pelo teste Platelia Aspergillus EIA® não parece ser útil para o diagnóstico da aspergilose em pinguins naturalmente infectados.

_Palavras chave:_ galactomannana, teste imunoenzimático, micoses, aves.

1. Introduction

Aspergillus species have been known for decades as important pathogens of birds, leading to high mortality rates, including the Spheniscidae family, being about 90-95% of the cases of aspergillosis caused by Aspergillus section Fumigati (Cray et al., 2009b; Cabana, 2013; Xavier et al., 2011). The diagnosis ante-mortem of invasive aspergillosis (IA) in birds is limited and traditional techniques, such as blood tests, biochemical tests and imaging studies may reveal only nonspecific changes (Xavier et al., 2011; 2008; 2007).

Mycological classic tests have low sensitivity and/or specificity, and use of serological tests, although indicated in the literature (Cabana et al., 2015; Tell, 2005) it is not in routine use, as the gold standard is still restricted to histopathological and mycological post-mortem (Cray et al., 2009a, b).

Modern techniques for the diagnosis of aspergillosis by direct detection of antigen galactomannan (GM) in clinical samples have been increasingly used for the diagnosis of IA in humans from different species. The GM is a polysaccharide present in the fungal cell wall of the genus Aspergillus, a family of derivatives galactofuranose antigens. Their release into the bloodstream occurs during the growth of hyphae in tissue invasion process (Mennink-Kersten et al., 2004; Nucci and Colombo, 2012). This molecule can be considered as an important biomarker for the determination of invasive fungal infections by Aspergillus spp. in different clinical samples to be water soluble (Maertens et al., 2007; Xavier et al., 2011).

The Platelia Aspergillus EIA® (Bio-Rad USA) is a commercially available diagnostic kit which is based on sandwich ELISA for detection of galactomannan (Maertens et al., 2007; Xavier et al., 2011). This test is standard for blood serum and bronchoalveolar lavage of human and neutropenic patients, and when performed serially, anticipates the diagnosis of aspergillosis within one week. Some limitations are described, and the rates of false-negative and false-positive results fluctuate around 10% (Mennink-Kersten et al., 2004; Nucci and Colombo, 2012).

Although the ELISA sandwich test for GM detection is considered an important diagnostic tool for AI in humans. Studies have shown that can also contribute to the diagnosis of other species such as dogs, cattle and poultry (Arca-Ruibal et al., 2006; Billen et al., 2009; Cray et al., 2009a; Franca et al., 2012; Garcia et al., 2001; Garcia et al., 2008; Guillot et al., 1999; Jones and Orosz, 2000; Nucci and Colombo, 2012; Xavier et al., 2011). However, there are protocols and indications for its use in penguins.

Due to the high incidence of aspergillosis in penguins determining high mortality rates in these animals in captivity as well as the difficulty of the definitive diagnosis of ante-mortem disease in this species, this study aim to evaluate the effective of Platelia Aspergillus EIA® test (Bio-Rad-US) the diagnosis of aspergillosis in naturally infected Magellanic penguins, determining sensitivity, specificity, and positive and negative predictive values for different cutoff points.

2. Material and Methods

They were included in the study, blood serum samples of Magellanic penguins that died of aspergillosis during the rehabilitation period in the Centro de Recuperação de Animais Marinhos of Rio Grande - CRAM-FURG (n=29). The samples are stored in the mycology laboratory - FAMED-FURG, and all cases were confirmed from post-mortem examinations with mycological culture and histopathological examination. As a control group, were included over 23 serum samples from healthy Penguins CRAM-FURG, which they were rehabilitated and released to their natural habitat. All samples were aliquoted in biosafety cabinet to prevent contamination by airborne conidia and found themselves stored at -20 ° C.
A single blood sample each animal was included and collected from venipuncture of the cephalic vein. All samples were obtained in a maximum period of 60 days (7-69) before death (case group) and/or release (control).

The GM detection was performed on all serum samples animals included in the study according to the manufacturer’s instructions. In brief, 300ul of sample was added to 100ul of Platelia Aspergillus EIA® treatment solution into microtubes and subsequently arranged in the thermoblock for heat treatment for six minutes at 120° C. Then the wells were centrifuged at 10,000xg for 10 minutes. In sequence the strips were filled with 50ul conjugate and the sample supernatant and then the plate was incubated for 90 minutes at 37° C. Then the wells were centrifuged at 10,000xg for 10 minutes. In sequence the strips were filled with 50ul conjugate and the sample supernatant and then the plate was incubated for 90 minutes at 37° C. The reaction was terminated by addition of 1.5 N solution of sulfuric acid and reading the optical density (OD) at 450 nm with 620 nm reference filter. The tests were conducted in duplicate and reactions were all positive control samples used, and the negative cutoff point provided by the kits diagnostic. The GM index was obtained by dividing the average value of OD of the duplicate of the clinical sample by duplicate OD of the average value of the cut-off sample provided by the kit.

From the database Laboratório de Micologia, It was obtained information about the presence of anti-Aspergillus fumigatus antibodies detected by agar gel immunodiffusion (AGID) for all serum samples.

Results were analyzed using chi-square test and Kruskal-Wallis from SPSS 20.0, IBM®. ROC curve was obtained and from this, rates of sensitivity, specificity, positive and negative predictive values were also calculated based on four different cutoff points (0.5, 1.0, 1.5 e 2.0).

3. Results

The serum GM index did not differ between animals in the case group and control ($p_{KW} = 0.097$). The penguins with aspergillosis (n = 29) GM index ranged from 0.29 to 6.5, with a median of 0.91 and mean of 1.71, while the healthy animals (n = 23) the median was 0.75 and 1.13 average (varying from 0.31 to 4.83) (Figure 1).

From these values it was determined the ROC (Receiver Operating Characteristic) for serum GM detection in the diagnosis of aspergillosis in penguins, where the x-axis (x) is the true positive (sensitivity) and the ordinate axis (y) is false positive (1 - specificity) (Figure 2). The analysis of the test demonstrated a precision index value of the area under the curve of 0.635 (0.482 to 0.788 CI) ($p = 0.097$).

From the values determined by the coordinate of the curve, four different cut points (0.5, 1.0, 1.5 and 2.0) were analyzed, resulting in sensitivity rates ranging from 86.2% to 34.5% and specificity between 91.3% and 26.1% (Table 1).

Of the 52 animals studied, only four had positive IGA, all of them belonging to the group case. Comparing the serum GM index in group case as the presence or absence of antibodies detected by AGID was no significant difference ($p = 0.503$), mean and median values of 2.59 and 1.76 (± 2.80), respectively, in animals with positive AGID, and 1.36 and 0.89 (± 1.20), respectively, in animals with antibodies to A. fumigatus.

4. Discussion

This study evaluated for the first time the applicability of the commercial Aspergillus EIA® Platelia kit for the diagnosis of aspergillosis in naturally infected penguins using different cut-off.

Table 1. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of serum GM detection in the diagnosis of aspergillosis in naturally infected penguins using different cut-off.

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 2.0</td>
<td>34.5</td>
<td>91.3</td>
<td>76</td>
<td>51</td>
</tr>
<tr>
<td>≥ 1.5</td>
<td>41.4</td>
<td>82.6</td>
<td>75</td>
<td>47</td>
</tr>
<tr>
<td>≥ 1.0</td>
<td>44.8</td>
<td>60.9</td>
<td>60</td>
<td>51</td>
</tr>
<tr>
<td>≥ 0.5</td>
<td>86.2</td>
<td>26.1</td>
<td>59</td>
<td>40</td>
</tr>
</tbody>
</table>

Figure 1. Serum GM indices in healthy penguins (0) and aspergillosis (1).

Figure 2. ROC curve of serum GM detection to diagnosis of aspergillosis in penguins.
between animals with and without the disease. A single study the this population with a similar was described by Cray et al. (2009b), however the authors included 56 birds with aspergillosis, of which only three were penguins, it is not possible to extrapolate the test efficacy results obtained by the authors for Sphenisciformes family.

In domestic and wild birds this different families and orders, including some species of raptors, Psittaciformes, Anseriformes and Galliformes. Described studies regarding the effectiveness of GM detection to diagnosis of aspergillosis, and demonstrate sensitivity rates ranging from 12 to 67% and specificity ranging 73 and 95% using 0.5 as the cutoff point (Arca-Ruibal et al., 2006; Cray et al., 2009a, b; Dhama et al., 2013; Franca et al., 2012; Fischer et al., 2014). These results do not match those found in our study with penguins, when considering this same cutoff value was detected high sensitivity rate (86.2%) but low specificity (26.1%). Similar rates of the authors mentioned above were found in our study only using a cutoff point four times (2.0), in this case the sensitivity was 34.5% and specificity of 91.3%.

Factors such as different animals species included in the studies, duration and development of aspergillosis in these birds, as well as clinical presentation of the disease (infection site) and immune response of different species of birds (Deem, 2003; Tell, 2005) may be related to conflicting results found in our study compared to others in the literature.

The high rate of false-positive results found in our study may be related to colonization of the respiratory tract by Aspergillus species Fumigati section, exposure to environmental strains or mainly to the presence of a cross-reactive antigen has not been elucidated, which is also suggested by other authors (Le Loch et al., 2005). On the other hand, false negative rates in the Platelia Aspergillus EIA® are generally related to encapsulation of the infection, immunocomplex formation by anti-Aspergillus antibodies or prior exposure to antifungal agents (for prophylaxis) (Xavier et al., 2011). However, none of these hypotheses can be extrapolated to our study, whereas all penguins with aspergillosis included had lesions spread through the respiratory tract to the post-mortem examination, not characterizing frame encapsulation of the infection and were not in antifungal treatment, moreover, no significant difference in the GM results comparing animals with and without antibodies to Aspergillus sp. (PKW = 0.449).

However, the interference factors in the test for this animal species (penguins) are not well been elucidated, as well as other animals (Arca-Ruibal et al., 2006; Fischer et al., 2014). In attempt to reduce the false-positive rate, authors recommend testing in at least two serum collections (Arca-Ruibal et al., 2006; Cray et al., 2009a, b; Verweij et al., 1995). In our study, only one clinical sample per animal was included, being this a limitation, in that it was not possible to evaluate the test results when performed as serial monitoring of serum levels of GM penguins.

Our results show that serum GM detection by Platelia Aspergillus EIA® does not seem to be useful for the diagnosis of aspergillosis in naturally infected penguins, with high rates of false-positive results with cut-off 0.5 (indicated by the manufacturer) and false negatives in high cut-off.

References


