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Maternal Transfer of Anti-*Aspergillus* spp. Immunoglobulins in African Black-footed Penguins (*Spheniscus demersus*)

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ABSTRACT: Anti-*Aspergillus* spp. antibody (Ab) levels of seven mating pairs of captive African penguins (*Spheniscus demersus*) and the maternal Ab levels of their embryonated and unembryonated eggs were determined by ELISA. The egg Ab levels were significantly higher than the Ab levels of their female or male parents. Maternal Ab of unembryonated eggs and Ab of the embryos were significantly correlated with the Ab levels of their female parents. Because eggs can be sampled where adult penguins are not accessible, this technique can be applied for prediction of *Aspergillus* spp. Ab titers of penguin females in wild populations.

Key words: Maternal antibodies, African penguins, *Spheniscus demersus*, *Aspergillus* spp., aspergillosis, ELISA.

The fungi, *Aspergillus* spp., cause significant mortality in birds (Redig et al., 1993). Aspergillosis is the most important air-borne disease of captive indoor penguins (Redig et al., 1993); however, serological studies on these pathogens in penguins do not exist. The egg-yolk transfer of maternal antibodies (MAb) was described in ducklings (*Anas platyrhynchos*) (Fraser et al., 1934) and in chickens (Schmidt et al., 1989). The maternal transfer of immunoglobulins against *Plasmodium* spp. in African penguins, (*Spheniscus demersus*) was described previously (Graczyk et al., 1994a). Development of rabbit-anti-*S. demersus* immunoglobulin G (IgG) labeled with alkaline phosphatase for the use in the enzyme-linked immunosorbent assay (ELISA) (Graczyk et al., 1994b) facilitates studies on humoral responses of penguins to various pathogens. Our objective was to determine whether the passage of MAb against *Aspergillus* spp. exists in African penguins and, if so, to characterize this phenomenon.

Seven pairs of adult (3- to 18-yr-old), captive *S. demersus* from the Baltimore Zoo, (Baltimore, Maryland, USA) were used. The penguins were maintained on the same diet (Stoskopf et al., 1980). The sex of birds was known from the previous year's breeding records. Each pair of birds produced two eggs. The second egg was laid within 3 days after the first one (Beall et al., 1994). The female and male of each pair were bled on the day of laying the first egg. The collection and processing of the blood samples for ELISA followed previously described protocol (Graczyk et al., 1994b). According to the directive of the Species Survival Plan (Beall et al., 1994) on reduction of the population of captive African penguins in the USA, 14 eggs were removed from the colony. Four of the eggs, 4-wk-old at the time of introduction of the restriction, were embryonated; the remaining 10 eggs were unembryonated. The yolks of unembryonated eggs were biopsed (Schmittle, 1950), and treated prior to the ELISA as described by Graczyk et al. (1994a). Four embryonated eggs were opened longitudinally, and the yolk-sacs and heart of embryos were sampled. The samples, taken from the egg-yolk, embryos, and the adult penguins were analyzed on the same ELISA plate (Dynatech Laboratories, Inc., Chantilly, Virginia, USA).

Cell wall galactoman and glucan were removed from a purified, alcohol-precipitated, mycelial antigen of *A. fumigatus*, *A. niger*, and *A. flavus* (Meridian Diagnostics, Inc., Cincinnati, Ohio, USA) by Concanavalin A-Sepharose 4B (Sigma Chemical Company, St. Louis, Missouri, USA) affinity column (de Repentigny et al., 1991). Components of the antigen were separated by SDS-PAGE and silver stained (De Repentigny et al., 1991). The 55 kilodalton (kDa) antigen (Fig. 1) was electro-

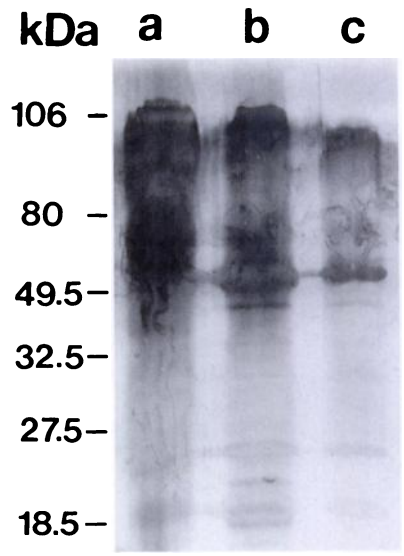

FIGURE 1. SDS-PAGE analysis of a purified, alcohol-precipitated, mycelial antigen of *Aspergillus fumigatus*, *A. niger*, and *A. flavus* stained with silver nitrate. Total mycelial homogenate (lane A) was purified one time (lane b) and two times (lane c) by Concanavalin A-Sepharose 4B chromatography. Molecular mass standard in kilodaltons (kDa).

eluted (Electro-Eluter M422, Bio-Rad Laboratories, Richmond, California, USA) and used to coat of ELISA plate. Protein concentration was determined as described by Graczyk et al. (1993). The wells were coated in triplicate with 100 μl (5.0 μg/ml) of antigen diluted with phosphate-buffered saline (PBS) (pH 7.4), and incubated for 3 hr at 41 C. Eight wells were not coated to determine nonspecific background values of absorbance. The plate was post-coated (Graczyk et al., 1993), and the wells were emptied, filled in triplicate with 100 μl of test sera and yolk samples diluted with PBS (1/100), and incubated for 3 hr at 41 C. Two negative control sera were used. The first was obtained by precipitation of the immunoglobulins and immunocomplexes from a pool of 14 penguin sera (Johnson et al., 1989). The second was a pool of the sera from four wild *S. demersus* from Boulders (33°26'S, 17°45'E) at Simons Town, The Republic of South Africa. The air-dried sera from the wild penguins were stored and shipped on filter paper. Filter paper storage does not diminish Ab binding capacity (Graczyk et al., 1993). The positive control serum, collected dried on filter paper, originated from captive *S. demersus* that died due to clinical aspergillosis. Four wells were filled with 100 μl of each of negative control serum, and eight wells were filled with 100 μl of positive control serum. The remained ELISA protocol followed the procedure of Graczyk et al. (1994b).

Analysis of variance (ANOVA) was used to determine the significance of the among-penguin-group effect (Sokal and Rohlf, 1981). The degree of linear association between Ab titers was compared using Pearson's correlation coefficient (r), and coefficient of determination (R), where $R = r^2 \times 100\%$ (Sokal and Rohlf, 1981).

The range of absorbance values for positive control serum was 0.61 to 0.73 with the mean ± SE of 0.66 ± 0.01. The two negative control sera had a similar range of absorbance, 0.10 to 0.12 with the mean of 0.11 ± 0.01 and the cutoff level of 0.14. Low nonspecific background values of absorbance (< 0.06) was observed.

All 14 eggs, and 12 of 14 penguins were positive for anti-*Aspergillus* spp. immunoglobulins. Absorbances of two males did not reach the cutoff levels. The levels of MAb of the unembryonated eggs were significantly higher than those in their female or male parents (ANOVA; $F = 27.11$, $P < 0.01$), and in the yolk-sacs of embryonated eggs (ANOVA; $F = 71.42$, $P < 0.01$) (Table 1). The levels of MAb of the unembryonated eggs were correlated significantly ($r = 0.87$, $F = 43.10$, $P < 0.01$) with Ab levels detected in their female parents. Based on a coefficient of determination, the unembryonated egg MAb levels was determined in females at 77%, and in males by 3%. The levels of MAb found in the yolk-sacs of the 4-wk-old embryos were non significantly correlated either with female or male parents ($r = 0.61$, $F = 3.78$, $P = 0.19$). The serum MAb levels of these embryos were significantly correlated with the Ab levels detected in their female parents (r

TABLE 1. Absorbance values obtained in the enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Aspergillus* spp. immunoglobulins in the mating pairs of adult African black-footed penguins (*Spheniscus demersus*) and their relative eggs.

	Unembryonated eggs			Embryonated eggs			
				4-wk-old embryos			
	Egg yolks (n = 10)	Female parents (n = 5)	Male parents (n = 5)	Yolk sacs (n = 4)	Serum (n = 4)	Female parents (n = 2)	Male parents (n = 2)
Mean	0.712(A,B,C)*	0.442(A)	0.313(B)	0.320(C)	0.724	0.557(B)	0.377(B)
SD	0.098	0.088	0.181	0.029	0.109	0.124	0.049
Range	0.497	0.325	0.120	0.301	0.594	0.445	0.321
	0.802	0.618	0.598	0.329	0.832	0.681	0.428

* Mean values followed by the same letter differed significantly ($P < 0.01$) with an analysis of variance.

= 0.93, $F = 102.81$, $P < 0.01$); MAb found in the serum of the 4-wk-old embryos was determined at 86% in the females, and at 16% in the males. The Ab levels were used to fit the binomial regression curves describing the level of MAb as penguin-female dependent; $y = 0.25 + 1.02x$ for unembryonated eggs, and $y = 0.29 + 0.83x$ for embryonated eggs, where x is the Ab level of the females, and y is the MAb in their relative eggs.

Maternal or parental Ab passages in birds can be achieved through egg-yolk or crop-milk (Rose and Orleans, 1981). The high level of *Aspergillus* spp. Ab in the yolk, and the significant correlation between Ab levels of the penguin females and their eggs, are evidence that *Aspergillus* spp. Ab were transmitted prenatally. Maternal Ab is transmitted from the yolk to the embryo via the vitelline and hepatic portal circulation (Rose and Orleans, 1981), and most maternal Ab passes from the yolk-sacs to the embryo circulation during the last 5 to 6 days of embryonic development (Rose and Orleans, 1981). A marked increment of uptake of Ab by embryos occurred during the last 4 days of chick development (Buxton, 1952). In chicks, transport of the yolk Ab across the yolk-sac splanchnopleure into the fetal circulation increases 3 days before hatching (Kowalczyk et al., 1985). The mean period of embryonic development in *S. demersus* is 38 days (Beall et al., 1994). Thus, the four, 4-wk-old embryos were dissected approximately 8 to 10 days before hatching, and at this stage most of the MAb already were transferred to the fetal circulation.

Maternal Ab protected poults against hemorrhagic enteritis (Fadly and Nazerian, 1989), avian leukosis virus (Fadly and Smith, 1991) and infectious bursal disease virus (IBDV) (Homer et al., 1992). Maternal Ab persists in the chicks up to 2 mo post-hatching (Rose and Orleans, 1981); MAb against *Plasmodium* spp. were detectable in African penguins up to 8 wk post-hatching (Graczyk et al., 1994a). Studies on the relationship between MAb titer to *Aspergillus* spp. in African penguins and the resistance of the birds to these pathogens do not exist. However, for the period of 1989 to 1994, aspergillosis was not observed in *S. demersus* younger than 4 wk despite the fact that the hatchlings were kept indoors, which enhances the risk of infection (Redig et al., 1993). This is evidence that MAb against *Aspergillus* spp. provided protection of *S. demersus* chicks against *Aspergillus* spp. during the first 4 wk PH.

There was no difference in the absorbance values between the negative control obtained by heating of the serum with citric acid, and the serum from the uninfected wild *S. demersus*. Thus, when unexposed penguin serum is unavailable, the

ELISA can utilize the negative control generated from *Aspergillus* spp.-positive serum.

The results of the present study support the observation in poults that egg-yolk Ab load reflects serum Ab load of the hens that laid these eggs (Brown et al., 1989). This phenomenon was used to predict serum Ab titer of hens against IBDV based on the egg-yolk samples (Brown et al., 1989). The restriction of the Species Survival Plan on reduction of the population of captive African penguins in the USA, ensures that more *S. demersus* eggs will be removed from the hatcheries. Thus, the penguin eggs, can be used for the prediction of female penguin Ab titer against avian malaria parasites (Graczyk et al., 1994a) and *Aspergillus* spp. Because eggs can be sampled where adult penguins are not accessible, this technique can be applied for prediction of *Plasmodium* spp. and *Aspergillus* spp. Ab titers of penguin females in wild populations.

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