Detection of Avian Malaria Infections in Wild and Captive Penguins

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ABSTRACT: Sera from wild African black-footed penguins (Spheniscus demersus L., 1758), Adelie penguins (Pygoscelis adeliae Houbron, 1841), Gentoo penguins (Pygoscelis papua Forster, 1781), king penguins (Aptenodytes patagonicus Miller, 1778), and little blue penguins (Eudyptula minor Forster, 1781) and from captive yellow-eyed penguins (Megadyptes antipodes Houbron, 1841) and Magellanic penguins (Spheniscus magellanicus Forster, 1781) were tested by enzyme-linked immunosorbent assays for the presence of avian malaria antibodies (Ab). Plasmodium falciparum sporozoite (R32tet1,) and gametocyte (P.F.R27) antigens were used. Specificity of anti-S. demersus, anti-duck, anti-chicken, and anti-turkey IgG labeled with alkaline phosphatase was determined for homologous and heterologous sera of 8 avian species (including 6 penguin species). The penguin conjugate was the most specific for the various penguin species immunoglobulins. It was possible to detect penguin immunoglobulins at a dilution of 10^{-4}. The relative binding of anti-S. demersus IgG was equal to relative binding of commercial conjugates. Kinetic profiles and overall magnitudes of malarial Ab detected by the 2 antigens were not significantly different. Antarctic P. adeliae were negative for malarial Ab, all New Zealand M. antipodes were positive, and the positivity prevalence of the remaining penguins ranged from 33 to 92%. Antibody titers and the prevalence of infection of wild S. demersus were significantly lower than those reported for captive North American S. demersus.

KEY WORDS: avian malaria, penguins, Plasmodium relictum, Plasmodium elongatum, ELISA, New Zealand, Antarctic

The African black-footed penguin, Spheniscus demersus, is an endangered species. Populations have been drastically decreasing along the southern coast of the Republic of South Africa (RSA) (Crawford et al., 1990) due to oil contamination, injuries, and diseases (Brossy, 1992). The first avian malaria case (Plasmodium relictum) in a penguin was discovered in S. demersus in 1927 (Fantham and Porter, 1944) from Saldanha Bay (32°26'S, 17°45'E) in the RSA. Later, the parasite was found in captive S. demersus in Europe, and the disease was associated with infected Culex pipiens mosquitoes (Rodhain, 1937). Over 60 yr later, Brossy (1992) reported a 0.7% prevalence of P. relictum infection in S. demersus from Saldanha Bay (RSA) and a markedly higher prevalence (22%) (with fatal outcome) in injured or oiled penguins along the southern coast of the RSA.

The malaria-related mortality of S. demersus in a North American Zoo (Baltimore, Maryland, U.S.A.) fluctuated between 75% (Stoskopf and Beier, 1979) and 50% (Cranfield et al., 1990). Recently, Cranfield et al. (1994) demonstrated that avian malaria infections, once acquired, last for the duration of the penguin’s lifetime. In the wild, or during transport from the natural habitat to captivity (i.e., from the Southern to Northern Hemisphere), such birds may die. This raised the question of whether mortality was caused by a newly acquired infection or recrudescence of a preexisting one. A high, posttransport, malaria-related mortality of nonparasitemic wild-caught penguins was reported by Fix et al. (1988). This problem has remained controversial because the results were based on the examination of blood smears. This technique determines only the prevalence of parasitemia, not the actual prevalence of infection.

The enzyme-linked immunosorbent assay (ELISA) developed for the diagnosis of avian...
malaria in captive *S. demersus* facilitates the evaluation of exposure of individual birds to parasites (Graczyk et al., 1994c). The assay utilizes anti- 

*S. demersus* IgG labeled with alkaline phosphatase. The purpose of the present study was to determine the applicability of this conjugate for the detection of immunoglobulins directed against avian malarial parasites in various species of penguins.

**Materials and Methods**

Blood samples were collected from 44 wild African black-footed penguins (*S. demersus*) at Boulders, Simon's Town Colony (33°26'S, 17°45'E), RSA; from 12 Gento o penguins (*Pygoscelis papua*) and 12 king penguins (*Aptenodytes patagonicus*) from Kerguelen (49°15'S, 70°10'E) and Crozet islands (46°21'S, 57°32'E), French Subantarctic Territories; from 5 yellow-eyed penguins (*Megadyptes antipodes*) from Dudenin (46°05'S, 17°23'E), New Zealand (NZ); and from 5 Adelie penguins (*Pygoscelis adeliae*) at Cape Birds, Ross Island (77°13'S, 166°29'E), Antarctica. Captive bird collection included samples from 12 little blue penguins (*Eudyptula minor*) from Napier Zoo (39°30'S, 176°40'E), Napier, NZ; 7 Magellanic penguins (*Spheniscus magellanicus*) from Sea World of California (32°40'N, 117°12'W), San Diego, U.S.A.; and 9 *S. demersus* from the Baltimore Zoo (39°21'N, 76°34'W), Baltimore, Maryland, U.S.A. All birds were adult. The blood was collected by heparinized syringe venipuncture from the jugular vein or brachial vein, centrifuged (1,200 × g, 10 min), and the plasma stored, air-dried, on filter paper as described in Graczyk et al. (1993).

To determine the specificity of anti- 

*S. demersus* IgG labeled with alkaline phosphatase to homologous and heterologous sera (6 penguin species, duck, chicken, turkey), a direct ELISA was performed according to the protocol of Graczyk et al. (1994c). The air-dried samples were eluted into buffer (Graczyk et al., 1994c) and a pooled sample for each species was prepared with 200 μl of the eluate from individual specimens. The 6 penguin serum pools were used at 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200, 1/6,400, and 1/12,800 dilutions in triplicate to coat the ELISA plate. The relative binding of anti- 

*S. demersus* conjugate was compared to anti-chicken IgG (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), anti-duck IgG, and anti-turkey IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, U.S.A.); all ligands were labeled with alkaline phosphatase. Human serum was used as a negative control (NC). The remaining eluate from an individual penguin sample (800 μl) was used for the indirect ELISA (Graczyk et al., 1994c) to determine the presence of anti- 

*Plasmodium* spp. immunoglobulins. Two antigens of *Plasmodium falciparum* were used: R32tet13 and P.F.R27. Immunoglobulins directed against *P. relictum* and *P. elongatum* recognized these antigens (Graczyk et al., 1993). These immunoglobulins did not cross-react with antigens from another avian hemoparasitic blood parasite, *Haemoproteus columbae* (Graczyk et al., 1994b), and anti- 

*H. columbae* immunoglobulins did not cross-react with R32tet13 or P.F.R27 antigens (Graczyk et al., 1994b). Additionally, sera of *S. demersus* infected with *Babesia* sp., as determined by Giemsa-stained thin blood smear, gave an ELISA-negative reaction with R32tet13 or P.F.R27 antigens (Graczyk et al., unpubl.). Pooled serum from 4 3-mo-old *S. demersus* chicks housed in indoors under mosquito-free conditions was used as a NC. At this age, maternally transmitted anti- 

*Plasmodium* spp. immunoglobulins were not detectable by ELISA (Graczyk et al., 1994a). The positive cutoff level was an absorbance greater than the mean ± 3 SD of 8 NC wells. A pool of serum from 2 captive, 2-yr-old *S. demersus* with clinical *P. relictum* and 3 2-yr-old penguins with clinical *P. elongatum* infections were used as a positive control (PC). The method of Schwartz et al. (1991) was used to compare the absorbance values from indirect ELISA trials.

Statistical analysis was performed with Analytical Software Statistix 3.5 (Analytical Software, St. Paul, Minnesota, U.S.A.). Analysis of variance (ANOVA) was performed to determine the significance of among-species effect. A 2-sample *t*-test was used to compare the mean absorbance values from different ELISA plates, paired *t*-test for the means derived from the same ELISA plate, and *G*-test to compare the prevalence of ELISA positivity among the penguin species. The degree of linear association among variables was compared using Pearson's correlation coefficient (r). Statistical significance was considered to be *P* < 0.05.

Other statistical treatment followed the procedures of Sokal and Rohlf (1981).

**Results**

The most specific conjugate for the detection of immunoglobulins in the 6 penguin species sera was anti- 

*S. demersus* IgG (Table 1). The mean absorbance value obtained by this conjugate for the penguins (0.945) was significantly higher (paired *t*-test; *t* = 12.24, *P* < 0.01) than the mean absorbance obtained by anti-duck IgG (0.296), anti-chicken IgG (0.594), or anti-turkey (0.414) IgG. Additionally, the mean absorbance for an individual penguin species was significantly elevated when compared to those obtained by the 3 other ligands (ANOVA test; *F* = 64.81, *P* < 0.01) (Table 1). The mean absorbance (±SD) of the penguin serum pool was 0.987 ± 0.086. When this pool was tested for relative binding of the 4 ligands, the mean absorbance was 0.809 for anti-chicken IgG, 0.699 for anti-turkey IgG, 0.607 for anti-penguin IgG, and 0.473 for anti-duck IgG. ANOVA showed that absorbances obtained with anti-penguin IgG were not significantly (*F* = 1.36, *P* = 0.31) lower than those obtained by the commercially available conjugates. The specificity of immunoglobulin detection in the penguin sera increased with incremental penguin serum dilution (up to 1/400). At a dilution of 1/400, the absorbances obtained by duck, chicken, and turkey conjugates did not reach the threshold ELISA
Table 1. Specificity of anti- _Spheniscus demersus_ IgG to the immunoglobulins in homologous and heterologous sera expressed by the mean absorbance values obtained at 405 nm. Sera diluted 1/100 with phosphate-buffered saline.

<table>
<thead>
<tr>
<th>Avian sera</th>
<th>Spheniscus demersus*</th>
<th>Duck†</th>
<th>Chicken*</th>
<th>Turkey†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Spheniscus demersus</em></td>
<td>1.033±</td>
<td>0.340</td>
<td>0.643</td>
<td>0.577</td>
</tr>
<tr>
<td><em>Pygoscelis adeliae</em></td>
<td>0.88±</td>
<td>0.300</td>
<td>0.630</td>
<td>0.393</td>
</tr>
<tr>
<td><em>Pygoscelis papua</em></td>
<td>0.980±</td>
<td>0.213</td>
<td>0.513</td>
<td>0.403</td>
</tr>
<tr>
<td><em>Aptenodytes patagonicus</em></td>
<td>0.895±</td>
<td>0.317</td>
<td>0.550</td>
<td>0.327</td>
</tr>
<tr>
<td><em>Megadyptes antipodes</em></td>
<td>0.950±</td>
<td>0.263</td>
<td>0.547</td>
<td>0.377</td>
</tr>
<tr>
<td><em>Spheniscus magellanicus</em></td>
<td>0.997±</td>
<td>0.327</td>
<td>0.667</td>
<td>0.420</td>
</tr>
<tr>
<td><em>Eudyptula minor</em></td>
<td>0.879±</td>
<td>0.310</td>
<td>0.610</td>
<td>0.401</td>
</tr>
<tr>
<td>Duck</td>
<td>0.387</td>
<td>0.727</td>
<td>0.713</td>
<td>0.426</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.577</td>
<td>0.403</td>
<td>1.112</td>
<td>0.803</td>
</tr>
<tr>
<td>Turkey</td>
<td>0.478</td>
<td>0.467</td>
<td>0.817</td>
<td>0.920</td>
</tr>
</tbody>
</table>

* Developed in rabbit.
† Developed in goat.
‡ ANOVA test; _F_ = 64.81, _P_ < 0.01.

The decreasing pattern of absorbance associated with the incremental serum dilutions was not significant (ANOVA; _F_ = 1.25, _P_ = 0.345) among the 7 penguin species.

In the indirect ELISA, the range of PC serum absorbances was 1.010–1.15 (_x_ = 1.077 ± 0.043) for R32tet_2 _and 1.09–0.899 (_x_ = 0.971 ± 0.060) for P.F.R27. The range of NC absorbances was 0.882–0.120 (_x_ = 0.905 ± 0.013), and the cutoff level was 0.134. All the Antarctic _P. adeliae_ sera were negative for anti- _P. relictum_ or anti- _P. elongatum_ immunoglobulins, and all _M._ _antarctica_ species were positive (Table 2). The prevalence of ELISA positivity ranges between 33 and 92% among the remaining 5 penguin species (Table 2). The mean absorbance of positive penguin sera was significantly elevated (2-sample _t_-test; _t_ = 4.21, _P_ < 0.05) when compared to the mean NC absorbance. The mean absorbance of wild ELISA-positive _S. demersus_ (0.356) was significantly lower (2-sample _t_-test; _t_ = 3.04, _P_ < 0.05) than the mean absorbance (1.024) of captive _S. demersus_ with clinical _P. relictum_ and _P. elongatum_ infections. Kinetic profiles of Ab detected by R32tet_2 were similar to those detected by P.F.R27 for all penguin species (0.826 < _r_ < 0.965, _P_ < 0.05). The overall magnitudes of Ab titers detected by these antigens were not significantly different from each other (2-sample _t_-test; _t_ = 1.31, _P_ = 0.13).

The prevalence of ELISA positivity among species of wild penguins was significantly different (_G_-test; _G_ = 7.14, _P_ < 0.05). The same effect was seen between species of captive penguins (_G_-test; _G_ = 22.60, _P_ < 0.05).

**Discussion**

The positivity for malarial Ab of wild penguins is generated by a single contact with the parasites, because the infection is acquired for a lifetime.

Table 2. The mean absorbance values (±SE) obtained at 405 nm in the indirect ELISA for detection of immunoglobulins against _Plasmodium relictum_ or _P. elongatum_ in the penguin sera diluted 1/100 with phosphate-buffered saline.

<table>
<thead>
<tr>
<th>Plasmodium falciparum antigen</th>
<th>Sporozoite (R32tet_2)</th>
<th>Gametocyte (P.F.R27)</th>
<th>Positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penguin species</td>
<td><em>x</em> ± SE</td>
<td><em>x</em> ± SE</td>
<td>(%)</td>
</tr>
<tr>
<td><em>Spheniscus demersus</em> (n = 44)</td>
<td>0.402 ± 0.034</td>
<td>0.313 ± 0.027</td>
<td>52</td>
</tr>
<tr>
<td><em>Pygoscelis papua</em> (n = 12)</td>
<td>0.544 ± 0.053</td>
<td>0.391 ± 0.021</td>
<td>33</td>
</tr>
<tr>
<td><em>Aptenodytes patagonicus</em> (n = 12)</td>
<td>0.488 ± 0.018</td>
<td>0.437 ± 0.021</td>
<td>58</td>
</tr>
<tr>
<td><em>Megadyptes antipodes</em> (n = 5)</td>
<td>0.782 ± 0.022</td>
<td>0.661 ± 0.038</td>
<td>100</td>
</tr>
<tr>
<td><em>Spheniscus magellanicus</em> (n = 7)</td>
<td>0.301 ± 0.031</td>
<td>0.241 ± 0.012</td>
<td>43</td>
</tr>
<tr>
<td><em>Eudyptula minor</em> (n = 12)</td>
<td>0.402 ± 0.019</td>
<td>0.444 ± 0.021</td>
<td>92</td>
</tr>
</tbody>
</table>

* Above the cutoff level of 0.134.
Figure 1. Mean absorbance values in a direct ELISA for detection of immunoglobulins in homologous and heterologous penguin sera by anti-\textit{Spheniscus demersus} IgG labeled with alkaline phosphatase. Human serum was used as a NC.
The detection of maternally transmitted Ab (Graczyk et al., 1994a) is excluded in the present study because all the birds were adults. The global distribution of warm-water penguins (Davis and Darby, 1990) is overlapped by the occurrence of Culex spp. mosquitoes (Knight and Stone, 1977). The contact with vectors is less likely for the cold-water penguins whose distribution is only partially covered by the occurrence of Culex spp. ELISA positivity of penguins in the areas with documented mosquito absence may be explained by exposure of birds during migration.

The prevalences of parasitemias of wild S. demersus at the southern coast of RSA were 7%, 5% (Fantham and Porter, 1944), and 0.7% (Brossy, 1992); however, the parasitemia prevalence of injured or oiled penguins increased to 22% (Brossy, 1992). The occurrence of Culex spp. had been reported in this region (34°41'S) by Edwards (1941). The Ab titers of wild S. demersus were significantly lower (2-sample t-test; t = 2.22, P < 0.05) than those reported by Graczyk et al. (1994c) in subclinically infected, captive S. demersus and were markedly lower than Ab titers of parasitic penguins in the present study. The prevalence of ELISA-positive wild African penguins (52%) was significantly lower (G-test; G = 49.86, P < 0.05) when compared to 100% positivity among captive birds (Graczyk et al., 1994c). These facts may indicate that the malarial infections of wild birds were subclinical. Additionally, 22% of parasitemia prevalence (Brossy, 1992) among oiled and injured wild penguins is still significantly lower (G-test; G = 51.41, P < 0.05) than the >50% reported by Cranfield et al. (1990) and 62% (Graczyk et al., 1994d).

In addition to avian malaria, Babesia sp. (Brossy, 1992) and Babesia percella (Earle et al., 1993) were reported from wild S. demersus from the RSA. Sera of S. demersus infected with Babesia sp., as determined by Giemsa-stained thin blood smear, gave ELISA-negative reaction with P. falciiparum R32tet13 or P.F.R27 antigens (Graczyk et al., unpubl.). The potential cross-reactivity between these antigens and the closely related avian hemoplasmonic blood parasite (Haemoproteus columbae) was excluded by Graczyk et al. (1994b).

No Culex spp. mosquitoes were found at Crozet (46°21'S) and Kerguelen (49°15'S) islands (Crafford et al., 1986). Chastel et al. (1993) reported a tick species (Ixodes uriae) parasitizing the penguins. However, based on blood smear examination, Fantham and Porter (1944) reported P. relictum spheniscide from A. patagonicus from the higher latitudes, South Georgia Island (54°15'S), and Culex spp. mosquitoes have been reported from southern coastal points of Argentina (Knight and Stone, 1977). Twenty percent of rock-hopper penguins (Eudyptes crestatus) at Gough Island (41°31'S) were parasitic with P. r. spheniscide (Fantham and Porter, 1944). The ELISA positivity of P. papua and A. patagonicus from Crozet and Kerguelen islands may reflect exposure to the parasites during migration. Bost and Jouventin (1990) reported that banded Gentoo penguin females were not seen on the Crozet Islands for up to 5 mo.

The lack of anti–P. relictum or anti–P. elongatum immunoglobulins in malaria-susceptible Antarctic P. adeliae is a consequence of lack of exposure due to the absence of the vectors. Antarctic P. adeliae that breed on the shores migrate northward in winter but remain in the southern oceans around the continent (Cockrem, 1990). Therefore, it is not likely that ELISA-negative penguins may have been infected with other than P. relictum or P. elongatum parasites. However, they may develop disease in areas of vector presence. Sladen et al. (1979) reported 46% malaria-induced mortality of Antarctic C. crestatus in North America.

The prevalence of P. r. spheniscide parasitemia in NZ yellow-eyed penguins (E. antipodes) from Stewart Island (47°12'S) was 10% (Fantham and Porter, 1944). Garnham (1966) reported P. relictum in NZ penguins but never in birds that remained in their Antarctic haunts. Plasmodial parasites from the NZ penguins were also reported by Laird (1950). Two indigenous (C. pervigilans and C. astelae) and 1 exotic (C. quinquiefractatus) avian malaria vector in NZ had been reported frequently (see Laird, 1990) from the beginning of this century (Miller, 1920). Culex pervigilans and C. quinquiefractatus have a remarkable wide range of tolerance in NZ; however, the latter is primarily confined to the coastal area (Laird, 1990). The 100% ELISA positivity of M. antipodes in the present study, and the highest absorbance compared to other penguin species, indicate intense exposure to the malarial parasites. The mainland M. antipodes has been reported to have unidentified disease problems (Gill and Darby, 1993) not observed in Antarctic P. adeliae.
The results of the present study showed that the ELISA developed for captive *S. demersus* can be utilized for diagnostic surveys of exposure to *P. relictum* or *P. elongatum* in wild warm- and cold-water penguins. The overall magnitudes of ELISA-detected Ab were not significantly different for the 2 antigens used. Consequently, the test can be simplified by the elimination of 1 antigen. The ELISA wells that gave absorbance of 0.120 or higher (the cutoff level was 0.136) at 405 nm wavelength can be clearly visually distinguished (particularly on a white background) from the negative wells. Thus, the need for an automated ELISA reader is eliminated, making this method suitable for field surveys.

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Literature Cited


Second Seminar on Food-Borne Parasitic Zoonoses: Current Problems, Epidemiology, Food Safety, and Control

Because the first seminar was a success, the SEAMEO-TROPMED PROJECT is organizing a Second Seminar on Food-borne Parasitic Zoonoses to be held in Khon Kaen, Thailand, 6–9 December 1995. In addition to scientific sessions, a 1-day trip will be made into Laos. Additional information can be obtained from the SEAMEO-TROPMED PROJECT, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand, or from Dr. John H. Cross; Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; phone (301) 295-3139; fax (301) 295-1971.