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Detection of Avian Malaria Infections in Wild and Captive Penguins

THADDEUS K. GRACZYK,[1,6] MICHAEL R. CRANFIELD,[2] JEAN J. BROSSY,[3] JOHN F. COCKREM,[4] PIERRE JOUVENTIN,[5] AND PHILLIP J. SEDDON[4]

[1] The Johns Hopkins University, School of Hygiene and Public Health, Department of Molecular Microbiology and Immunology, 615 North Wolfe Street, Baltimore, Maryland 21205; e-mail: tgraczyk@jhuhyg.sph.jhu.edu,
[2] The Baltimore Zoo, Medical Department, Druid Hill Park, Baltimore, Maryland 21217,
[3] University of Cape Town, Medical School, Department of Anatomy and Cell Biology, Observatory 7925, Cape Town, Republic of South Africa,
[4] Massey University, Faculty of Veterinary Science, Palmerston North, New Zealand and
[5] Centre National de la Recherche Scientifique, Centre d'Etudes Biologiques de Chize, F-79360, Villers en Bois, France

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 ($R32tet_{32}$) 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 $10^{-4.11}$. 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°455′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

[6] Corresponding author.

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 Gentoo 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, 171°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 penguin 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: $R32tet_{32}$ 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 hemosporidian blood parasite, *Haemoproteus columbae* (Graczyk et al., 1994b), and anti–*H. columbae* immunoglobulins did not cross-react with $R32tet_{32}$ 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 $R32tet_{32}$ 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.

Avian sera	Alkaline phosphatase–labeled conjugates			
	*Spheniscus demersus**	Duck†	Chicken*	Turkey†
Spheniscus demersus	1.033‡	0.340	0.643	0.577
Pygoscelis adeliae	0.883‡	0.300	0.630	0.393
Pygoscelis papua	0.980‡	0.213	0.513	0.403
Aptenodytes patagonicus	0.895‡	0.317	0.550	0.327
Megadyptes antipodes	0.950‡	0.263	0.547	0.377
Spheniscus magellanicus	0.997‡	0.327	0.667	0.420
Eudyptula minor	0.879‡	0.310	0.610	0.401
Duck	0.387	0.727	0.713	0.426
Chicken	0.577	0.403	1.112	0.803
Turkey	0.478	0.467	0.817	0.920

* Developed in rabbit.
† Developed in goat.
‡ ANOVA test; $F = 64.81$, $P < 0.01$.

cutoff level (0.134). However, a 1/400 dilution of penguin serum significantly diminished (2-sample t-test; $t = 3.45$, $P < 0.05$) the detection of avian malaria antibodies (Ab) from an absorbance of 1.033 (dilution 1/100) (Table 1) to 0.565 (Fig. 1). Because the 3 other conjugates were not used for detection of avian malaria Ab, the dilution of 1/100 of penguin serum was selected for the ELISA. Using anti–*S. demersus* alkaline phosphatase–labeled IgG it was possible to detect penguin immunoglobulins up to a dilution of 1/12,800 ($10^{-4.11}$) (Fig. 1). 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 ($\bar{x} = 1.077 \pm 0.043$) for $R32tet_{32}$ and 1.09–0.899 ($\bar{x} = 0.971 \pm 0.060$) for P.F.R27. The range of NC absorbances was 0.082–0.120 ($\bar{x} = 0.095 \pm 0.013$), 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. antipodes* 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 $R32tet_{32}$ 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.

Penguin species	*Plasmodium falciparum* antigen		
	Sporozoite ($R32tet_{32}$) $\bar{x} \pm$ SE	Gametocyte (P.F.R27) $\bar{x} \pm$ SE	Positive* (%)
Spheniscus demersus ($n = 44$)	0.402 ± 0.034	0.313 ± 0.027	52
Pygoscelis papua ($n = 12$)	0.544 ± 0.053	0.391 ± 0.021	33
Aptenodytes patagonicus ($n = 12$)	0.488 ± 0.018	0.437 ± 0.021	58
Megadyptes antipodes ($n = 5$)	0.782 ± 0.022	0.661 ± 0.038	100
Spheniscus magellanicus ($n = 7$)	0.301 ± 0.031	0.241 ± 0.012	43
Eudyptula minor ($n = 12$)	0.402 ± 0.019	0.444 ± 0.021	92

* Above the cutoff level of 0.134.

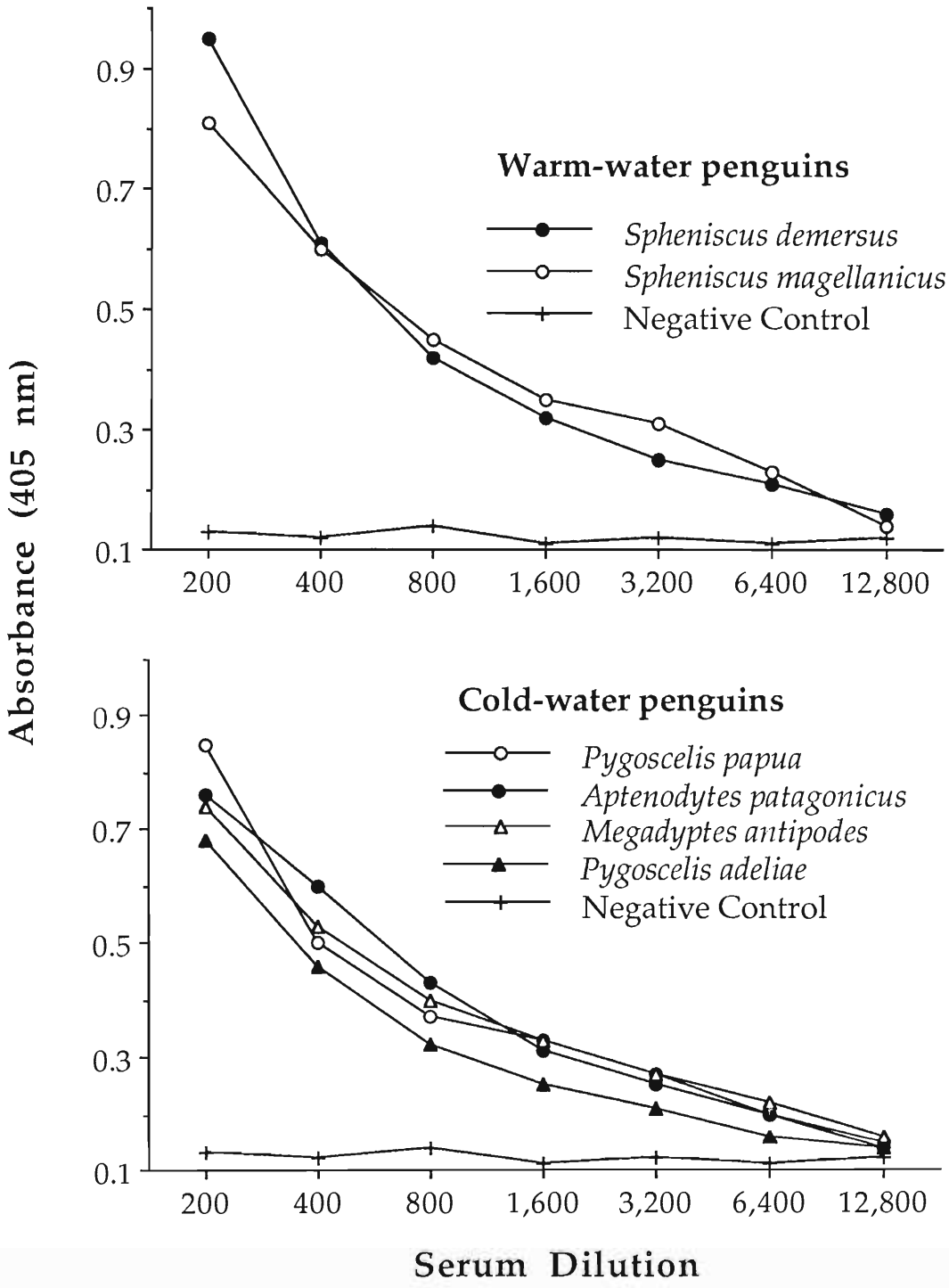

Figure 1. Mean absorbance values in a direct ELISA for detection of immunoglobulins in homologous and heterologous penguin sera by anti-*Spheniscus demersus* IgG labeled with alkaline phosphatase. Human serum was used as a NC.

(Cranfield et al., 1994). 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 parasitemic 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 percei* (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. falciparum* $R32tet_{32}$ or P.F.R27 antigens (Graczyk et al., unpubl.). The potential cross-reactivity between these antigens and the closely related avian hemosporidian 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 parasitemic 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 *E. 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. asteliae*) and 1 exotic (*C. quinquefasciatus*) avian malaria vector in NZ had been reported frequently (see Laird, 1990) from the beginning of this century (Miller, 1920). *Culex pervigilans* and *C. quinquefasciatus* 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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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.