Journal of Wildlife Diseases, 30(3), 1994, pp. 365–371

MATERNAL ANTIBODIES AGAINST *PLASMODIUM* SPP. IN AFRICAN BLACK-FOOTED PENGUIN (*SPHENISCUS DEMERSUS*) CHICKS

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ABSTRACT: Anti-*Plasmodium* spp. antibody titers of mating pairs of adult, captive-reared, African black-footed penguins (*Spheniscus demersus*) and their chicks were determined using the enzyme-linked-immunosorbent assay (ELISA). Two *Plasmodium falciparum* antigens were used for the ELISA: $R32tet_{32}$ (sporozoite antigen), and crude red blood cell extract (CRBCE). Eighteen chicks were bled weekly for ten weeks starting with their day of hatching. The yolk sacs of two penguin eggs were biopsed for ELISA-detectable maternal antibodies (MAB). None of the 28 adult penguins were parasitemic by Giemsa-stained thin blood smear; however, all had anti-*Plasmodium* spp. immunoglobulins reacting with *P. falciparum* antigens. All 18 newly hatched chicks had anti-*Plasmodium* spp. MAB while housed in a mosquito-free environment. The level of MAB in the newly hatched chicks was correlated significantly ($P < 0.001$) with antibody level detected in their female parents ($R32tet_{32}$: $r = 0.87$, CRBCE: $r = 0.89$). No correlation was found between antibody titers of the newly hatched chicks and their male parents. The level of maternal-fetal antibodies was regressed significantly ($P < 0.001$) over the 10-week period. Penguin chicks over 10 weeks of age had no anti-*Plasmodium* spp. MAB. Egg-yolk samples had significantly ($P < 0.03$) higher MAB titers than female parents that laid these eggs.

Key words: Maternal-fetal antibody, African penguins, avian malaria, *Plasmodium relictum*, *Plasmodium elongatum*, ELISA test, *Spheniscus demersus*.

INTRODUCTION

Avian malaria is a mosquito transmitted disease affecting captive penguins in open-air colonies (Fleischman et al., 1968). From 1969 to 1979, mortality due to *Plasmodium relictum* and *Plasmodium elongatum* infections among African black-footed penguins (*Spheniscus demersus*) in the Baltimore Zoo, Baltimore, Maryland (USA) was estimated to be 75% in juveniles, and 64% among the adult birds (Stoskopf and Beier, 1979). From 1980 to 1990, Graczyk et al. (1994) reported a 50% mortality among the untreated juvenile African penguins.

Cross-reactivity of anti-*P. relictum* and anti-*P. elongatum* immunoglobulins with *Plasmodium falciparum* antigens in the enzyme-linked-immunosorbent assay ELISA) Graczyk et al., 1994) facilitates the study of penguin humoral responses to avian malarial parasites. Previously, we observed that older penguins had a lower level of anti-*Plasmodium* spp. immunoglobulins than those exposed in open-air colony for one or two seasons (Graczyk et al., 1994), and speculated that there is a maternal antibody transfer to the young in penguins.

Cases of maternal-fetal antibody transfer have been described in chickens (Brambell, 1970), mallard ducklings (*Anas platyrhynchos*) (Liu and Higgins, 1990), cockatoos (*Cacatua alba, C. sulphurea*) and parrots (*Psittacus erithacus, Amazona ochrocephala oratrix*) (Ritchie et al., 1992), and in pigeons (*Columbia livia*) (Rose and Orleans, 1981). However, there is no information about maternal-fetal immunoglobulin transfer among penguins. Our objective was to determine whether the transfer of maternal antibodies occurs in African penguins, and to characterize this phenomenon.

MATERIALS AND METHODS

Fourteen pairs of adult (six to 15 yr old), captive-reared African black-footed penguins

from a colony maintained by the Baltimore Zoo, Baltimore, Maryland were used. Birds were marked individually by a metal flipper tag with an attached color-coded plastic tape for precise identification from a distance. All penguins were kept together in an outdoor exhibit from May to October 1992 in the avian malaria epizootiological habitat described by Beier (1980). From November 1992 to February 1993, the penguins were kept inside in a mosquito-free environment. The malaria vector-free environment was maintained by anti-mosquito screen-doors (Gerberg, 1970); plastic curtains (Griner, 1974) to exclude mosquitoes; Avermectin B_{1A}/ AvermectinB_{1B} (Boyle-Midway Household Products, Inc., Wayne, New Jersey, USA) baitstrips located in predicted mosquito-resting surfaces; night-flying insect traps "Spinsect" (AMPSCO Corp., Columbus, Ohio, USA) located in the penguin rooms; and elimination of all potential internal breeding areas for mosquitoes, *Culex* spp. (Clements, 1992). The insecticide baits and the light traps were examined weekly for the presence of mosquitoes. The nesting area was equipped with numbered animal cages (75 × 65 × 50 cm) (Carolina Biological Supply Company, Burlington, North Carolina, USA) filled at the depth of 10 cm with flushable cat-litter substrate (Bio-plus Inc., Ashburn, Georgia, USA) to facilitate egg-laying and egg-hatching. All adult penguins were maintained on the same diet (Stoskopf et al., 1980). The sex of birds in each pair was known from the previous year's records. One pair of adult penguins produced three chicks, two penguin pairs produced two chicks, and only one chick was obtained from 11 pairs. The penguin parents were allowed to feed the chicks with regurgitated fish. Eighteen chicks and their respective parents were bled at the day of hatching. The three female penguins that produced more than one chick were bled only once; each male parent was bled each time along with the newly hatched chick. Collection of blood from newly hatched chicks and the adult penguins was performed between 1000 and 1100 hr (Stoskopf and Beier, 1979), and followed the protocol of Graczyk et al. (1994). Thin smears prepared from the adult penguin blood were Giemsa-stained and examined according to the protocol of Stoskopf and Beier (1979). Ten weeks before the transfer of penguins to the outdoor colony (early May), 18 juvenile penguins were bled every week.

Collected blood samples were evaluated by the protocol of Lana et al. (1983), as modified by Graczyk et al. (1993) prior to performing the indirect ELISA. The blood samples taken from the chicks and their parents were evaluated on the same ELISA plate. The blood samples collected from an individual chick were run on the same ELISA plate. The method of Graczyk et al. (1994) was used to determine the number of antibody titration units (ATU) in the test serum.

Two antigens of *Plasmodium falciparum* were used: $R32tet_{32}$ and crude red blood cell extract (CRBCE). The $R32tet_{32}$ is a high antigenic portion of circumsporozoite (CS) protein presented by *P. falciparum* sporozoites (Cerami et al., 1992). The $R32tet_{32}$ antigen was received from the SmithKline Beecham Pharmaceuticals (King of Prussia, Pennsylvania, USA). The CRBCE was obtained by the method of Graczyk et al. (1993). The indirect ELISA assay followed the protocol of Graczyk et al. (1994).

Two cull penguin eggs originating from different penguin pairs were determined by the Baltimore Zoo bird curator as genetically not valuable. The eggs were biopsed with a 40-mm needle syringe according to the protocol of Schmittle (1950), and the yolk samples were collected on filter paper. Yolk samples were eluted into phosphate-buffered saline (PBS) (pH 7.4) prior to ELISA in the same manner as the blood (Graczyk et al., 1994). Ten 0.1-ml samples from the yolk sac were taken from each egg because antibodies are laid down in the yolk in a series of concentric circles (Brambell, 1970). Albumen was not sampled because of the small amount of antibodies occurring there (Rose et al., 1974). We used the ELISA protocol described for the blood of Peking ducklings of Graczyk et al. (1993).

Statistical analysis was performed with the Analytical Software Statistix 3.5 (Analytical Software, St. Paul, Minnesota, USA). Analysis of variance (ANOVA) was performed to determine the significance of the among-penguin-group effect (female parents, chicks, male parents). A two sample t-test was used to compare the results from different ELISA plates, and the paired t-test was performed for the data obtained on the same ELISA plate. Chick antibody titers were compared with parent antibody titers by a simple correlation coefficient (r), and coefficient of determination (R), where $R = r^2 \times 100\%$ (Sokal and Rohlf, 1981). A discrete (Poisson) regression test (Sokal and Rohlf, 1981) was used to determine the significance of the decrease of antibody titer in juvenile penguins over time.

RESULTS

No *Culex* spp. mosquitoes were found in either light traps and in the insecticide baits.

All Giemsa-stained thin blood films were negative for plasmodial parasites; howev-

TABLE 1. Anti-*Plasmodium* spp. geometrical mean titer expressed in antibody titration units (ATU) for the African black-footed penguins (*Spheniscus demersus*).

	Plasmodium falciparum antigens					
	$R32tet_{32}$			Crude red blood cell extract		
	Chicks (n = 18)	Female parents (n = 14)	Male parents (n = 18)	Chicks (n = 18)	Female parents (n = 14)	Male parents (n = 18)
Mean	70.98[a,d]	38.11[e]	59.77[a]	37.82[b,c,d]	31.94[b,e]	26.66[c]
SE	4.11	3.75	4.18	2.87	2.91	1.90
Range	47.90	27.40	41.73	21.37	15.30	18.35
	96.75	80.20	88.84	55.65	54.10	42.34

[a] These values differed significantly ($P < 0.002$) with a paired t-test.
[b] These values differed significantly ($P < 0.02$) with a paired t-test.
[c] These values differed significantly ($P < 0.05$) with a paired t-test.
[d] These values differed significantly ($P < 0.04$) with a two-sample t-test.
[e] These values differed significantly ($P < 0.004$) with a two-sample t-test.

er, based on the ELISA tests all adult penguins had anti-*Plasmodium* spp. immunoglobulins reacting with *Plasmodium falciparum* antigens (Fig. 1, Table 1). The levels of MAB captured by $R32tet_{32}$ and CRBCE varied widely within the group of 28 adult birds, and within the group of 18 chicks (Fig. 1). Based on ANOVA, there was a significant among-groups (chicks, mothers, fathers) effect in antibody titration units (ATU) for $R32tet_{32}$ ($F = 3.93$, $P < 0.03$), and for the CRBCE ($F = 5.39$, $P < 0.01$). The newly hatched chicks had a higher GMT for $R32tet_{32}$ and CRBCE than analogous GMT's of their parents (Table 1). Based on a paired t-test, the difference in the GMT for $R32tet_{32}$ between the newly hatched chicks and their female parents was significant ($P < 0.02$) as was the difference between newly hatched chicks and their male parents ($P < 0.002$, Table 1). Using a paired t-test for CRBCE titers, there also were significant ($P < 0.05$) differences between female parents and their newly hatched chicks ($P < 0.03$) and between newly hatched chicks and their male parents ($P < 0.05$, Table 1). Using a two-sample t-test, the differences between GMT's obtained for $R32tet_{32}$ and CRBCE were significant ($P < 0.05$) for newly hatched chicks and for female parents ($P < 0.004$) (Table 1); however, they were not significant ($P = 0.17$) between the chicks and their male parents.

The levels of MAB in the newly hatched chicks was correlated significantly ($P < 0.001$) with antibody levels detected in

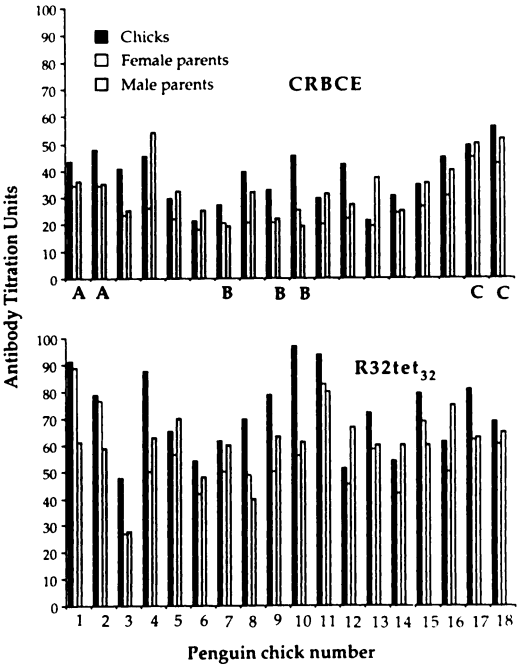

FIGURE 1. The levels of anti-*Plasmodium* spp. immunoglobulins in newly hatched African black-footed penguin (*Spheniscus demersus*) chicks (n = 18) and their female (n = 14) and male (n = 18) parents in a mosquito-free environment. A, B, and C are chicks originated from the same parents.

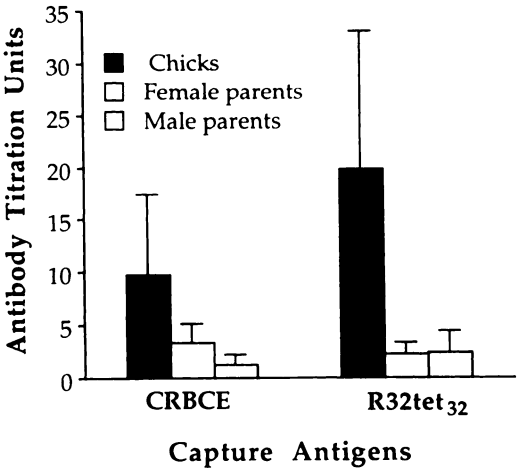

FIGURE 2. Comparison of the mean range ± SE of the levels of anti-*Plasmodium* spp. immunoglobulins in the African black-footed penguin (*Spheniscus demersus*) chicks (n = 7) originating from the same parents with the mean range of antibody levels detected in their parents (n = 7).

their female parents either against $R32tet_{32}$ (r = 0.87), and CRBCE (r = 0.89). The levels of MAB in the newly hatched chicks and the levels of anti-*Plasmodium* spp. immunoglobulins in their female parents were used to fit the model curve: $R32tet_{32}$, y = 19.82 + 0.87x; CRBCE, y = 1.34 + 1.34x, where y = ATU in newly hatched chicks, and x = ATU in the female parents. No correlation was found between MAB levels in the newly hatched chicks and their male parents ($R32tet_{32}$, r = 0.37; CRBCE, r = 0.41). Based on a coefficient of determination, female antibody levels and chick MAB levels varied with the differences in antibody levels exhibited by female parents in 78% for $R32tet_{32}$, and in 78% for CRBCE. Relations in male parent-chick antibody levels were due to the differences in males only in 13% for $R32tet_{32}$, and in 17% for CRBCE.

The mean range of ATU obtained for the three pairs of penguin-parents which gave more than one chick (Fig. 1), and the mean ATU's range in the same parent and chicks are presented in Fig. 2. Differences in the MAB ranges in the same-parent newly hatched chicks were significantly higher by two sample t-test ($R32tet_{32}$; $P < 0.0001$, CRBCE; $P < 0.01$) than the differences in the ranges of antibody levels in their parents.

The flock of 18 juvenile penguins showed the decrease of the mean (±SE) GMT over the 10-wk period from 35.7 ± 6.2 ATU, to 7.1 ± 0.4 ATU for $R32tet_{32}$, and from 20.6 ± 3.5 ATU to 8.6 ± 0.4 ATU for CRBCE. The ATU range decreased over the 10-wk period from 70.6 to 3.0 for $R32tet_{32}$, and from 46.0 to 5.0 for CRBCE. Based on the discrete (Poisson) regression test, the decreases of the chick MAB levels were significantly correlated to the sequence in bleeding time points ($R32tet_{32}$; $P < 0.03$, CRBCE; $P < 0.0004$). The MAB levels were used to fit the binomial model curve describing the age-decreasing antibody titer: $R32tet_{32}$, y = 34.62 − 2.95x; CRBCE, y = 19.71 − 1.23x, where y is the chick MAB level and x is the bleeding time point. The two groups of ATU values ($R32tet_{32}$, CRBCE) had significant between-groups effects with an ANOVA test ($P < 0.0004$). At the end of the 10-wk period, the difference between GMT for $R32tet_{32}$ and CRBCE was not significant (P = 0.34) by two sample t-test; however, this difference was significant ($P < 0.001$) at the beginning of the 10-wk period.

The mean (±SE) (range) in yolk sac samples was 96.7 (±7.2) ATU (71.0 to 157.0) for $R32tet_{32}$, and 62.1 (±4.6) ATU (37.0 to 91.0) for CRBCE. The mean level of antibodies in the egg-yolk was significantly higher by two sample t-test than those levels in the chicks and their parents for $R32tet_{32}$ ($P < 0.01$) and CRBCE ($P < 0.03$).

DISCUSSION

These findings support the observations of Graczyk et al. (1994) that adult African penguins were not parasitemic in winter; however, all birds had antibodies reacting with *Plasmodium falciparum* antigens. In adults, anti-*Plasmodium* spp. immunoglobulins are generated by a low-level exoerythrocytic infection (Sergent and Sergent, 1956). In our study, however, all

penguin neonates were positive for anti-*Plasmodium* spp. immunoglobulins while housed in a mosquito-free environment. This is evidence for the transference of immunity against plasmodial parasites in African penguins.

Maternal or parental antibody transfers are potential mechanisms of equipping chicks with parent-derived immunoglobulins. Parental transfer of antibodies with crop-milk is characteristic for Columbiformes (Rose and Orleans, 1981) as a parallel mechanism to egg-yolk antibody transfer. This is characteristic only in birds having a well-developed crop (King and McLelland, 1979). African penguins have a simple nonsacculated crop (Stoskopf and Kennedy-Stoskopf, 1986). However, the high level of anti-*Plasmodium* spp. immunoglobulins in egg yolk sacs, and the significantly high correlation in penguin female-chick antibody titer, are evidence that anti-*Plasmodium* spp. immunoglobulins were transmitted prenatally.

The hatching period for African penguins occurred in the Baltimore Zoo from December to February. After hatching, the chicks had a high anti-*Plasmodium* spp. antibody titer; however, after 2 mo the level of MAB was close to zero. Consequently, at the moment of outdoor exposure (late April to May) juvenile penguins are *Plasmodium* spp.-naive birds. This moment correlated with the peak of parasitemia in wild birds (Beier, 1980) abundantly present in the penguin open-air habitat. Confirmed mosquito-vectors (*Culex pipiens, Culex restuans*) also are present in this area (Beier, 1980; Beier and Stoskopf, 1980) and 3% of the biting mosquito population transmit avian malaria (Beier and Trpis, 1981). The high mortality (Stoskopf and Beier, 1979; Graczyk et al., 1994) of juvenile African penguins is a result of these epizootiological conditions.

Serological data concerning the relationship between antibody titer to *Plasmodium* spp. and the resistance of birds to plasmodial parasites do not exist. Maternal antibodies protected poults against clinical hemorrhagic enteritis (Fadly and Nazerian, 1989), against avian leukosis virus (Fadly and Smith, 1991), and against infectious bursal disease (Komine et al., 1989; Homer et al., 1992). Graczyk et al. (1994) found that older penguins had a lower level of anti-*Plasmodium* spp. immunoglobulins than birds exposed in the open-air colony for one or two seasons. However, those older penguins were the only birds used for reproduction. We conclude that penguin chicks with anti-*Plasmodium* spp. antibodies can be obtained by using females for reproduction which produce high titers of anti-*Plasmodium* spp. antibodies.

In the present study, newly hatched chicks from the same females had significantly ($P < 0.01$) different antibody titers than their female or male parents. Similar observations in MAB against infectious bursal disease virus were made by Bumstead et al. (1993). Bumstead et al. (1993), and Pardue et al. (1990) explained the antibody titer differences by the variable ability of individual chicks to absorb intestinal yolk sac MAB. This phenomenon generates wide variability in the flock of juvenile penguins.

The ELISA method used herein is based on cross-reactivity between anti-*P. relictum* or anti-*P. elongatum* immunoglobulins and *P. falciparum* antigens (Graczyk et al., 1993). In experimental infections of ducklings, birds infected with *P. elongatum* had higher levels of antibodies than those infected with *P. relictum* (Graczyk et al., 1993). This finding cannot be extrapolated to the penguins with acquired natural infection. However, the higher titers for anti-R32tet$_{32}$ than anti-CRBCE obtained in this study, probably are related to the stage-specific differences in antigenicity of *Plasmodium* spp. infections.

Avian malaria infections still are the most important diseases of penguins in open-air exhibitions. Because the immunological tools to investigate anti-*Plasmodium* spp. humoral responses have been developed

(Graczyk et al., 1993, 1994), we believe that serological profiles of individual penguins should be an integral component of breeding programs that involve outdoor colonies. The most important elements of the applied program should consider using brood stock composed of females with high anti-*Plasmodium* spp. antibody titer for reproduction, shortening the interval between egg-hatching and the outdoor exposure, and determining the protective levels of anti-*Plasmodium* spp. immunoglobulins.

ACKNOWLEDGMENTS

We gratefully acknowledge G. Johnson, D. Heyl, S. Sarro, and S. Maltese for their dedication to this study. We thank SmithKline Beecham Pharmaceuticals for providing us with *Plasmodium falciparum* R32tet$_{32}$ antigen. This study received financial support from the Baltimore Zoo Executive Director, B. A. Rutledge, The Maryland Zoological Society, and from the AKC Fund of New York.

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Received for publication 3 September 1993.