

## IMMUNOLOGICAL SURVEY OF BABESIOSIS (*BABESIA PEIRCEI*) AND TOXOPLASMOSIS IN JACKASS PENGUINS IN SOUTH AFRICA

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### Summary :

*Babesia peircei* was extracted from nucleated erythrocytes of naturally infected Jackass penguin (*Spheniscus demersus*) from South Africa (SA). *Babesia peircei* glycoprotein-enriched fractions were obtained by concanavalin A-Sepharose affinity column chromatography and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). At least 14 protein bands (9, 11, 13, 20, 22, 23, 24, 43, 62, 90, 120, 204, and 205 kDa) were observed, with the major protein at 25 kDa. Blood samples of 191 adult *S. demersus* were tested by enzyme-linked immunosorbent assay (ELISA) utilizing *B. peircei* glycoprotein-enriched fractions to detect anti-*B. peircei* IgG. The samples originated from three groups of free-ranging penguins ( $n = 110$ ), 1 group of penguins ( $n = 66$ ) which were rescued after offshore oil-spill contaminations and rehabilitated at the South African National Foundation for the Conservation of Coastal Birds (SANCCOB), and the final group from SANCCOB-resident penguins ( $n = 15$ ). The overall *B. peircei* seroprevalence was 65 %, and the mean seropositivity ranged from 60 to 71 % among the five penguin groups. The ELISA appeared to be specific for *B. peircei* IgG as tested against *Haemoproteus columbae* IgG and avian malaria (*Plasmodium relictum*, and *P. elongatum*) IgG. *Toxoplasma gondii* antibody (Ab) were detected by the direct agglutination test using killed *T. gondii* tachyzoites. All birds were seronegative for *T. gondii* Ab. The lack of *T. gondii*-positive penguins was due to the appropriate sanitary conditions and anti-*Toxoplasma* prevention procedures utilized by the SANCCOB.

**KEY WORDS :** *Babesia peircei*, Jackass penguins, African black-footed penguin, *Spheniscus demersus*, *Toxoplasma gondii*, ELISA, SDS-PAGE.

**MOTS CLÉS :** *Babesia peircei*, manchot, *Spheniscus demersus*, *Toxoplasma gondii*, ELISA, SDS-PAGE.

### Résumé : EXTRACTION ET UTILISATION D'ANTIGÈNE DE *BABESIA PEIRCEI* EN ELISA ET SÉRONEGATIVITÉ POUR *TOXOPLASMA GONDII* DE *SPHENISCUS DEMERSUS* EN AFRIQUE DU SUD

*Babesia peircei* a été extrait d'érythrocytes nucléés provenant de *Spheniscus demersus* originaires d'Afrique du Sud infectés naturellement. Des fractions de *Babesia peircei* enrichies en glycoprotéines ont été obtenues par chromatographie sur colonne d'affinité concanavaleine A-Sepharose et séparées par électrophorèse en gel de polyacrylamide-dodécylsulfate de sodium (SDS-PAGE). Quatorze bandes protéiques au minimum ont été observées (9, 11, 13, 20, 22, 23, 24, 43, 62, 90, 120, 204, et 205 kDa), la protéine majeure étant de 25 kDa. Des prélèvements sanguins provenant de 191 *S. demersus* adultes ont été testés par ELISA en utilisant les fractions de *B. peircei* enrichies en glycoprotéines pour détecter les IgG dirigées contre *B. peircei*. Les prélèvements provenaient de trois groupes de manchots sauvages ( $n = 110$ ), d'un groupe de manchots ( $n = 66$ ) ayant été secourus après avoir été contaminés par une marée noire en mer et soignés à la Fondation Nationale Sud Africaine pour la Conservation des Oiseaux Littoraux (SANCCOB), et d'un dernier groupe issu des manchots pensionnaires du SANCCOB ( $n = 15$ ). La prévalence globale pour *B. peircei* était de 65 %, et la séropositivité moyenne s'échelonnait de 60 à 71 % parmi les cinq groupes de manchots. L'ELISA apparaissait spécifique pour les IgG dirigées contre *B. peircei* lorsque testée pour les IgG contre *Haemoproteus columbae* et les IgG contre le paludisme aviaire (*Plasmodium relictum*, et *P. elongatum*). Les anticorps (Ac) dirigés contre *Toxoplasma gondii* ont été détectés par le test d'agglutination directe utilisant des tachyzoïtes de *T. gondii* tués. Tous les oiseaux étaient séronégatifs pour les Ac dirigés contre *T. gondii*. L'absence de manchots positifs pour *T. gondii* était due aux conditions sanitaires appropriées et aux méthodes de prévention contre *T. gondii* utilisées par le SANCCOB.

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The Jackass or African black-footed penguin, *Spheniscus demersus*, is an endangered species with decreasing populations in its natural habitat on coastal islets of South Africa (SA) (Crawford *et al.*, 1990). Frequent offshore oil-spill contamination presents the most devastating hazards for existing wild populations. The spills debilitate, injure, and induce disease in penguins (Erasmus *et al.*, 1981; Kerley *et al.*, 1985). The disabled penguins are rehabilitated in the rescue station of the South African National Foundation for the Conservation of Coastal Birds (SANCCOB) in Milnerton (33°55'S, 18°22'E), Cape Town, SA, and released later into the wild.

An intraerythrocytic piroplasm of free-ranging *S. demersus* originally described as *Aegyptianella* sp. (Coles, 1941) was reidentified as *Babesia* sp. (Bennett *et al.*, 1992) and named *Babesia peircei* (Earlé *et al.*, 1993). *Babesia peircei* associated babesiosis is common and endemic in wild *S. demersus* and contributes to illness when associated with avian malaria (Brossy, 1992; 1993). The prevalence of *B. peircei* parasitemia in *S. demersus* rehabilitated at SANCCOB varied from 11 to 15%, and was 4 % in free-ranging birds (Brossy, 1993). A putative *Babesia* sp. infection that caused regenerative anaemia in juvenile little penguins (*Eudyptula minor*) in Australia has been reported by Clarke & Knowles (1993).

Fatal *Toxoplasma gondii* associated toxoplasmosis was reported in captive Humboldt's penguins (*S. humboldti*), Magellanic penguins (*S. magellanicus*), and *S. demersus* in North America (Ratcliff & Worth, 1951). Another case of fatal toxoplasmosis, diagnosed histologically and immunologically, occurred in Tasmania, Australia, in *E. minor* (Mason *et al.*, 1991) fed with sheep meat (Clarke & Knowles, 1993) by a person who boarded many cats (Mason *et al.*, 1991). *Toxoplasma gondii* infections are common in many domestic and wild bird species (Dubey & Beattie, 1988).

The availability of penguins undergoing rehabilitation at SANCCOB and access to free-ranging birds offered an opportunity for serological screening of the birds for pathogens. It is crucial in penguin management to assess accurately naturally occurring exposure to the pathogens in free-ranging *S. demersus* in order to determine the impact of exposure to oil contamination on the penguin's immunological competence, and to evaluate the risk of releasing possibly infected birds into the wild after rehabilitation. Availability of the *S. demersus*-specific conjugate for use in enzyme-linked immunosorbent assay (ELISA) (Graczyk *et al.*, 1994a) facilitates serological monitoring of Jackass penguins for infections with parasites. The direct agglutination test, using mercaptoethanol (Dubey & Desmonts, 1987), appears to be the most specific for detecting active *T. gondii* infections, especially in avian species.

The primary purpose of the present study was to develop and characterize the glycoprotein antigen of *B. peircei* for use in ELISA, and to determine the exposure of free-ranging *S. demersus* to this pathogen by detection of anti-*B. peircei* IgG. Additionally, we serologically screened penguins rehabilitated at SANCCOB facilities and free-ranging birds for infection with *T. gondii*.

## MATERIALS AND METHODS

A total of 191 blood samples from five groups of adult *S. demersus* were collected from December 1993 to July 1994 at SANCCOB

(Brossy, 1992), air-dried, and stored on filter paper as described by Graczyk *et al.* (1993). The samples originated from three groups of free-ranging penguins from offshore islands (Dassen Island and Robben Island) and mainland colony (Boulders, Simons Town), from penguins rescued after offshore oil-spill contaminations and rehabilitated at SANCCOB, and from SANCCOB-resident penguins. The penguins were screened for blood parasites by Giemsa-stained thin blood smears (Brossy, 1992), and a total of 10 ml of blood was collected from four penguins with the highest *B. peircei* parasitemia. Five thin blood smears were prepared and examined (Graczyk *et al.*, 1994b) from the blood sample, and 10 (30 µl each) blood samples were air-dried and stored on filter paper. Parasites were identified based on the description of Earlé *et al.* (1993). The parasitemia was presented as a mean % ( $\pm$  SD) of parasitized erythrocytes per 2,000 red blood cells (RBC) derived from five counts.

The blood for parasite extraction was centrifuged (10,000 g, 10 min) at 4° C, and the plasma was stored at -70° C. The packed RBC were subjected to two freeze/thaw (-70° C/41° C) cycles and resuspended in 10 ml of phosphate buffered saline (PBS) (pH 7.4). Two hundred µl of N,N',N'-polyoxyethylene(10)-N-tallow-1,3-diaminopropane (EDT-20) (Sigma Chemical Co., St. Louis, Missouri, USA), diluted 1/1 with deionized water, was added per 1.0 ml of the preparation, and the solution was incubated for 20 min at 41° C. EDT-20 is a water-soluble cationic detergent that targets lipoproteins and lipids without denaturing proteins (Schuttle & Kula, 1990). The remainder of the protocol for parasite extraction including a 4-step Percoll gradient centrifugation (4,000 g, 30 min) followed that of Graczyk *et al.* (1994c). The pellet containing the particles of RBC and the supernatant from the final parasite centrifugation were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity of *B. peircei* preparation.

The preparation of *B. peircei* went through a freeze/thaw cycle (-70° C/41° C) five times, sonication (6 x 15 sec) (VirSonic Cell Disrupter, The Virtic Co., Gardiner, New York, USA), and centrifugation (10,000 g, 10 min). The antigenic extract (AE) was collected and dialyzed overnight against 0.02 M Tris PBS (pH 7.4) at 4° C and cleared by centrifugation (10,000 g, 10 min). Protein concentration determined by bovine serum albumin (BSA) assay (Pierce, Rockford, Illinois, USA) was 3.1 µg/µl. Glycoprotein-enriched fractions of the *B. peircei* AE for use in the ELISA were obtained by concanavalin agglutinin (Con A) - Sepharose 4B (Sigma Chemical Co., St. Louis, Missouri, USA) affinity column chromatography (De Repentigny *et al.*, 1991). Two glycoprotein-enri-

ched fractions were obtained following the primary and secondary elution of the column (Fig. 1). The first and the second fraction contained 1.5 and 1.1  $\mu\text{g}/\mu\text{l}$  proteins, respectively, as determined by the BSA assay. Con A-bound fractions of *B. peircei* AE were incubated for 5 min at 95°C and separated by SDS-PAGE with a 15% gel (Jule Biotechnologies Inc., New Haven, Connecticut, USA) run at 100 V constant voltage for 7 h at 21°C in electrode buffer (De Repentigny *et al.*, 1991) and stained with Coomassie blue (Laemmli, 1970). To determine the purity of the *B. peircei* AE, the RBC extraction-derived particles were processed by Con A-Sepharose 4B affinity column chromatography in the same manner. Prestained broad range molecular weight standards (Bio-Rad Laboratories, Hercules, California, USA) were used. To further determine purity of the antigen, the glycoprotein enriched fractions of *B. peircei* AE and RBC were separately electroeluted (Electro-Eluter M422, Bio-Rad Laboratories, Richmond, California, USA) and, after determination of protein concentration, eluted fractions were used to coat the ELISA plate at the same concentration (Graczyk & Cranfield, 1995).

For the indirect ELISA detection of anti-*B. peircei* IgG, whole blood, air-dried on filter paper and eluted into buffer, was used (Graczyk *et al.*, 1993). Filter paper storage does not diminish IgG binding capacity (Graczyk *et al.*, 1993). ELISA was performed with Immulon 2 microtitration plates (Dynatech Laboratories Inc., Chantilly, Virginia, USA). The pooled Con A-bound fractions were used as an antigen. The optimal antigen concentration for the ELISA with the positive control (PC) at a fixed 1/100 dilution with PBS and diluted 1/1,000 with PBS alkaline phosphatase-labelled rabbit-anti-*S. demersus* IgG (Graczyk *et al.*, 1994a) was determined by serial antigen dilutions. The PC was whole blood eluted from filter paper of the penguin from which *B. peircei* was extracted. The penguin samples were assayed in duplicate, eight wells were not coated with antigen to determine nonspecific background absorbance (NBA), and eight wells were filled with each of PC and negative control (NC). The NC was a blood pool from three, adult captive-reared *S. demersus* kept indoors. The positive cutoff level was established as an absorbance greater than the mean  $\pm$  3 SD of 8 NC. Sensitivity of the ELISA was determined by testing PC at dilution of 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200, and 1/6,400. The remainder of the ELISA protocol followed previously described procedures (Graczyk *et al.*, 1994a), and the method of Schwartz *et al.* (1991) was used to compare the absorbance values from different ELISA plates.

To determine the specificity of the indirect ELISA, the plate was coated with *B. peircei* antigen. Following post-coating with casein blocking buffer (Graczyk *et al.*, 1993), the wells were filled in duplicate with PC and with two blood

samples of 2-wk-old Peking ducklings (*Anas platyrhynchos*) experimentally infected with *Plasmodium relictum* and *P. elongatum*, respectively, and blood sample from Rock dove pigeon (*Columba livia*) naturally infected with *Haemoproteus columbae*. Additionally, the wells were filled with blood samples of two captive adult *S. demersus* naturally clinically infected with *P. relictum* and *P. elongatum*, respectively, and the blood sample from wild *S. demersus* known to be positive for malarial IgG (Graczyk *et al.*, 1995). All blood samples were air-dried and stored on filter paper, eluted into the buffer and diluted 1/100 with PBS. Duckling, pigeon, and captive penguins infections were determined by ELISA (Graczyk *et al.*, 1994c) and were confirmed by parasitemia found on the Giemsa-stained thin blood films. The remainder ELISA protocol followed previous procedures (Graczyk *et al.*, 1994c) utilizing bird species-specific conjugates.

To determine the presence of *T. gondii* agglutinating antibody (Ab), a modified direct agglutination test with killed *T. gondii* tachyzoites was carried out (Desmonts & Remington, 1980). In a modified test, 2-mercaptoethanol was incorporated into the antigen instead of into the blood sample to avoid prolonged exposure to toxic fumes (Dubey & Desmonts, 1987). Positive and NC controls were prepared as described previously (Dubey & Desmonts, 1987). All blood samples were assayed at 1/25, 1/50, and 1/500 dilution with PBS (pH 7.4) on U-bottom well microtitre plates (Dynatech Laboratories Inc., Chantilly, Virginia, USA) with *T. gondii* PC and NC included on each plate.

Statistical analysis was carried out with Analytical Software Statistix 4.1 (Analytical Software, St. Paul, Minnesota, USA). The absorbance values of ELISA-positive penguins were tested by the approximate Wilk-Shapiro statistic to determine if their distribution conforms to a normal distribution. Analysis of variance (ANOVA) was carried out to determine the significance of among-penguin group effects in absorbance values. A two-sample *t*-test was used to compare the mean absorbance between penguin groups, and the *G*-heterogeneity test (Sokal & Rohlf, 1981) was applied to compare *B. peircei* seroprevalence among penguin groups. The absorbance reference range for *B. peircei* parasitemia was established according to the protocol of Lumeij (1994) as mean ( $\bar{x}$ ) ( $\pm$  3 SD) of 48 absorbance values obtained in the indirect ELISA for the parasitemic penguin blood. Statistical significance was considered to be  $P < 0.05$ .

## RESULTS

**B**abesiosis parasitemia in the 10-ml blood samples varied within the limits of 78 to 87%; with a mean ( $\pm$  SD) = 81.6  $\pm$  4.1%.



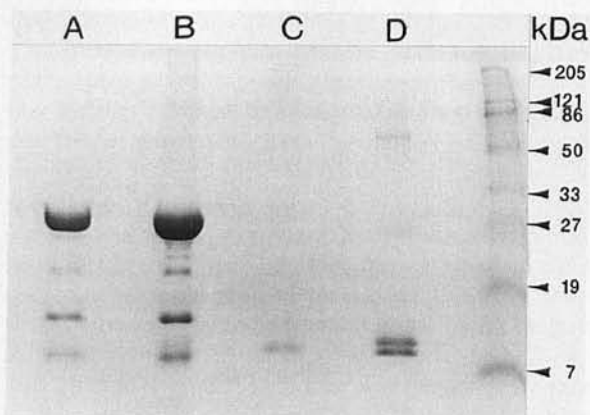


Fig. 1. — Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of glycoprotein-enriched fractions obtained by concanavalin A-Sepharose 4B affinity column chromatography with intraerythrocytic stages of *Babesia peircei* extracted from naturally infected Jackass penguins (*Spheniscus demersus*) rescued after offshore oil-spill contamination and rehabilitated at the Rescue Station of the South African National Foundation for the Conservation of Coastal Birds (SANCCOB) in Milnerton, Cape Town, South Africa. *Babesia peircei* antigenic fractions obtained by the primary (lane B) and secondary elution of the column (lane A). Lane C, supernatant from the final step of extraction. Lane D, fraction of erythrocytes from which the parasites were extracted. Prestained broad range molecular weight standards stained with Coomassie blue in kilodaltons (kDa).

Two parasites per erythrocyte were observed in 38.5–42.5% ( $\bar{x}$ ,  $40.2 \pm 1.8\%$ ) of examined cells. In the majority of singly and doubly infected RBC, the parasites were situated on the polar end of the erythrocyte or displayed bilateral polar symmetry of location. The round, spherical, ovoid, and elongated merozoites were the most common form of the parasite. Divided elongated merozoites (presented in a low-parasitemia sample) which formed tetrads, were rare, occurred singly in erythrocytes, and were usually not on the RBC

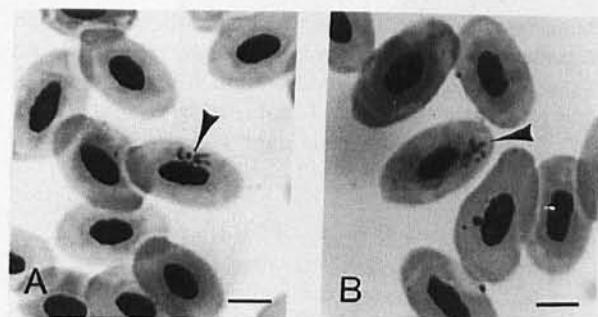


Fig. 2. — Two forms (A, B) of divided merozoites of *Babesia peircei* which formed a tetrad (arrows) in the erythrocytes of naturally infected Jackass penguin (*Spheniscus demersus*) rescued after offshore oil-spill contamination and rehabilitated at the Rescue Station of the South African National Foundation for the Conservation of Coastal Birds (SANCCOB) in Milnerton, Cape Town, South Africa. Giemsa-stained thin blood smear. Scale bar = 5  $\mu$ m.

polar end (Fig. 1). Two forms of the tetrad were observed in the Giemsa-stained thin blood films (Fig. 1). Extracellular stages of *B. peircei* were not observed.

Analysis of *B. peircei* protein showed that at least 14 bands ranging from 9 to 205 kDa were seen in SDS-PAGE and Coomassie blue staining of the first glycoprotein-enriched fraction. The molecular sizes of these glycoproteins were 9, 11, 13, 20, 22, 23, 24, 43, 62, 90, 120, 204, and 205 kDa, with the major glycoprotein 25 kDa (Fig. 2). All bands > 25 kDa were faint. Erythrocyte particles derived from *B. peircei* extraction process displayed two major comigrating bands (10 and 12 kDa), and two bands of 26 and 60 kDa. Erythrocyte proteins eluted for the gel produced negative results when used in the ELISA.

The reference absorbance range for *B. peircei* parasitemia was 0.45 to 0.81. Using optimal antigen concentration of 5  $\mu$ g/ml, it was possible to detect *B. peircei* IgG at the PC dilution of 1/3,200. The estimates of

Geographical origin of penguins			Penguins			Absorbance	
			Total number	Positive* number	(%)	Range	Mean $\pm$ SD
** Dassen Island	33°26'S	18°04'E	30	18	(60)	0.23-0.89	0.38 $\pm$ 0.15
** Robben Island	33°49'S	18°22'E	46	29	(63)	0.27-1.00	0.53 $\pm$ 0.19
** Mainland	34°14'S	18°26'E	34	22	(65)	0.23-0.77	0.44 $\pm$ 0.15
*** SANCCOB	34°14'S	18°26'E	66	47	(71)	0.20-1.00	0.47 $\pm$ 0.19
**** SANCCOB	34°14'S	18°26'E	15	9	(60)	0.41-0.93	0.61 $\pm$ 0.19

\* Above the cutoff level of 0.16.

\*\* Free-ranging penguins.

\*\*\* Penguins rescued after offshore oil-spill contamination.

\*\*\*\* Resident penguin population.

Table 1. — Seroprevalence for *Babesia peircei* IgG determined by the indirect enzyme-linked immunosorbent assay (ELISA) of adult Jackass penguins (*Spheniscus demersus*) at the Rescue Station of the South African National Foundation for the Conservation of Coastal Birds (SANCCOB) in Milnerton, Cape Town, South Africa.

Wilk-Shapiro statistics for absorbance values (0.74, 0.96, 0.94, 0.89, and 0.84) for the five penguin groups listed in Table I, respectively, showed that the absorbance values were normally distributed. The seroprevalence of *B. peircei* IgG ranged from 60 to 71 % among the five penguin groups (Table I), with an overall seroprevalence of 65 % (125 of 191). The *G*-heterogeneity test showed a nonsignificant effect in the prevalences of *B. peircei* seropositivity among these groups ( $G = 1.07$ ,  $P > 0.05$ ). The absorbance values of positive penguins varied within the limits of 0.20 to 1.00, with the total mean absorbance ( $\pm$  SD) of  $0.48 \pm 0.19$ . The absorbance values varied nonsignificantly among the three groups of free-ranging *S. demersus* (ANOVA;  $F = 1.89$ ,  $P > 0.05$ ). The absorbance values of 59 of 191 (31 %) penguins exceeded the lower reference range of 0.45 established for *B. peircei* parasitemia. Those were: 3 of 18 (17 %) of Dassen Island, 18 of 29 (62 %) of Robben Island, 11 of 22 (50 %) of Boulders penguins, and 19 of 48 (40 %) of oiled birds, and 8 of 9 (89 %) of penguin-resident at SANCCOB. The *G*-heterogeneity test showed a significant effect among five penguin groups in the percentage of birds having absorbance values exceeding the reference range of *B. peircei* parasitemia ( $G = 10.63$ ,  $P < 0.5$ ). The blood samples of *C. livia* naturally infected with *H. columbae*, Peking ducklings experimentally infected with *P. relictum* and *P. elongatum*, and *S. demersus* naturally infected with avian malaria gave negative results in the indirect ELISA utilizing *B. peircei* antigen. *Toxoplasma gondii* Ab were not detected in penguin plasmas.

## DISCUSSION

More in depth immunologic studies on avian babesiosis are needed to interpret the prevalence range of *B. peircei* seroconversion of Jackass penguins. A comprehensive review of penguin diseases and parasites (Clarke & Knowles, 1993) listed six species of *Ixodes* and three species of *Ornithodoros* ticks from 14 species of penguins; however, only *Ornithodoros capensis* was reported from free-ranging *S. demersus* (Daturi, 1986; Duffy & Daturi, 1987; Brossy, 1993). It was documented that penguins that reuse the same colony and nest sites for generations are particularly vulnerable to ticks and tick-transmitted pathogens (Duffy, 1991). *Ixodes uriae*, implicated as a vector for *B. peircei* (Earlé *et al.*, 1993) is distributed worldwide having even been reported from the Antarctic Peninsula (Clarke & Knowles, 1993). However, only *O. capensis* is present in the natural habitat of Jackass penguins (Daturi, 1986; Duffy & Daturi, 1987; Brossy, 1993) indicating that *O. capensis*

is probably a transmitting agent of *B. peircei*. The mean tick load was  $1.0 \pm 1.64$  per adult *S. demersus* and 2.0 (range 1-50) per nestling on the Marcus Island, SA (Duffy, 1991). The low tick infestation in that study was explained by the presence of ants (Duffy, 1991), but weather patterns play an important role in tick prevalence.

The ELISA results in our studies indicated that the seroprevalence of *B. peircei* was uniformly distributed among the five populations of Jackass penguins. If the bovine/equine model (Spickett & Fivaz, 1992; Fivaz & De Waal, 1993) of babesiosis is applicable to free-ranging penguins, the observed uniformity of seroconversion may reflect the uniformity in the exposure to the tick.

Glycoproteins of a number of the protozoans parasitizing erythrocytes have been shown to be parasite stage-specific, and to be the most immunogenic fraction of the parasite surface proteins (Mitchell & Anders, 1982). The penguin RBC-derived glycoproteins in this study showed two major comigrating bands (10 and 12 kDa) of close molecular weight to the minor glycoproteins of the pathogen a fraction (9, 11, and 13 kDa). Therefore, *B. peircei* extract should be assayed by SDS-PAGE before use in the ELISA in order to determine if it is contaminated with foreign glycoproteins.

The antigenic extract contained the proteins of at least nine intraerythrocytic stages of *B. peircei* (Earlé *et al.*, 1993), and IgG responses against other avian blood parasitic protozoa (*H. columbae*, *P. relictum* and *P. elongatum*) were not specific to *B. peircei*, as determined by the ELISA. We conclude that the humoral responses were directed against blood stages of *B. peircei*. However, except in the penguins from which *B. peircei* was extracted, the relationship between IgG magnitude and parasitemia level remain unknown. Serological studies on captive *S. demersus* naturally infected with avian malaria show that IgG responses do not correlate with parasitemia (Graczyk *et al.*, 1994d). Further studies are necessary to investigate if this is also the case for *B. peircei* infections.

In non-oiled penguins, ticks are primarily around the eyes, at the commissures of the mouth, on the webs between the toes, and around the cloaca (Clarke & Knowles, 1993). Thus, we do not feel oil-impaired feathers make Jackass penguins more vulnerable to ticks feeding. However, oiled Jackass penguins can develop fatal infections with *Salmonella typhimurium* and *Staphylococcus aureus* (Westphal & Rowan, 1970), and mount weaker Ab responses against avian malaria (Graczyk *et al.*, 1995). Interestingly, *B. peircei* was described as a new species from oiled Jackass penguins (Earlé *et al.*, 1993).

Toxoplasmosis is always a concern in bird centers because high density of birds favor transmission of the

pathogen and the fatal cases, including penguins, were reported from such centers (Ratcliff & Worth, 1951; Hubbard *et al.*, 1986; Hartley & Dubey, 1991). Ratcliff & Worth (1951) reported fatal toxoplasmosis in four *S. humboldti*, two *S. magellanicus*, and seven *S. demersus* at the Philadelphia Zoo (USA). The seven *S. demersus* were healthy but became ill one week after arrival at the Zoo; it is most likely that penguins acquired toxoplasmosis at the Zoo.

In the present study, Ab to *T. gondii* in diluted penguin sera were tested by the direct agglutination test. Unlike the Sabin-Feldman dye test, the direct agglutination test has proved to be highly sensitive for the detection of *T. gondii* Ab in many avian species including owls, quails, turkeys, chickens, pheasants, and partridges experimentally infected with *T. gondii* (Dubey *et al.*, 1995). Many avian species including chickens and turkeys do not develop appropriate Ab for the dye test (Frenkel, 1981).

The negative outcome in our *Toxoplasma* survey indicates that none of the penguins had contracted *T. gondii* tachyzoites at the time of blood sample collection. The lack of *T. gondii*-positive penguins in SANCCOB facilities indicates appropriate sanitary conditions and successful anti-*Toxoplasma* management prevention procedures used by the center.

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