AVIAN MALARIA AND MAREK'S DISEASE: POTENTIAL THREATS TO GALAPAGOS PENGUINS *SPHENISCUS MENDICULUS*

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SUMMARY

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Blood samples from Galapagos Penguins *Spheniscus mendiculus* were screened for avian malaria and the avian herpesvirus Marek's Disease Virus (MDV) by the polymerase chain reaction (PCR), using *Plasmodium* and MDV-specific primers, respectively. Malaria was considered as a potential threat to these seabirds, in light of the fact that *Culex quinquefasciatus*, a known mosquito vector of *Plasmodium*, has recently been recovered in the Galapagos Islands, and because penguins are considered to be especially susceptible to this disease. The screening for MDV was undertaken because of the recent Marek's Disease epizootic in Galapagos domestic poultry. No evidence of existing infection with either of these pathogens was detected. However, the impact of introduced malaria on endemic birds in island systems such as Hawaii, and the death of over 800 chickens in Galapagos due to Marek's Disease, underscores the need for continued monitoring of exotic disease agents and the need to consider them as threats to endangered wildlife.

Key words: Galapagos Penguin, *Spheniscus mendiculus*, avian malaria, Marek's Disease

INTRODUCTION

The Galapagos Penguin *Spheniscus mendiculus* is endemic to the Galapagos Islands. This seabird is considered Endangered according to World Conservation Union (IUCN) criteria, because of its small and declining population size, as well as its restricted distribution, limited to the Galapagos Islands (Cepeda & Cruz 1994, Ellis *et al.* 1998, BirdLife International 2000). Prior to the 1997–1998 El Niño event, which may have reduced the population by as much as 50% (Vargas 1999) the total population for this species was estimated to be between 1700 and 8500 individuals (Boersma 1998). Mills & Vargas (1997) and Boersma (1998) identify climatic change, a fluctuating food supply, possible disturbance by tourist visits and illegal fishing activities as continuing threats to the well being of Galapagos Penguins.

Additionally, novel pathogens introduced as a consequence of human activity could threaten Galapagos wildlife, including penguins. Many common avian diseases are spread either directly or indirectly through human agency (Alexander 1982) and like other endemic species, penguins may be particularly vulnerable to exotic disease agents (Clarke & Kerry 1993). The recent detection of neutralizing antibodies to infectious bursal disease virus, a pathogen of domestic chickens, in Adélie Penguins *Pygoscelis adeliae* (Gardner *et al.* 1997) highlights the threat to penguins posed by introduced pathogens.

Here we report on our use of the polymerase chain reaction (PCR) to determine if *Plasmodium*, the causative agent of avian malaria, is present in Galapagos Penguins, and thus poses a significant risk to this already vulnerable species. Avian *Plasmodium* has a worldwide distribution, and is transmitted wherever appropriate mosquito vectors are found (Redig *et al.* 1993). It is considered to be the most important cause of mortality in outdoor zoological penguin exhibits, causing over 50% mortality in untreated juvenile and previously unexposed adults (Cranfield *et al.* 1991). Less is known about the presence of *Plasmodium* in wild penguins. *Plasmodium* has been reported in Magellanic Penguins *Spheniscus magellanicus* (Fix *et al.* 1988), but these birds, while wild-caught, are thought to have acquired their infections in captivity. This protozoan parasite has also been detected in a variety of temperate and sub-Antarctic species, including African *S. demersus*, Yellow-eyed *Megadyptes antipodes*, Rockhopper *Eudyptes chrysocome* and Chinstrap *Pygoscelis antarctica* Penguins (Clarke & Kerry 1993).

Although *Plasmodium* infection can be diagnosed by blood smear examination, this technique is unreliable, because there is often limited erythrocytic involvement in birds (Redig *et al.* 1993). This may be especially true in penguins (Cranfield *et al.* 1994, Graczyk *et. al.* 1995). Feldman *et al.* (1995) found that PCR was a significantly more sensitive screening technique than were standard histological methods for a variety of passerine and non-passerine birds.

It is currently unknown if avian *Plasmodium* is present in the Galapagos Islands. The only mosquito historically recorded in the

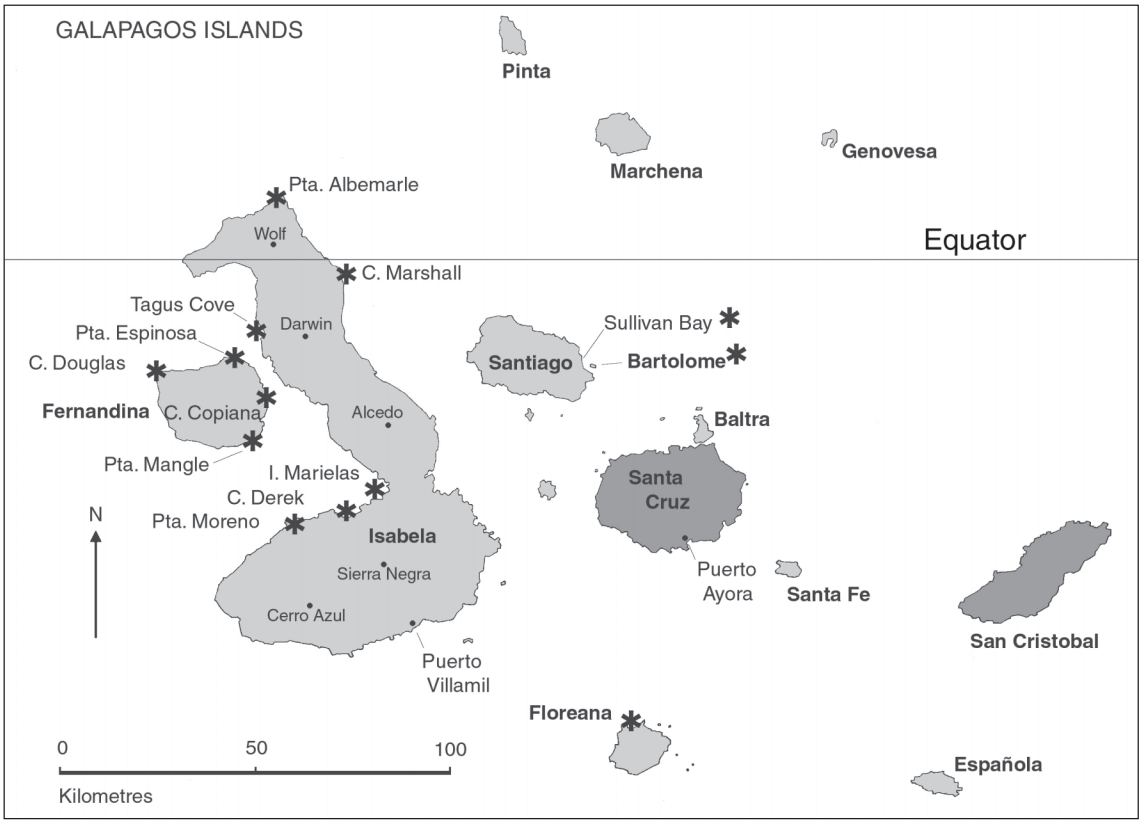

Fig. 1. *The Galapagos Islands. Asterisks indicate sites from which penguin blood samples were collected. Darker shading indicates that the malaria vector* Culex quinquefasciatus *has been collected from these islands.*

islands, *Aedes taeniorhynchus* (Linsley & Usinger 1966), is not considered a *Plasmodium* vector. However, with the more recent establishment of *Culex quinquefasciatus* in the Galapagos (S. Peck pers. comm.), it appears likely that avian malaria either currently exists, or eventually will be detected in endemic island avifauna.

Additionally, in late 1995 and early 1996, Marek's Disease, a disease of poultry caused by an avian herpesvirus (MDV), was reported for the first time in the Galapagos Islands, ultimately killing an estimated 800 domestic chickens (Vargas & Snell 1997). In light of this epizootic, and because of concern that this pathogen might spread to endemic birds, we also screened Galapagos Penguins for MDV, using PCR-based techniques.

METHODS

Genomic DNA

Blood samples were collected from a total of 109 penguins at all documented Galapagos Penguin habitats (Mills & Vargas 1997) in May and June 1996 (Fig. 1). Each bird, netted from an inflatable boat, was weighed and measured, and a small (1–2 ml) blood sample was drawn from the tarsal–metatarsal vein. Blood was expelled directly into Queen's lysis buffer composed of 10 mM NaCl, 10 mM EDTA, and 2.0% SDS (modified from Seutin *et al*. 1990). Genomic DNA was extracted from the blood using standard phenol/chloroform extraction procedures (Sambrook *et al.* 1988).

Plasmodium screening

DNA samples were screened for *Plasmodium* using a two-step PCR procedure developed by Perkins *et al*. (1998). In the first reaction, genus-specific oligonucleotide primers from Li *et al*. (1995) (5'-CGACTTCTCCTTCCTTTAAAAGATAGG-3' and 5'-GGATAACTACGGAAAAGCTGTAGC-3') were used to amplify an approximately 1200-bp region of the 18S *Plasmodium* ribosomal sub-unit gene. Each 25-µl reaction contained one 'Ready-To-Go' Bead (Amersham Pharmacia Biotech), 1 mM of each primer, and 200 ng of target DNA. PCR were run at five minutes at 95°C, followed by 35 cycles of 60 s at 95°C, 60 s at 48°C, and two minutes at 72°C. The second reaction utilized the nested primers 5'-TAACACAAGGAAGTTTAAGGC-3' and 5'-TATTGATAAAGATTACCTA-3' (Li *et al.* 1995). Each 25-µl reaction again received 1µl of each primer and 1 'Ready-To-Go' bead, as well as 1µl of product from the first reaction, serving as target DNA. Thermocycling conditions were identical to those in the first reaction, except that the annealing temperature was raised to 50°C. The products were run out on an acrylamide gel, stained with ethidium bromide, and were scored positive if a band of approximately 420-bp, indicative of the 18S *Plasmodium* ribosomal sub-unit gene, was apparent.

Ninety-four Galapagos Penguins were screened. Two positive controls (DNA from the Western Fence Lizard *Scelaperus occidentalis* infected with *Plasmodium mexicanum*, furnished by S.L. Perkins and from a Humboldt Penguin *Spheniscus humboldti* that died from malaria, provided by R. Wallace) and one negative

control were run with each group of samples. The presumptive *Plasmodium* DNA in the positive penguin control was cloned, sequenced, and confirmed as *P. relictum*, by comparing it to sequence data available through 'GenBank'. We also performed control PCR reactions with 200 ng of sample penguin DNA, deliberately spiked with 200 ng of positive control DNA, to verify that avian DNA in our samples did not interfere with the amplification of *Plasmodium* DNA under the conditions used.

Marek's Disease Virus screening

DNA samples were screened for MDV with PCR, using oligonucleotide primers (5'-GCAAGTCATTATGCGTGAC-3' and 5'-TGTTTCCATTCTGTCTCCAAGA-3') specific for the MDV glycoprotein C (Coussens & Velicer 1988). These primers specifically amplify a 200-bp product from the MDV genome. PCR reaction conditions included 3.5 mM MgCl, and 200 ng of penguin genomic, or control DNA (see below). PCR reactions were performed for 35 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. Following PCR, all samples, including positive controls, were analyzed by polyacrylamide gel electrophoresis, stained with ethidium bromide. A total of 109 samples was screened.

A positive control of RB1B (Schat *et al.* 1982) DNA from MDV, grown in domestic chicken embryo fibroblasts, was included with each set of PCR reactions. Control PCR reactions with 100 ng of penguin DNA mixed with 100 ng of MDV infected chicken embryo fibroblast DNA were also performed, to insure that penguin DNA was not interfering with the MDV DNA amplification.

RESULTS

No evidence of infection with *Plasmodium* was detected in any of the Galapagos Penguin samples. The positive controls, both from the infected lizard and the Humboldt Penguin, gave consistently strong positive results. As mentioned above, the 400-bp gene segment from the captive Humboldt Penguin positive control was sequenced and confirmed as *P. relictum.* Galapagos Penguin samples contaminated with positive control DNA yielded positive results, suggesting that penguin DNA will not interfere the amplification of the parasite's DNA, and thereby increase the likelihood of false negative results.

Likewise, none of the Galapagos Penguin samples screened for MDV showed evidence of infection. A 200-bp fragment was consistently amplified in all positive controls, as well as in penguin DNA contaminated with MDV DNA, indicating that penguin DNA does not interfere with the amplification of the viral DNA.

DISCUSSION

The 109 Galapagos Penguin blood samples screened for MDV, and the 94 samples screened for *Plasmodium*, represent, depending on the estimate of population accepted, between 2 and 10% of the total population of this species. Included in this study were birds from Floreana, Bartolomé, and Santiago Islands (Fig. 1), all of which either have permanent human populations or are heavily visited by tourists. This relatively large sample size, combined with the fact that even those birds most likely to interact with humans tested negative for these two pathogens, indicates that in all likelihood, neither *Plasmodium* nor MDV is currently present in the Galapagos Penguin population.

Marek's Disease Virus is certainly still present in the Galapagos domestic chicken population, because herpesviruses are known to infect their reservoirs indefinitely (White & Fenner 1994). The question remains as to whether or not MDV may pose a threat to penguins or other indigenous avifauna. This may be unlikely as herpesviruses in general have relatively narrow host ranges (White & Fenner 1994). However, in light of the recent epizootic among Galapagos chickens, continued screening for this virus, especially in domestic chickens as well as in wild birds that may have contact with chickens, is warranted.

Of far greater concern is avian malaria. This is especially true when considering how vulnerable penguins are to this disease (Cranfield *et al*. 1994), as well as the disastrous consequences that introduced malaria can have for immunologically naïve, endemic birds in island systems such as Hawaii (Van Riper *et al*. 1988, Feldman *et al*. 1995).

It is unclear if *Plasmodium* is present in the Galapagos, and to what degree, if any, it has affected native birds to date. Our data suggest that, thus far, *Plasmodium* is not currently present in the endemic penguin population. It is unlikely that we were simply unable to detect infected birds, because the nested PCR technique we employed has been shown to be effective at rates of parasitemia as low as 1 parasite/10 000 erythrocytes (Perkins *et al*. 1998). Furthermore, the only known malaria vector in the Galapagos, *Culex quinquefasciatus*, is seemingly a recent arrival in the islands. This mosquito was unknown in Galapagos prior to 1989, and has currently only been recovered on San Cristobal and Santa Cruz Islands (S. Peck pers. comm.). Although San Cristobal has never been known to have resident penguins, birds have historically been present along the north shore of Santa Cruz Island (H. Snell pers. comm.). Despite intensive searching along this coastline, we found no penguins currently resident on Santa Cruz (Fig. 1). Whether or not the apparent disappearance of penguins from Santa Cruz is related to the recent introduction of *C. quinquefasciatus* awaits verification.

Regardless of whether or not *Plasmodium* has, to date, impacted Galapagos avifauna including penguins, the continued screening of endemic birds for this pathogen is prudent. A prudent strategy might be to combine PCR techniques with serological surveys in order to obtain evidence of both current and prior infection. All future screening efforts should include both domestic poultry serving as potential reservoirs, as well as appropriate mosquito vectors. In this way, the risk of an outbreak at any given time could be assessed, and in the event of disease transmission, measures to contain disease spread could quickly be considered.

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