

Novel relapsing fever *Borrelia* detected in African penguins (*Spheniscus demersus*) admitted to two rehabilitation centers in South Africa

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Abstract The African penguin, *Spheniscus demersus*, the only penguin species that breeds in Africa, is endangered, and several diseases including avian malaria, babesiosis, and aspergillosis are common in some populations. From 2002 to 2010, spirochetes morphologically consistent with *Borrelia* were observed on thin blood smears from 115 of 8,343 (1.4%) African penguins admitted to rehabilitation centers in the Western Cape and Eastern Cape provinces of South Africa. Prevalence rates were significantly higher among chicks and juveniles compared with adults and for birds sampled during the summer months of October to February compared with winter months. The majority of infected birds were ultimately released, despite lack of antibiotic treatment; however, at least one bird is believed to have died of borreliosis based on characteristic gross and microscopic lesions. Analysis of partial *flaB* gene sequen-

ces indicated this was a relapsing fever *Borrelia* most similar to a *Borrelia* sp. detected in soft ticks from a seabird colony in Japan. This represents the fourth report of a relapsing fever *Borrelia* sp. in an avian species and highlights the need for additional studies of potentially pathogenic organisms infecting the African penguin in South Africa.

Introduction

The African penguin, *Spheniscus demersus*, is the only penguin species that is found in Africa. It breeds on 25 islands and four mainland localities across southwestern Africa (Kemper et al., 2007). In South Africa, penguins breed in the Western Cape and Eastern Cape provinces, separated by approximately 600km. The total population was estimated at over 1.45 million individuals at the start of the 20th century (Shannon and Crawford 1999) however numbers decreased to 141,000 breeding pairs in 1956 and subsequently to 26,000 breeding pairs in 2009 (Crawford et al., 2011). The IUCN conservation status of the species has been re-classified from Vulnerable to Endangered (BirdLife International 2010). Historical threats to the African penguin included guano and egg collection and current threats include oil spills, human disturbance, reduced food availability due to increased commercial fishing, and diseases such as aspergillosis and avian malaria (Westphal and Rowan 1970; Brossy 1992; Crawford et al. 1995; Graczyk et al. 1995; BirdLife International 2010).

Several vector-borne pathogens have been reported from African penguins including *Babesia peircei*, *Plasmodium relictum spheniscidae*, *Plasmodium juxtannucleare*, and *Leucocytozoon tawaki* (Bennett et al. 1992; Brossy 1992;

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Earle et al. 1993; Grim et al. 2003). Among free-ranging penguins, hemoparasite infections have only been reported from temperate penguin species from South Africa, Australia, New Zealand, and some regions of the South Atlantic. All surveys for hemoparasites in penguins from the sub-Antarctic and Antarctic have been negative, presumably because of a lack of appropriate vectors (Jones and Shellam 1999).

The emergence, establishment, or increased transmission of vector-borne pathogens is a complex relationship between the host, vector(s), and environment and can result from numerous changes such as the introduction of novel vectors or reservoir hosts, increased host densities that increase transmission, environmental changes that increase vector densities, or exposure of hosts to vectors. Additionally, stress due to oiling, lack of food, or degraded habitat can predispose penguins to develop clinical disease resulting from infection with a parasite that might not have been pathogenic in an immunocompetent healthy bird.

Spirochetes morphologically similar to *Borrelia* have been detected in blood smears of African penguins admitted to rehabilitation centers in South Africa. The current study was initiated to determine the prevalence of this spirochete in the African penguin and to characterize the organisms by sequence analysis of the *flaB* gene.

Materials and methods

From January 2002 to December 2010, blood smears were made from 8,343 African penguins admitted to the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) facility in Cape Town (Western Cape) ($n=7,693$) and the Penguins- Eastern Cape (PEC) facility in Cape St. Francis (Eastern Cape) ($n=650$). Smears were stained with either eosin–methylene blue or a modified Wright-Giemsa stain (Kyro-Quick stain, Kyron Laboratories, Benrose, South Africa) and examined for blood parasites. Upon admission, each penguin is given a full physical exam, and blood and fecal samples are collected for parasite analysis and birds are treated with a topical insecticide. Ectoparasites were observed on some birds but were not collected systematically. Soft ticks that were examined were identified as *Carios* sp. Chi-square analysis with Yates correction was used to detect differences in prevalence among month of sampling, season, and age.

To molecularly characterize the spirochete observed in blood smears, we conducted polymerase chain reaction (PCR) for *Borrelia* spp. on blood ($\sim 20 \mu\text{l}$) from two penguins from 2009. Blood was spotted on glass slides, allowed to dry, and fixed with ethanol. The dried blood was prepared for DNA extraction by scraping the blood from the slide and mixing with $50 \mu\text{l}$ of phosphate-buffered saline.

DNA was extracted from the solution using QIAGEN DNA extraction kit (QIAGEN Inc., Valencia, CA).

A 330-bp region of the flagellin gene (*flaB*) was targeted using the external primers FLALL and FLARL and the internal primers FLALS and FLARS in a nested PCR assay previously described (Barbour et al. 1996). Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination. DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas. A negative water control was included in each set of DNA extractions, and one water control was included in each set of primary and secondary PCRs.

Sequences obtained from this study and from other *Borrelia* spp. stored in GenBank were aligned using the multisequence alignment ClustalX program (Thompson et al. 1994). Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis program version 2.1 (Kumar et al. 2004) using the neighbor-joining algorithm using the Kimura 2-parameter model and maximum parsimony using a heuristic search. The GenBank accession number for the *flaB* gene sequence of the *Borrelia* sp. from the African penguins is JN402326.

Results

Spirochetes morphologically compatible with *Borrelia* (Fig. 1) were observed in blood smears from 115 of 8,343 (1.4%) African penguins. Significant differences in prevalence rates were noted by age with higher prevalence rates being noted in chicks ($p<0.0001$) and juveniles ($p<0.0004$) compared with adult penguins (Table 1). Of the four infected adult penguins detected, at least three were believed to be

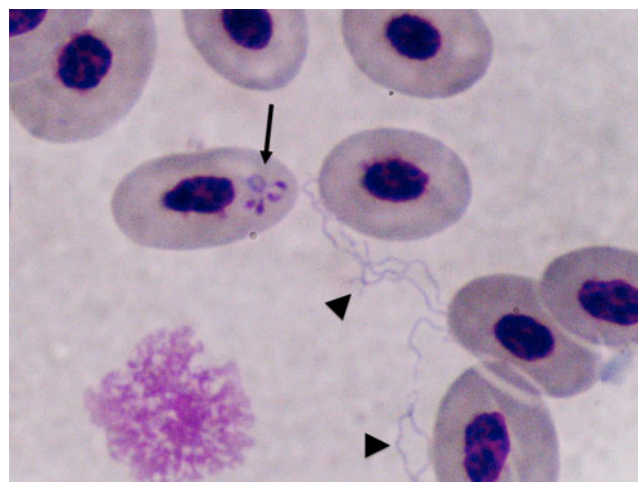


Fig. 1 Eosin–methylene blue-stained thin blood smear from an African penguin with a co-infection of *B. peircei* (arrow) and relapsing fever *Borrelia* (arrowheads to two groups of spirochetes)

Table 1 Seasonal and age-related difference in the prevalence of a relapsing fever *Borrelia* infection in African penguins admitted to two rehabilitation centers in South Africa

| Facility | Years | No. positive/No. tested (%) | | | | | |
|----------|-----------|-----------------------------|-----------------|----------------------|----------------------|---------------------|--|
| | | Season | | Age | | | |
| | | Summer | Winter | Chick | Juvenile | Adult | |
| SANCCOB | 2002–2008 | 82/2,699 (3.04)* | 4/3,630 (0.11)* | 68/1,833 (3.71)**,** | 15/1,800 (0.83)**,** | 3/2,696 (0.11)**,** | |
| PEC | 2008–2009 | 5/252 (1.98) | 1/255 (0.39) | 0/29 | 5/404 (1.2) | 1/74 (1.4) | |

* $p < 0.0001$, ** $p < 0.0001$, *** $p < 0.0001$, **** $p = 0.0004$

immunocompromised because two were oiled and one was molting prior to submission. The fourth adult penguin was admitted with neurologic signs probably related to clinical borreliosis (see below). A significantly higher prevalence was noted in summer months (Oct–Feb) compared with winter months (March–Sept) (Table 1). Among the summer months, penguins sampled in November (5.1%) and December (4.8%) had significantly ($p < 0.0001$) higher prevalence rates compared to all other months and accounted for 84 of the 115 (73%) total positives. At SANCCOB, prevalence rates have increased significantly since 2002 ($\chi^2 = 37.17$, $df = 6$, $p < 0.00001$) with prevalence rates in 2002 and 2003 being significantly less than in 2004, 2006–2008, and 2010 (all $p < 0.007$) (Fig. 2). No differences in prevalence between years were noted at PEC (Fig. 2). Interestingly, not all penguins were blood smear positive immediately upon admission. At SANCCOB, 55 (50%) penguins were negative at admission but were first noted to be positive >6 days post admission with 10 penguins only being noted as positive after being in the facility for >30 days.

At SANCCOB, the majority of *Borrelia*-infected penguins ($n = 82$, 75%) did not receive antibiotic treatment;

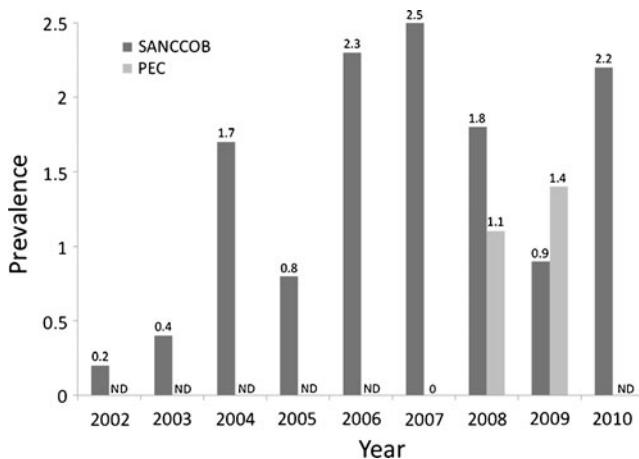


Fig. 2 Prevalence of *Borrelia* on thin blood smears from African penguins from South Africa admitted to two rehabilitation centers. Samples were collected at SANCCOB from 2002 to 2010 and at PEC from 2007 to 2009

however, the majority of these infected birds ($n = 60$, 73%) were ultimately released. Numerous causes of death were determined for 33 *Borrelia*-infected penguins including pneumonia, airsacculitis, aspergillosis, babesiosis, gastroenteritis, hematoma, blindness, malaria, and one possible case of borreliosis. Cause of death was not determined for the remainder because post mortem examinations were not conducted. At PEC, one penguin was admitted with a history of unsteady gait, circling, and torticollis. The penguin was given supportive care and 15 mg enrofloxacin daily per os (Baytril, Bayer) but died 4 days after admission. Grossly, the penguin had splenomegaly and possibly hepatomegaly. Histologically, splenic reticuloendothelial hyperplasia with hemosiderosis; edema in the lungs; and moderate, subacute, lymphocytic meningoencephalitis were noted. In response to this death, the other *Borrelia*-positive penguins at PEC received 25–50 mg/kg doxycycline daily per os for at least 10 days.

Coinfections with other vector-borne parasites were noted on blood smear analysis at both rehabilitation centers with 5 and 40 penguins being infected with *Plasmodium* spp. and *B. peircei*, respectively, at SANCCOB and three of six penguins from PEC being infected with *B. peircei* (Fig. 1). Only 1 of the 650 penguins from PEC was positive for *Plasmodium* spp.

A 327-bp amplicon was amplified and sequenced from two *Borrelia* blood smear-positive penguins. The two sequences were identical and were most similar (99%) to *Borrelia* sp. K64 previously detected in *Carios sawaii* ticks removed from seabirds in Japan (Takano et al. 2009) followed by numerous *Borrelia turicatae* sequences (97.9%). Phylogenetic analyses indicated that the penguin *Borrelia* sp. was included in a well-supported clade with *Borrelia* sp. K64 and *B. turicatae* (Fig. 3).

Discussion

Several different species of *Borrelia* have been previously reported from numerous avian species including *Borrelia anserina*, the causative agent of fowl spirochetosis, and

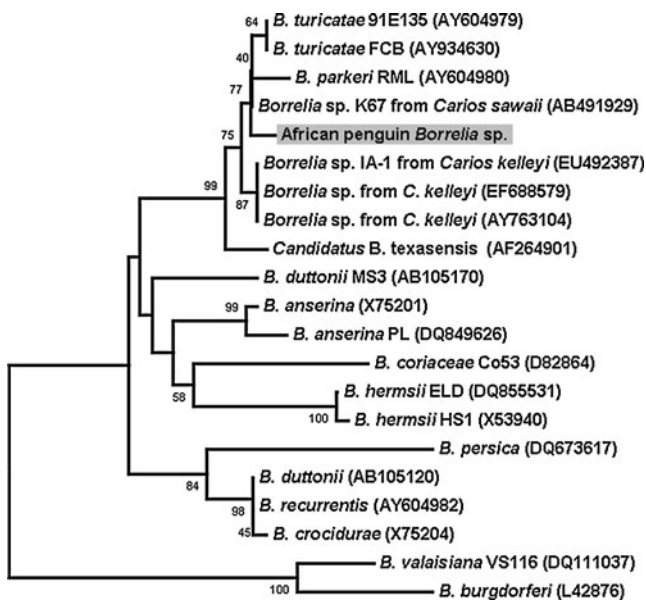


Fig. 3 Neighbor-joining phylogenetic analysis of a relapsing fever *Borrelia* sp. detected in African penguins

Borrelia burgdorferi sensu lato (including *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia valaisiana*, each causative agents of Lyme disease). *B. anserina* is found worldwide and was once a significant pathogen of domestic poultry (McNeil et al. 1949). Changes in poultry rearing have resulted in the elimination of soft tick infestations, but borreliosis can still be a problem in small or backyard flocks (Ataliba et al. 2007). Worldwide, passerines are common hosts for different genospecies of *B. burgdorferi* sensu lato (Richter et al. 2000; Michalik et al. 2008). Enzootic cycles of *B. garinii* are maintained in numerous species of seabirds throughout the world by *Ixodes uriae*, a common tick species infesting seabirds (Olsén et al. 1993; Gylfe et al. 1999). Exposure of seabirds appears to be variable by host species and by locality which may be related to differential behavior of hosts (nesting density), habitat suitability for ticks, or host reaction to *Borrelia* infections (Staszewski et al. 2008).

Among penguins, only a single study has reported antibodies to a *Borrelia* species (*B. burgdorferi*) in king penguins (*Aptenodytes patagonicus*) from the Crozet Archipelago in the southern Indian Ocean (Gauthier-Clerc et al. 1999). Because no spirochetes were isolated and characterized, the species of *Borrelia* infecting the king penguins is unknown. Based on our phylogenetic studies, the *Borrelia* sp. we detected in the African penguins from eastern South Africa was clearly a relapsing fever *Borrelia* (RFB) and was distinct from other RFB including *B. anserina*. This finding represents only the fourth report of a RFB in an avian species. The first report was in a northern spotted owl (*Strix occidentalis caurina*) that died from a systemic RFB infection caused by *Borrelia hermsii*, a zoonotic RFB that utilizes rodents as natural hosts (Thomas

et al. 2002; Bunikis et al. 2004; Fischer et al. 2009). Domestic chickens are naturally infected with two RFBs, *B. anserina* and *Borrelia duttonii*. Although *B. anserina* can be a significant pathogen, *B. duttonii* has not been shown to cause clinical disease in experimental or naturally infected chickens (Kervan 1947; McCall et al. 2007).

Tick-borne relapsing fever of humans is a zoonotic infection caused by 15 species of RFB and is associated with recurrent periods of pyrexia and spirochetemia. Each species of RFB is associated with a specific species of soft tick (*Ornithodoros* sp.) vector. In many parts of Africa, diagnosis is complicated because of the high prevalence of malaria that has a similar clinical presentation (Nordstrand et al. 2007). In western Africa, *Borrelia crocidurae*, which utilizes rodents and shrews as reservoir hosts, is most often associated with human disease (Godeluck et al. 1994). *B. duttonii* is common in eastern and central Africa and is rarely found in western Africa (Fukunaga et al. 2001; Nordstrand et al. 2007). In Tanzania, natural infections with *B. duttonii* have been reported in pigs and chickens (McCall et al. 2007), but no wildlife reservoir has been identified. In addition, molecular characterization of *Borrelia* from human RFB cases and ticks in Tanzania indicates that another undescribed species is endemic (Kisinzia et al. 2003; Mitani et al. 2004). Although rare human RFB cases have been reported in South Africa since 1912, the *Borrelia* species has not been determined (Rosenthal 1982). Two other RFBs, *Borrelia graingeri* and *Borrelia tillae*, have been isolated from *Ornithodoros* spp. in southern Africa, but these species have not been associated with human infections (Zumpt and Organ 1961) nor have they been genetically characterized. Thus, we do not know the relatedness of the penguin RFB to the RFB agent in South African humans, *B. graingeri* or *B. tillae*. We detected *Carios* (formerly *Ornithodoros*) sp. ticks on some penguins but none were tested for *Borrelia* because they were blood-fed. Future studies should investigate the vector potential of this *Carios* sp. for the penguin *Borrelia* sp.

The zoonotic potential of the RFB detected in African penguins is unknown, but many RFB species are zoonotic. The *Borrelia* sp. from the African penguin was most closely related to a RFB detected in soft ticks (*C. sawaii*) from a colony of Swinhoe's storm petrels (*Oceanodroma monorhis*) and streaked shearwaters (*Calonectris leucomelas*) in Japan (Takano et al. 2009). Ticks from this bird colony have been associated with a disease consistent with RFB in humans (Tsurumi et al. 2002).

Currently, little is known about this *Borrelia* species; for example, is the African penguin the natural host, is it emerging in penguins, or is it a possible pathogen for penguins or humans? Although infections of penguins were noted at one facility annually since 2002, there was a general trend of increasing prevalence at both rehabilitation

centers. The pathogenicity of this RFB for penguins is unknown, but it is likely not highly pathogenic to immunocompetent penguins as the majority of infected penguins were released without any antibiotic treatment. In general, immunocompromised or naive individuals are more likely to become infected, and in this study, we found a higher prevalence in chicks and in adults with concurrent issues. One of the *Borrelia*-positive penguins that died did have neurologic signs and lesions similar to those reported in the fatal infection of an owl with *B. hermsii* and in domestic fowl with *B. anserina* infections (McNeil et al. 1949; Dickie and Barrera 1964; Thomas et al. 2002; Bunikis et al. 2004). Similarly, a fatal case of RFB infection in a *Pipistrellus* bat from southern England had anemia, splenomegaly, hepatomegaly, and blood-tinged pleural fluid (Evans et al. 2009). Further studies are needed to determine the other possible hosts (avian or mammalian), vector, distribution, pathogenicity, and prevalence of this *Borrelia* sp. in African penguins throughout their range. Because populations are declining significantly, the emergence of any potential pathogen represents an important threat to the long-term recovery of the endangered African penguin population.

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