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Short communication

First record of *Babesia* sp. in Antarctic penguins

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ABSTRACT

This is the first reported case of *Babesia* sp. in Antarctic penguins, specifically a population of Chinstrap penguins (*Pygoscelis antarctica*) in the Vapour Col penguin rookery in Deception Island, South Shetlands, Antarctica. We collected peripheral blood from 50 adult and 30 chick Chinstrap penguins. Examination of the samples by microscopy showed intraerythrocytic forms morphologically similar to other avian *Babesia* species in 12 Chinstrap penguin adults and seven chicks. The estimated parasitaemias ranged from 0.25×10^{-2}% to 0.75×10^{-2}%. Despite the low number of parasites found in blood smears, semi-nested PCR assays yielded a 274 bp fragment in 12 of the 19 positive blood samples found by microscopy. Sequencing revealed that the fragment was 97% similar to *Babesia* sp. 18S rRNA from Australian Little Penguins (*Eudyptula minor*) confirming presence of the parasite. Parasite prevalence estimated by microscopy in adults and chicks was higher (24% vs. 23.3%, respectively) than found by semi-nested PCR (16% vs. 13.3% respectively). Although sampled penguins were apparently healthy, the effect of *Babesia* infection in these penguins is unknown. The identification of *Babesia* sp. in Antarctic penguins is an important finding. *Ixodes uriae*, as the only tick species present in the Antarctic Peninsula, is the key to understanding the natural history of this parasite. Future work should address the transmission dynamics and pathogenicity of *Babesia* sp. in Chinstrap penguin as well as in other penguin species, such as Gentoo penguin (*Pygoscelis papua*) and Adélie penguin (*Pygoscelis adeliae*), present within the tick distribution range in the Antarctic Peninsula.

1. Introduction

The genus *Babesia* (Phylum Apicomplexa) comprises more than 100 species of tick-borne parasites that infect erythrocytes in a wide variety of vertebrate hosts (Spielman et al., 1985). Several species of *Babesia* are significant pathogens of veterinary and medical importance. However, despite their prevalence in mammals, there is little information concerning their incidence in avian hosts (Schnittger et al., 2012). Currently, there are about 15 species of birds from which *Babesia* spp. have been reported; four of these are seabirds, two of which are penguins (Yabsley et al., 2009), the Jackass penguin (*Spheniscus demersus*) and the Little Penguin (*Eudiptula minor*) (Earle et al., 1993; Peirce, 2000; Vanstreels et al., 2015). The likely presence of *Babesia* spp. in Antarctic penguins has not been given enough attention, probably due to the idea that no suitable vectors, such as ticks, are present in this continent. There are no previous data in the literature on *Babesia* in Antarctic birds (Barbosa and Palacios, 2009). Although the presence of the tick *Ixodes uriae* was known in Antarctica (Lee and Baust, 1982), its distribution and relative abundance was not established until very recently (Barbosa et al., 2011), when it was shown to have wide distribution along the Antarctic Peninsula. The presence of *I. uriae* in the Antarctic Peninsula has now been recorded on King George Island (62°15′ S 58°37′ W), Livingston Island (62°39′ S 60°36′ W and 62°40′ S 61°13′ W), Deception Island (63°00′ S 60°40′ W), Ronge Island (64°40′ S 62°40′ W), Humble Island (64°46′ S 64°06′ W), Doumer Island (64°51′ S 63°55′ W) and Petermann Island (65°11′ S 64°10′ W) (Barbosa et al., 2011).

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Considering this wider distribution of ticks and their potential to transmit pathogens, it is expected that this *Babesia* sp. and other tick-borne pathogens may be present in Antarctic penguins. The focus of this study was to identify and report on the presence of *Babesia* sp. in Antarctic penguins, specifically in a population of Chinstrap penguins (*Pygoscelis antarctica*) in the Vapour Col penguin rookery in Deception Island, South Shetlands, Antarctica.

2. Materials and methods

The study was conducted at the Vapour Col Chinstrap penguin (*P. antarctica*) rookery on Deception Island, South Shetlands (63°00′ S 60°40′ W), Antarctica, from December to January during the austral summer of 2012–2013. In light of previous information regarding the distribution of ticks in this colony (Barbosa et al., 2011), we sampled randomly 50 adult Chinstrap penguins and 30 chicks in three sub-colonies where ticks were present. Sampling was carried out during the guard phase in late December and early January. Penguins were carefully captured by hand on the nest. When adults were captured, the chicks were taken out of the nest and kept safe from predation. After blood collection, adults and chicks were replaced in the nest and adults were observed to immediately resume care of their progeny. Blood samples were taken from a foot vein with a heparinized capillary tube immediately after capture. One drop of blood was then smeared on individually marked microscope slides, air-dried and fixed in 96° ethanol for 5 min. The remaining sample was later centrifuged at 12,000 rpm for 10 min to separate plasma from red blood cells (RBCs), and frozen at −20 °C until further analyses.

Blood smears were stained with Giemsa Stain Modified Solution (Sigma–Aldrich, Missouri, USA) for 30 min and examined with an Axioskop 2® microscope (Carl Zeiss, New York, USA) at a magnification of 1000× under oil immersion. Each smear was examined for 30 min for the presence of blood parasites. Nineteen smears were considered positive and subsequently re-examined by counting nearly 40,000 RBCs per slide using a DMI3000 B® microscope (Leica, Wetzlar, Germany) and 60× magnification objective under oil immersion. Images were taken with a digital camera using LAS v4.0® software (Leica). Morphometric measurements of each form were calculated using a screw micrometer calibrated against a standard stage micrometer.

Packed RBCs from the positive blood samples were diluted 1:10 in Phosphate Buffered Saline and homogenized with the TissueRuptor® (Qiagen, Valencia, USA). Genomic DNA was isolated from 200 μL of each homogenized sample using the QIAamp DNATissue Mini Kit® (Qiagen) and the QIAcube robotic workstation (Qiagen), following to manufacturer's instructions. Then, a 1873 bp product of the *P. antarctica* 18S rRNA gene was amplified (GenBank accession no KP875235) using four of the DNA samples and the eukaryotic 18S rRNA gene primers 18SPF (5′AACCTGGTTGATCCTGCCAGT3′) and 18SPR (5′TGATCCTTCTGCAGGTTCACC3′) (Medlin et al., 1988). To prevent unspecific fragments being amplified, the 1873 bp from the *P. antarctica* 18S rRNA gene was aligned, using ClustalW2, with sequences from other *Babesia* spp. 18S rRNA genes obtained from the GenBank database. Therefore, two specific primers named BF10 (5′GGTGACCTAAACCCTCACCAG3′) and BR12 (5′ATGCTGAAGTATTCAAGACAAAAGT3′) were designed to amplify a specific fragment of the *Babesia* spp. 18S rRNA gene by semi-nested PCR. Then, packed RBCs from the 19 positive blood samples were examined for *Babesia* sp. by semi-nested PCR. The first PCR was performed using the primers BF10 and BabRLBR (Vanstreels et al., 2015) in a 25 μL final reaction volume of containing 200–500 ng of gDNA, 1× PCR buffer with 2 mM MgCl2 (Biotools B&M Labs, Madrid, Spain), 200 μM of each deoxynucleoside triphosphate (Biotools), 0.4 μM of each primer (Biotools) and 1 unit of Taq polymerase (Biotools) with 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. The second PCR was carried out using 1 μL of the first PCR and the primers BF10-BR12 for the semi-nested amplification. The reagents and conditions were the same as in the first PCR. DNA from *Babesia divergens* (Bd Rouen 1986 strain) was used as a positive control and included in all PCR tests. The amplified product was purified using a QIAquick Gel Extraction Kit (Qiagen) and sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City California).

Phylogenetic relationships of *Babesia* sequences obtained in the present study were inferred by using 57 sequences of the *Babesia* spp. and 4 sequences of *Theileria* spp. retrieved from GenBank. These sequences and the *Babesia* sp. from *P. antarctica* were aligned in ClustalX using the default settings. The resulting alignment was checked and adjusted with Se-Al v2.0a11 (Rambaut, 2002). Consequently, a matrix with the final alignment was generated.

Phylogenetic relationships for the matrix were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian Inference (BI). Twenty million generations in two parallel runs were performed, sampling trees at 2000 generation intervals. The first 25% of sampled trees were discarded, and the remaining trees were used to calculate the posterior probabilities.

3. Results and discussion

Intraerythrocytic forms compatible with *Babesia* sp. were observed in the peripheral blood of 12 Chinstrap penguin adults and 7 chicks. The parasites were detected only inside the RBCs, and usually in small numbers. There was one form for each parasitized cell which did not displace the nucleus of the RBC. The estimated parasitaemias ranged from 0.25×10^{-2}% to 0.75×10^{-2}%. Hemozoin pigment was not detected within the forms. The spherical, elongated and amoeboid forms observed were similar to the rings previously described for the avian *Babesia percei* (Earle et al., 1993), *Babesia poelea* (Work and Rameyer, 1997), *Babesia uriae* (Yabsley et al., 2009), *Babesia ugwidiensis* (Peirce and Parsons, 2012) and *Babesia moshkovskii* (Merino et al., 2002) piroplasms. These forms had a pale cytoplasm that occasionally looked like a refractile vacuole. Spherical forms ($n = 17$) were 1.59 ± 0.39 μm with a central cytoplasm and a single chromatin dot on one side, two dots on different sides or chromatin spread around the parasite edge. The elongated forms ($n = 15$) having a diameter of 1.97 ± 0.57 μm, showed a chromatin dot in a distal position. Amoeboid shapes ($n = 6$) measuring 2.24 ± 0.40 μm were less frequent, and showed the chromatin spread around the periphery. The increasing size of these amoeboid forms and their chromatin distribution may indicate division of chromatin as described for *B. percei* (Earle et al., 1993) and *B. ugwidiensis* (Peirce and Parsons, 2012). Although, one possible tetrad precursor (3.48 μm of diameter) similar to those described for *B. ugwidiensis* (Peirce and Parsons, 2012) was observed at the periphery of the host cell (Fig. 1 and Supplementary file S1). Further development of precursors into tetrad forms was not detected. The prevalence of all these forms, at 24% and 23.3% did not significantly differ between Chinstrap adults (12/50) and Chinstrap chicks (7/30) (Chi-square test = 0.004, $p = 0.94$) respectively. All forms were morphologically indistinguishable from *Babesia* spp.

Therefore, the 19 RBC samples, which showed intraerythrocytic forms under microscopy, were tested by semi-nested PCR. A 274 bp fragment was amplified in 12 of the total of 19 samples. No DNA amplification was detected for the Chinstrap penguin. A BLASTN search (Zhang et al., 2000) revealed that the nucleotide sequences of the amplified product were clearly assigned to *Babesia* spp. The nucleotide sequencing (GenBank accession no. KT800053) showed