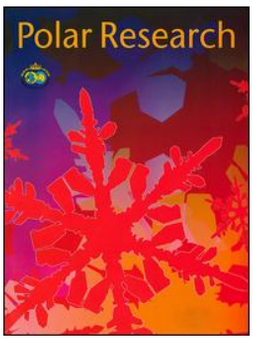

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RESEARCH NOTE

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Investigation of *Babesia* sp. in pygoscelid penguins at the South Shetland Islands

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ABSTRACT

Babesia spp. are tick-borne parasites, and 16 avian-infecting species have been described to date, including one species (*Babesia peircei*) that infects penguins. Considering the results of a recent study reporting *Babesia* sp. in penguins on Deception Island, South Shetland Islands, we re-examined the samples obtained in a previous investigation on the occurrence of blood parasites in adult Adélie (*Pygoscelis adeliae*), chinstrap (*Pygoscelis antarcticus*) and gentoo penguins (*Pygoscelis papua*) on King George and Elephant islands, South Shetland Islands. Notwithstanding a comprehensive re-examination of the blood smears, *Babesia* sp. was not detected. When we employed two nested PCR tests targeting the *18S rRNA* gene of *Babesia*, a considerable proportion of the samples produced positive results; however, gene sequencing revealed these were due to cross-amplification of non-target organisms. We therefore did not detect *Babesia* sp. infection in penguins on King George and Elephant islands. Additional studies will be valuable to clarify the distribution and epidemiology of tick-borne pathogens in sub-Antarctic and Antarctic seabirds.

KEYWORDS
Antarctica; blood; seabird; Spheniscidae; tick-borne pathogen

ABBREVIATION
PCR: polymerase chain reaction technique

Babesia spp. are tick-borne protozoan parasites (Apicomplexa: Piroplasmida) that infect birds and mammals. Sixteen avian-infecting species have been described to date (Peirce 2000; Yabsley et al. 2009; Peirce & Parsons 2012), and one species, *Babesia peircei*, is known to infect penguins (Earlé et al. 1993). In a previous study (Vanstreels et al. 2013), we investigated the occurrence of blood parasites in Adélie (*Pygoscelis adeliae*), chinstrap (*Pygoscelis antarcticus*) and gentoo penguins (*Pygoscelis papua*) sampled in the South Shetlands Islands in 2011/12. We found no evidence of parasites in blood smears, and did not detect DNA from *Haemoproteus* and *Plasmodium* through nested PCR testing targeting the *cyt-b* gene. Considering that previous studies examining blood smears of pygoscelid penguins in the South Shetland Islands and along the Antarctic Peninsula had failed to detect any blood parasites (Myrcha & Kostelecka-Myrcha 1980; Zinsmeister & Van Der Heyden 1987; Merino et al. 1996; Moreno et al. 1998; Vleck et al. 2000; González-Acuña et al. 2013), this led us to suggest that blood parasites were likely absent in penguins breeding in the archipelago.

However, Montero et al. (2016) found evidence of the occurrence of *Babesia* sp. in chinstrap penguins at Deception Island, South Shetland Islands, while examining samples collected during the 2012/13 breeding season. Intraerythrocytic inclusions consistent with *Babesia* sp. were seen in the blood smears of 23% of chicks (7/30) and 24% of adults (12/50). When the samples from smear-positive individuals were tested with a newly designed semi-nested PCR targeting a fragment of the *18S rRNA* gene, however, only 13% of chicks (4/30) and 16% of adults (8/50) were confirmed as positive. Phylogenetic analysis of a short fragment of the *18S rRNA* gene revealed a high similarity to *Babesia* sp. from little penguins (*Eudyptula minor*) from Australia. Intrigued by these results, we thoroughly re-evaluated the samples from our original study (Vanstreels et al. 2013), this time also employing molecular methods specifically targeting *Babesia* sp.

Materials and methods

We re-evaluated blood smears from 64 adult pygoscelid penguins sampled in 2011/2012 in the South Shetland Islands: Keller Peninsula (62°4′55″S 58°24′32″W; seven *P. antarcticus*, 18 *P. papua*), Demay Point (62°12′25″S

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58°27′7″W; 10 *P. antarcticus*) and Thomas Point (62° 10′23″S 58°28′31″W; two *P. adeliae*, one *P. antarcticus*, two *P. papua*) at Admiralty Bay, King George Island, and Stinker Point, Elephant Island (61°13′20″S 55° 21′35″W; 24 *P. papua*). An experienced observer (R.E.T. Vanstreels) evaluated an additional 10^5 cells (in the original study we had examined 3×10^4 cells) under 1000× magnification.

Frozen blood samples were available for a subset of these individuals (two *P. adeliae*, 17 *P. antarcticus*, 19 *P. papua*; all from King George Island). DNA was extracted from the blood samples in the laboratory using a standard phenol/chloroform and proteinase K extraction followed by ethanol precipitation (Sambrook et al. 2001) and non-phenolic extraction (Carvalho 2010); DNA extraction was verified and quantified with Nanodrop 2000 spectrophotometry (ThermoScientific). Quality of extracted DNA was further verified through molecular sexing (see Brummelhaus et al. 2015). Samples were tested with two nested PCR tests targeting the *18S rRNA* gene of *Babesia*: (a) nested PCR_1 employs primers Bab5.1 and BabB (ca. 1800 bp amplicon) followed by BabRLBF and BabRLBR to amplify a 460–520 bp fragment, and (b) nested PCR_2 employs primers Bab5.1 and BabB followed by Bab5.1v2 and Bab3.1 to amplify a 1620–1720 bp fragment. PCR protocols were identical to those described by Vanstreels et al. (2015). Gel electrophoresis was conducted to visualize amplification products, using 2% agarose gel electrophoresis with SYBR Safe (S33102, Invitrogen) and 6% polyacrylamide gel electrophoresis with silver staining. PCR amplification products of positive samples were purified with Polyethylene Glycol 8000. Bi-directional Sanger sequencing with dye-terminator fluorescent labelling (Applied Biosystems 4 337 455, Life Technologies) was performed through automated sequencing (ABI Prism 3100, Applied Biosystems). Traditional precautions to avoid DNA contamination (laminar flow hoods, separate work areas for reaction mixture preparation, DNA extraction, primary and secondary amplification reactions and gel electrophoresis) were used to prevent carry-over of amplified products. Blood from a Cape cormorant (*Phalacrocorax capensis*) naturally infected with *Babesia ugwidiensis* and blood from a chicken raised in an arthropod-free environment were used as controls in all reactions; the amplification products of the positive control were sequenced to verify the reaction.

Results

We did not observe any parasites while re-examining the blood smears. Nested PCR_1 identified 75% of the chinstrap penguins (12/16), 37% of the gentoo penguins (7/19) and none of the Adélie penguins (0/2) as positive, with electrophoretic bands of approximately 530 bp (whereas the positive control had approximately 510 bp). Nested PCR_2 agreed with nested PCR_1 for all but one individual: 69% of the chinstrap penguins (11/16), 37% of the gentoo penguins (7/19) and none of the Adélie penguins (0/2) were positive, with electrophoretic bands of approximately 1660 bp (whereas the positive control had approximately 1560 bp). These results were consistent when all nested PCR tests were repeated. However, despite two to four attempts to sequence the amplification products from each sample, gene sequencing was largely unsuccessful, as samples failed to produce chromatograms with acceptable quality. For three samples, however, partial sequences (112–401 bp) with high similarity to the *18S rRNA* gene of fungi (98– 99% sequence identity to *Saccharomyces cerevisiae*) were produced (GenBank MG745326–8), indicating cross-reactivity of the primers with non-target organisms, presumably fungal contaminants.

Discussion

Despite our thorough re-examination of the samples from our previous study (Vanstreels et al. 2013), we could not find any morphological or molecular evidence of *Babesia* infections in Adélie, chinstrap and gentoo penguins on King George and Elephant islands.

The fact that the primers used to target the *18S rRNA* gene of piroplasmids can also amplify the gene sequences of non-target fungal organisms is not unexpected considering that they were originally intended for phylogenetic studies and were designed to target highly conserved regions (Medlin et al. 1988; Gubbels et al. 1999; Criado-Fornelio et al. 2003), hence they were not designed to be genus-specific for diagnostic purposes. Previous studies have shown that primers targeting the *18S rRNA* gene of avian piroplasmids may also occasionally cross-amplify host DNA (Vanstreels et al. 2015; Montero et al. 2016). Our results underscore that when using primers targeting highly conserved sequences such as the *18S rRNA* gene, it is imperative to sequence amplification products, and only sequencing-confirmed results should be considered as positive in estimates of piroplasmid prevalence.

Despite their inherent limitations in terms of specificity, the primers targeting the *18S rRNA* gene employed in this study have been successfully employed in other studies to detect *Babesia* spp. in birds (Yabsley et al. 2006; Yabsley et al. 2009), including penguins (Vanstreels et al. 2015; Yabsley et al. 2017). Unfortunately, the *18S rRNA* gene sequence produced by Montero et al. (2016) for *Babesia* sp. from chinstrap penguins at Deception Island was very short (228 bp excluding primers) and does not cover the annealing sites of the primers employed in this study. It is therefore not possible at present to determine the sequence identity between the primers employed in this study and that of the *Babesia* strain reported by Montero et al. (2016). However, considering that the partial *18S rRNA* gene

sequence produced by Montero et al. (2016) has a high identity to the sequence of *Babesia* sp. from little penguins (226/228 nucleotides; 99.1%) that was produced using the same methods as in this study (see Vanstreels et al. 2015), it seems reasonable to assume that the primers and protocols employed in this study would have been successful in detecting the *Babesia* sp. strain reported by Montero et al. (2016) if it had been present in the studied samples.

The fact that we did not detect *Babesia* sp. in this study could be related to our relatively small sample size and/or to a lower prevalence of ticks at the studied colonies than on Deception Island. Barbosa et al. (2011) found that the abundance of stones with ticks was much higher in the Deception Island penguin colony (26%) than at other colonies along the South Shetland Islands (0 – 10%), including Stranger Point (9%). It is therefore plausible that the remarkably high prevalence of *Babesia* sp. found by Montero et al. (2016) at penguins sampled at Deception Island might be related to the abundance and distribution of ticks at that colony. Alternatively, it is also possible that the high *Babesia* prevalence reported at Deception Island may have been an overestimation or represent an unusual epidemiological circumstance, i.e., an epizootic.

It should be noted that although the *Babesia*-positive penguins studied by Montero et al. (2016) were apparently healthy, there is evidence that *Babesia* infections can be pathogenic to seabirds. *Babesia* infection may lead to mild anaemia in little penguins (*Eudyptula minor*; Sergent et al. 2004) and is associated with mild anaemia, leukocytosis and impairment of hepatic function in African penguins (*Spheniscus demersus*; Parsons et al. 2016). Furthermore, there are cases of *Babesia* hyper-infections leading to the death of common murres (*Uria aalge*; Yabsley et al. 2009) and a king penguin (*Aptenodytes patagonicus*) during rehabilitation (Parsons et al. 2017). It is therefore clear that additional studies are necessary to provide insight into the epidemiology of tick-borne pathogens on Antarctic seabirds, especially considering their potential implications to the health and conservation of these species and the predictions that ticks will shift their distribution under climate change (Süss et al. 2008; Ogden & Lindsay 2016).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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