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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 protozoan parasites (Apicomplexa: Piroplasmida) that infect birds and mammals. Sixteen 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 pygosceld 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°45′S 58°24′32″W; seven P. antarcticus, 18 P. papua), Demay Point (62°12′25″S 58°45′55″W; 36 P. papua, five P. antarcticus).
58°27ʹ7ʺW; 10 P. antarcticus) and Thomas Point (62°
10ʹ23ʹʹS 58°28ʹ31ʹʺW; two P. adeliae, one P. antarcticus,
and Stinker Point, Elephant Island (61°13ʹ20ʹʺS 55°
21ʹ35ʹʺW; 24 P. papua). An experienced observer
(R.E.T. Vanstreels) evaluated an additional 10⁶ cells
(in the original study we had examined 3 × 10⁴ 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 PCR₁ 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 PCR₂ 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 sequenc-
ing 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 reac-
tions 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 PCR₁ identified 75% of the
chinstrap penguins (12/16), 37% of the gentoo pen-
guins (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 PCR₂ agreed with nested
PCR₁ 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 chromato-
grams 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 evi-
dence 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 piroplasms can also amplify the gene
sequences of non-target fungal organisms is not unex-
pected 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 pur-
Poses. Previous studies have shown that primers target-
the 18S rRNA gene of avian piroplasms 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 sequen-
cing-confirmed results should be considered as positive
in estimates of piroplasmid prevalence.

Despite their inherent limitations in terms of speci-
ficity, 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). Unfortu-
nately, 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 iden-
tity 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 Vansstreels 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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No potential conflict of interest was reported by the authors.

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### References


species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782–1789.