



Short communication

## First record of *Babesia* sp. in Antarctic penguins

Estrella Montero<sup>a,\*</sup>, Luis Miguel González<sup>a</sup>, Alberto Chaparro<sup>a</sup>, Jesús Benzal<sup>b</sup>, Marcelo Bertellotti<sup>c</sup>, José A. Masero<sup>d</sup>, Roger Colominas-Ciuró<sup>e</sup>, Virginia Vidal<sup>e</sup>, Andrés Barbosa<sup>e</sup>



<sup>a</sup> Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo Km 2,2, Majadahonda, 28220 Madrid, Spain

<sup>b</sup> Department Functional and Evolutionary Ecology, Estación Experimental de Zonas Áridas – CSIC, Carretera de Sacramento s/n, La Cañada de San Urbano, 04120 Almería, Spain

<sup>c</sup> Centro de Estudios de Sistemas Marinos CENPAT-CONICET, Bvd. Almirante Brown 2915, Puerto Madryn U9120ACD, Chubut, Argentina

<sup>d</sup> Zoología, Facultad de Ciencias, Universidad de Extremadura, Av. de Elvas s/n, 06071 Badajoz, Spain

<sup>e</sup> Dept. Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

### ARTICLE INFO

#### Article history:

Received 16 April 2015

Received in revised form 27 January 2016

Accepted 3 February 2016

Available online 10 February 2016

#### Keywords:

Antarctica

*Babesia* sp.

Deception Island

*Pygoscelis antarctica* (Chinstrap penguin)

Vapour Col penguin rookery

### 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  $0.25 \times 10^{-2}\%$  to  $0.75 \times 10^{-2}\%$ . 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.

© 2016 Elsevier GmbH. All rights reserved.

### 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

(*Eudyptula 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).

\* Corresponding author.

E-mail address: [estrella.montero@isci.es](mailto:estrella.montero@isci.es) (E. Montero).

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'TGATCCTTCTGCAGGTTACCC3') (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'GGTGACCTAACCCCTCACCAG3') 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 MgCl<sub>2</sub> (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).

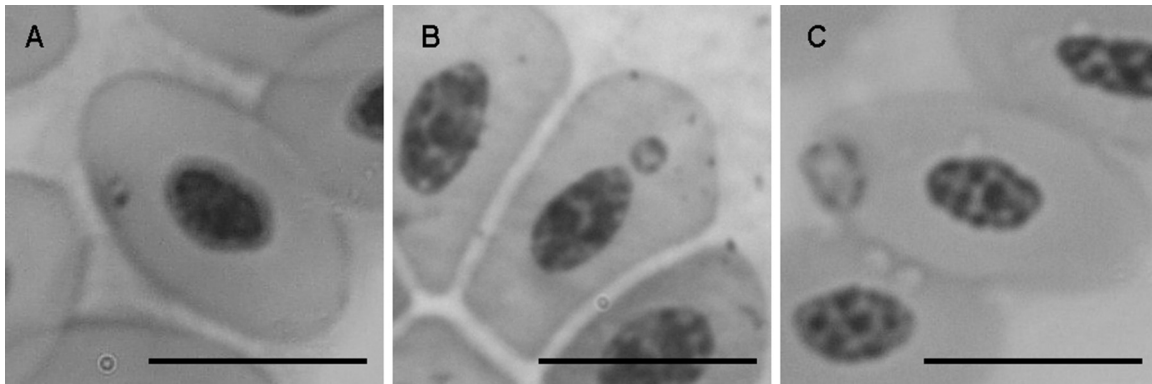
Phylogenetic relationships of *Babesia* sequences obtained in the present study were inferred by using 57 sequences of the *Babesia* spp. and 4 sequences of *Theileria* spp. retrieved from GenBank. These sequences and the *Babesia* sp. from *P. antarctica* were aligned in ClustalX using the default settings. The resulting alignment was checked and adjusted with Se-AI 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  $0.25 \times 10^{-2}\%$  to  $0.75 \times 10^{-2}\%$ . 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 perciei* (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  $1.59 \pm 0.39 \mu\text{m}$  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  $1.97 \pm 0.57 \mu\text{m}$ , showed a chromatin dot in a distal position. Amoeboid shapes ( $n=6$ ) measuring  $2.24 \pm 0.40 \mu\text{m}$  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. perciei* (Earle et al., 1993) and *B. ugwidiensis* (Peirce and Parsons, 2012). Although, one possible tetrad precursor ( $3.48 \mu\text{m}$  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.

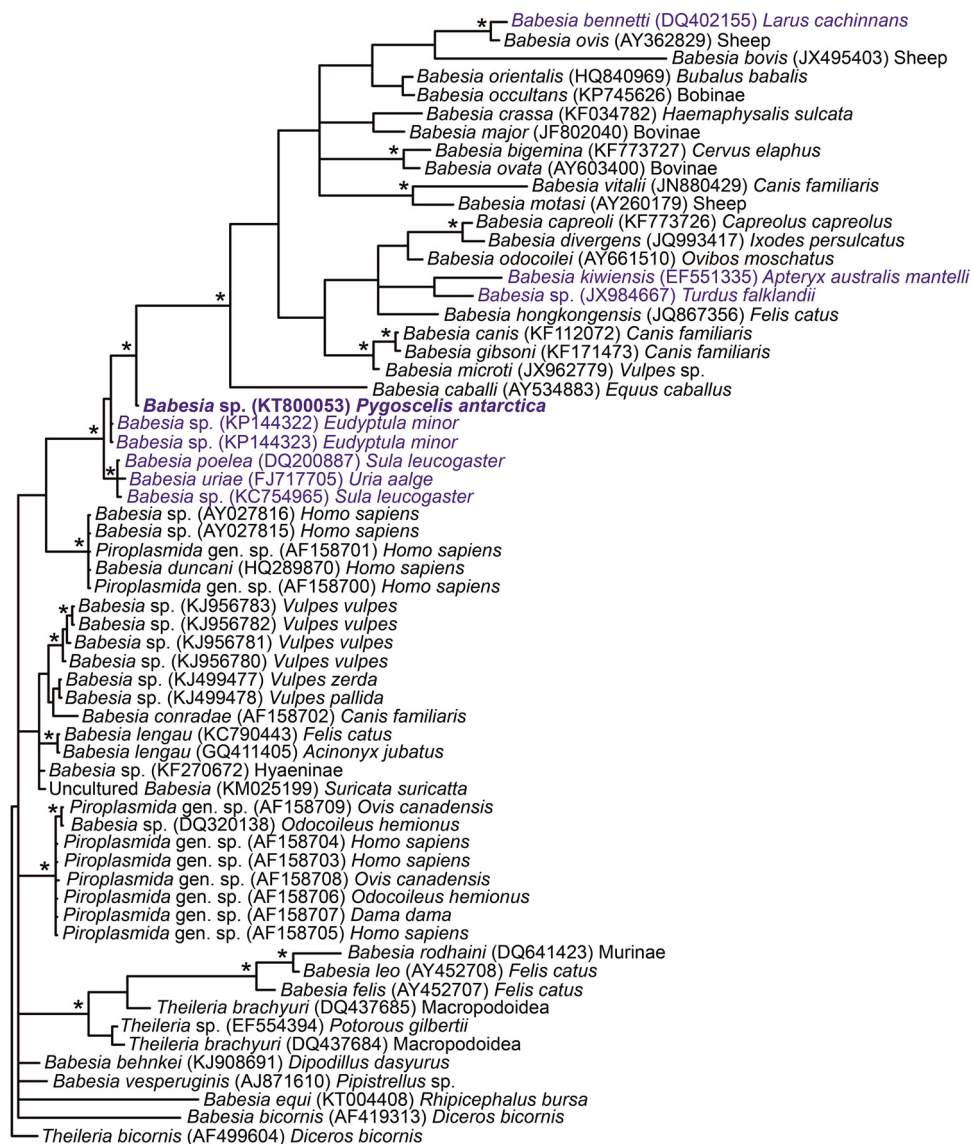
Therefore, the 19 RBC samples, which showed intraerythrocytic forms under microscopy, were tested by semi-nested PCR. A 274 bp fragment was amplified in 12 of the total of 19 samples. No DNA amplification was detected for the Chinstrap penguin. A BLASTN search (Zhang et al., 2000) revealed that the nucleotide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



**Fig. 1.** Intraerythrocytic *Babesia* sp. parasites in Chinstrap penguin RBCs. Photomicrograph of *Babesia* sp. merozoites from three Giemsa-stained thin blood smears of chinstrap penguins from Deception Island, Antarctica. These three samples were also positive by semi-nested PCR. (A–B) rings. (C) Possible tetrad precursor. Bar = 10 µm.

that the fragment was 97% similar to the *Babesia* sp. 18S rRNA gene from Australian Little Penguins (*Eudyptula minor*) (GenBank accession no. KP144323.1 and KP144322.1). The results of our phylogenetic analyses (Fig. 2) clearly indicate that the *Babesia* sequence

from Antarctic penguins is very close to a group of *Babesia* species from seabirds (Little Penguin, Brown booby and Common guillemot) as supported by a high bootstrap value. Considering that most *Babesia* spp. are host specific at the family or subfamily level (Peirce,



**Fig. 2.** Phylogenetic relationships of the 18S rDNA gene sequences of the studied *Babesia* lineages. Tree topology was inferred by Bayesian analyses. Stars mark clade supported by bootstrap values higher than 0.95. Blue color shows *Babesia* found in bird hosts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2000) and the higher distance between the *Babesia* in this study and *B. uriae* and *B. poelea*, the *Babesia* in the Antarctic penguins is potentially a new species.

Prevalence of *Babesia* sp. found by semi-nested PCR in *P. antarctica* was 16% in adults (8/50) and 13.3% in chicks (4/30) (chi-square test,  $X^2_1 = 0.08$ ,  $p = 0.78$ ) which was lower than with optical microscopy. These results could suggest the detection of false positives by optical microscopy, but also that the outcome of the semi-nested PCR could depend on the quality of the sample DNA. However, as other authors have noted (Garamszegi, 2010), the prevalence may differ between studies, not only due to different approaches used, but also because of natural variations in the blood samples. Therefore, each methodology used in this study provided estimated parasite prevalence with a certain amount of error. Future studies, using a larger number of blood samples from the penguin population, will be able to provide more reliable prevalence results.

*Babesia* spp. requires competent vertebrate and non-vertebrate hosts to maintain transmission cycles (Homer et al., 2000), and the primary vectors of *Babesia* are Ixodid ticks (Yabsley and Shock, 2013). It seems that ticks also act as vectors of avian *Babesia* spp. (Earle et al., 1993). In particular, *I. uriae* transmit various pathogenic agents (Nuttall et al., 1984; Olsen et al., 1995; Major et al., 2009), which may have negative effects on seabird populations (Chastel et al., 1987). *I. uriae* is notably the only tick species and potential vector of *Babesia* present along the Antarctic Peninsula (Barbosa and Palacios, 2009; Barbosa et al., 2011; McCoy et al., 2013). These ticks also transmit *Borrelia burgdorferi* to penguins (Barbosa, unpublished data). Other effects of ticks on the penguins remain unknown and deserve more attention. Babesiosis could affect host tissues and cause anemia (Yabsley et al., 2009), however, sampled penguins were apparently healthy. The harsh environmental conditions of the Antarctica Peninsula reduce the activity of the *Ixodes* vector, which only has one meal per season (Frenot et al., 2001). This activity is involved in the relationship between the infected *Ixodes* vector, the *Babesia* sp. parasite and the low parasitemia along with the absence of symptoms in its Chinstrap penguin host. More research on the health of Chinstrap penguins and *Babesia* sp. infection is warranted. Our morphological and molecular results show that *Babesia* sp. was present in *P. antarctica* RBCs. Semi-nested PCR is also in progress to find out the prevalence of *Babesia* infection in this penguin population. Future research will be focused on its pathogenicity of the *Babesia* sp., the possible involvement of *I. uriae* as a vector and the transmission dynamics of this pathogen in the Chinstrap and other penguin species, such as Gentoo (*Pygoscelis papua*) and Adélie penguin (*Pygoscelis adeliae*) present in the distribution range of ticks in the Antarctic Peninsula.

## Acknowledgments

We thank the Spanish Antarctic Base “Gabriel de Castilla”, the Spanish polar ship “Hespérides” and the Marine Technology Unit (CSIC) for hospitality and logistic support. Permission to work in the study area and for penguin handling was granted by the Spanish Polar Committee. RCC was supported by a PhD grant from the Spanish Ministry of Economy and Competitiveness (BES-2012-059299). This study was funded by the Spanish Ministry of Economy and Competitiveness projects (CTM2011-24427 and AGL2010-21774). This is a contribution to the PINGUCLIM project. We very much appreciate the help of Annie Machordom for the phylogenetic

analyses. We thank Aurelio Velasco and Deborah Fuldauer for reviewing the manuscript.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2016.02.006.

## References

- Barbosa, A., Benzal, J., Vidal, V., D'Amico, V., Coria, N., Diaz, J., Motas, M., Palacios, M.J., Cuervo, J.J., Ortiz, J., Chitimia, L., 2011. Seabird ticks (*Ixodes uriae*) distribution along the Antarctic Peninsula. *Polar Biol.* 34, 1621–1624.
- Barbosa, A., Palacios, M.J., 2009. Health of Antarctic birds: a review of their parasites, pathogens and diseases. *Polar Biol.* 32, 1095–1115.
- Chastel, C., Monnat, J.Y., Lelay, G., Balouet, G., 1987. Infestation and hyperinfestation of Kittiwake, Rissa-Tridactyla L, by ticks [*Ixodes*-(C)-*Uriae*, *Ornithodoros*-(A)-*Maritimus*] infected by Arboviruses – pathological effects. *Ann. Parasitol. Hum. Comp.* 62, 492–504.
- Earle, R.A., Huchzermeyer, F.W., Bennett, G.F., Brossy, J.J., 1993. *Babesia peircei* sp. nov. from the Jackass Penguin. *S. Afr. J. Zool.* 28, 88–90.
- Frenot, Y., de Oliveira, E., Gauthier-Clerc, M., Deunff, J., Bellido, A., Vernon, P., 2001. Life cycle of the tick *Ixodes uriae* in penguin colonies: relationships with host breeding activity. *Int. J. Parasitol.* 31, 1040–1047.
- Garamszegi, L.Z., 2010. The sensitivity of microscopy and PCR-based detection methods affecting estimates of prevalence of blood parasites in birds. *J. Parasitol.* 96, 1197–1203.
- Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. *Clin. Microbiol. Rev.* 13, 451–469.
- Lee, R.E., Baust, J.G., 1982. Respiratory metabolism of the Antarctic Tick, *Ixodes uriae*. *Comp. Biochem. Physiol. A-Physiol.* 72, 167–171.
- Major, L., La Linn, M., Slade, R.W., Schroder, W.A., Hyatt, A.D., Gardner, J., Cowley, J., Suhrbier, A., 2009. Ticks associated with Macquarie Island penguins carry Arboviruses from four genera. *Plos One* 4.
- McCoy, K.D., Leger, E., Dietrich, M., 2013. Host specialization in ticks and transmission of tick-borne diseases: a review. *Front. Cell. Infect. Microbiol.* 3.
- Medlin, L., Elwood, H.J., Stickel, S., Sogin, M.L., 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71, 491–499.
- Merino, S., Peirce, M.A., Fernandez, M., Lanzarot, P., 2002. Redescription of *Babesia moshkovskii* (Schurenkova) from the griffon vulture *Gyps fulvus* (Hablitzl). *J. Nat. Hist.* 36, 1635–1638.
- Nuttall, P.A., Kelly, T.C., Carey, D., Moss, S.R., Harrap, K.A., 1984. Mixed infections with tick-borne viruses in a seabird colony in Eire. *Arch. Virol.* 79, 35–44.
- Olsen, B., Duffy, D.C., Jaenson, T.G., Gylfe, A., Bonnedahl, J., Bergstrom, S., 1995. Transhemispheric exchange of Lyme disease spirochetes by seabirds. *J. Clin. Microbiol.* 33, 3270–3274.
- Peirce, M.A., 2000. A taxonomic review of avian piroplasmids of the genus *Babesia* starcovici, 1893 (Apicomplexa: Piroplasmorida: Babesiidae). *J. Nat. Hist.* 34, 317–332.
- Peirce, M.A., Parsons, N.J., 2012. *Babesia ugwidensis*, a new species of avian piroplasm from Phalacrocoracidae in South Africa. *Parasite* 19, 375–379.
- Rambaut, A., 2002. Se-Align Editor. v. 2.0a11. University of Oxford, Oxford, UK (Available at <http://tree.bio.ed.ac.uk/software/seal/>).
- Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.
- Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, D.A., 2012. *Babesia*: a world emerging. *Infect. Genet. Evol.* 12, 1788–1809.
- Spielman, A., Wilson, M.L., Levine, J.F., Piesman, J., 1985. Ecology of *Ixodes dammini*-borne human babesiosis and Lyme disease. *Annu. Rev. Entomol.* 30, 439–460.
- Vanstreels, R.E., Woehler, E.J., Ruoppolo, V., Vertigan, P., Carlie, N., Priddel, D., Finger, A., Dann, P., Herrin, K.V., Thompson, P., Ferreira Junior, F.C., Braga, E.M., Hurtado, R., Epiphonio, S., Catao-Dias, J.L., 2015. Epidemiology and molecular phylogeny of *Babesia* sp. in Little Penguins *Eudyptula minor* in Australia. *Int. J. Parasitol. Parasit. Wildl.* 4, 198–205.
- Work, T.M., Rameyer, R.A., 1997. Description and epizootiology of *Babesia poelea* n. sp. in brown boobies (*Sula leucogaster* (Boddaert)) on Sand Island, Johnston Atoll, Central Pacific. *J. Parasitol.* 83, 734–738.
- Yabsley, M.J., Greiner, E., Tseng, F.S., Garner, M.M., Nordhausen, R.W., Ziccardi, M.H., Borjesson, D.L., Zabolotzky, S., 2009. Description of novel *Babesia* species and associated lesions from common murre (*Uria aalge*) from California. *J. Parasitol.* 95, 1183–1188.
- Yabsley, M.J., Shock, B.C., 2013. Natural history of zoonotic Babesia: role of wildlife reservoirs. *Int. J. Parasitol. Parasit. Wildl.* 2, 18–31.
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 7, 203–214.