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Apparent absence of *Cryptosporidium, Giardia* and *Toxoplasma gondii* in three species of penguins along the Antarctic Peninsula

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Abstract: We carried out a study to investigate the presence of some protozoan parasites (*Cryptosporidium* sp., *Giardia* sp., *Toxoplasma gondii*) on three species of Antarctic penguins: Adélie (*Pygoscelis adeliae*), gentoo (*Pygoscelis papua*) and chinstrap (*Pygoscelis antarctica*) from different locations along the Antarctic Peninsula and the South Shetland Islands. Swabs and faeces samples were analysed by PCR assay for *Cryptosporidium* sp. and *Giardia* sp. while *Toxoplasma* was studied using serological methods from blood samples. We did not detect the presence of these organisms in the species studied. However, based on the upper values of the confidence intervals of the observed prevalence, their presence cannot be completely excluded.

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Introduction

Protozoan parasites are widely distributed and the cause of a range of different diseases in animals, including humans. Examples of these organisms are blood-inhabiting parasites, which affect body condition and reproduction of the hosts (Merino *et al*. 2000, Sanz *et al*. 2001), or coccidia, which are responsible for loss of body weight and diarrhoea (Dorrenstein 1997). Polar environments also contain these parasites. For instance, *Giardia* sp. is prevalent in faeces of both land and marine mammals in Arctic and Sub-Arctic areas (Roach *et al*. 1993, Olson *et al*. 1997). Coccidia, such as *Tyzzeria parvula* or *Toxoplasma gondii*, are also present in Arctic wildlife such as barnacle geese (*Branta leucopsis* Bechstein) and Arctic foxes (*Alopex lagopus* Miller) (Prestrud *et al*. 2007, Dolnik & Loonen 2007). In Antarctica, several protozoa have been reported. Some of them, such as blood parasites, are restricted to the sub-Antarctic region where suitable vectors are present (Merino *et al*. 1997, Jones & Shellam 1999, Barbosa & Palacios 2009). Others include *Cryptosporidium* sp. (Fredes *et al*. 2007a, 2008), *Eimeria pygosceli* (Golemanski 2003), *Sarcocystis* sp. (Ippen & Henne 1989) and *Isospora* sp. (Golemanski 2003). In general, information about diseases and parasites, including protozoa in Antarctic birds, is scarce and fragmented (Barbosa & Palacios 2009). Although penguins are among the best studied bird species in Antarctica, protozoa are one of the least studied groups of parasites in these birds (Clarke & Kerry 2000, Barbosa & Palacios 2009), and information is restricted to a few locations. A reasonable distributional map of these parasites is lacking and further information about the presence/absence of pathogens is therefore needed. Such information is crucial in evaluating whether their presence is due to a natural process or introduced by human activities.

For this paper, we studied the presence of the protozoan parasites *Cryptosporidium* sp., *Giardia* sp. and *Toxoplasma gondii* in three species of Antarctic penguins (chinstrap - *Pygoscelis antarctica* Forster, gentoo - *Pygoscelis papua* Forster and Adélie - *Pygoscelis adeliae* (Hombron & Jacquinot)) in different locations along the western part of the Antarctic Peninsula in order to assess the distribution of these parasites in the same hosts within a wide geographical area as a reference point for future studies.

Materials and methods

During January and February 2006 and 2007, we visited several breeding colonies of chinstrap, gentoo, and Adélie penguins along the west coast of the Antarctic Peninsula, covering a geographical range from 62°10'–67°46'S (Table I). Chinstrap penguins range from 56–65°S, gentoo penguin from 46–65°S and Adélie penguins from 54–77°S (Williams 1995). Therefore, our study covers the intermediate part of the Adélie penguin range, and the southern part of the ranges of chinstrap and gentoo penguins.

Adult penguins were captured at random on the beach in order to minimize disturbance in the breeding colonies (see Barbosa *et al*. 2007 for a similar approach). From each individual (see sample sizes in Table I) a blood sample was

Table I. Sample size analysed for *Cryptosporidium* sp., *Giardia* sp. and *Toxoplasma gondii* in each penguin species in the different locations. Mean percentage of population analysed = 1.27% (range = 0.06–5.64; own data except for Stranger Point where data were taken from Carlini *et al*. 2009)

Location	Latitude/Longitude	Species	*Cryptosporidium*	*Giardia*	*Toxoplasma*
King George Island (Stranger Point)	62°15'S; 58°37'W	*P. papua*	40	40	25
King George Island (Barton Point)	62°14'S; 58°46'W	*P. antarctica*	0	0	25
King George Island (Stranger Point)	62°15'S; 58°37'W	*P. adeliae*	0	0	25
Livingston Island (Hannah Point)	62°39'S; 60°36'W	*P. papua*	20	20	25
Livingston Island (Hannah Point)	62°39'S; 60°36'W	*P. antarctica*	10	10	26
Deception Island (Vapor Col)	63°00'S; 60°40'W	*P. antarctica*	209	209	80
Ronge Island (George Point)	64°40'S; 62°40'W	*P. papua*	20	20	25
Ronge Island (George Point)	64°40'S; 62°40'W	*P. antarctica*	20	20	25
Paradise Bay	64°49'S; 62°52'W	*P. papua*	20	20	0
Yalour Island	65°15'S; 64°11'W	*P. adeliae*	21	21	25
Avian Island	67°46'S; 68°43'W	*P. adeliae*	22	22	25

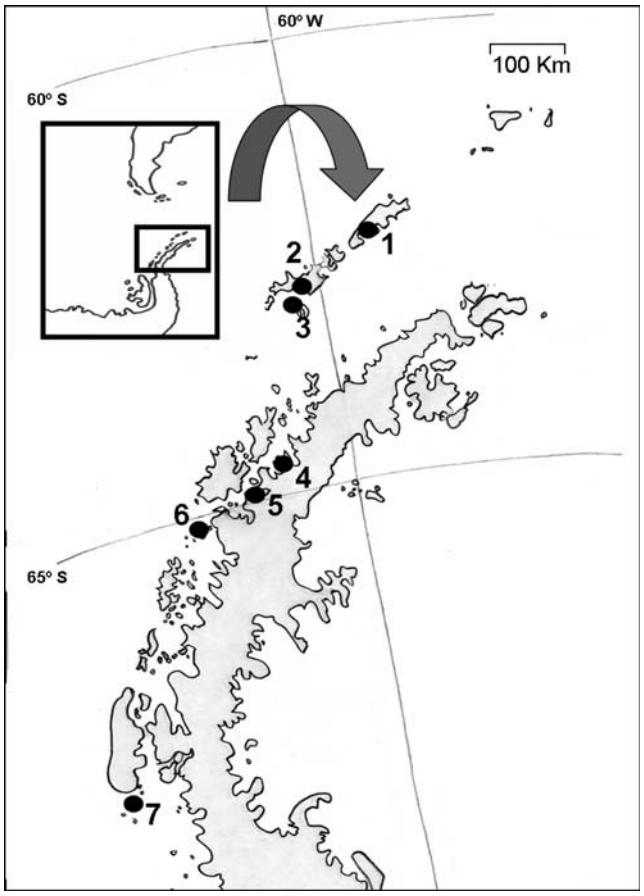

Fig. 1. Localities sampled. 1. King George Island (Stranger Point), 2. Livingston Island (Hannah Point), 3. Deception Island (Vapor Col), 4. Ronge Island (George Point), 5. Paradise Bay, 6. Yalour Island, 7. Avian Island.

taken from a foot vein with a heparinised capillary tube immediately after capture. This sample was later centrifuged at 12 000 rpm for 10 min (relative centrifugal force = 14 811 *g*) to separate plasma from red blood cells. After centrifugation both fractions were frozen for subsequent analyses. Cloacal swabs and faeces were also collected. Faeces were stored at -20°C and cloacal swabs were preserved in a sodium chloride–Tris–EDTA buffer (NaCl 0.5M, Tris 0.05M, EDTA 0.05M).

The number of samples recovered and analysed from the different penguin species in the different locations (Fig. 1) is summarized in Table I. Following Bush *et al*. (1997) 95% confidence intervals of prevalence were calculated. *Cryptosporidium* and *Giardia* were studied by means of PCR assay while *Toxoplasma* was studied by serological methods.

Cryptosporidium *and* Giardia *detection*

Oocyst/cyst disruption and DNA purification from faecal samples and cloacal swabs was performed as described in McLauchlin *et al*. (1999) and comprised disruption with zirconia beads in the presence of guanidinium thiocyanate followed by purification with activated silica (Boom *et al*. 1990). For *Cryptosporidium* detection and characterization, a nested PCR procedure was performed for amplification of an 827–840 bp polymorphic fragment of the 18S rDNA (Xiao *et al*. 1999, 2000). PCR products were amplified in 50 µl volumes containing 1x PCR buffer, 6 mM $MgCl_2$ (primary reaction) or 3 mM $MgCl_2$ (secondary reaction), 200 µM dNTPs, 10 pmoles of each primer, 2.5 units of DNA polymerase (Biotools, Madrid, Spain) and 5 µl of

template DNA (primary reaction) or 2 µl PCR product (secondary reaction). Templates were subjected to an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s, and a final extension at 72°C for 7 min. Primers used include 18SX1F: 5'-TTCTAGAGCTAATACATGCG-3' and 18SX1R: 5'-CC CATTTCCTTCGAAACAGGA-3' in the first step and 5 µl of the first step product and primers 18SX2F: 5'-GGAAG GGTTGTATTTATTAGATAAAG-3' and 18SX2R: 5'-AA GGAGTAAGGAACAACCTCCA-3' in the second step. Positive and negative controls were included in each batch of tests.

For *Giardia* detection and characterization, a nested procedure was performed to amplify a 511 bp fragment of the beta-giardin gene (Lalle *et al*. 2005). PCR products were amplified in 25 µl volumes containing 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 pmoles of each primer, 1.5 units of DNA polymerase (Biotools, Madrid, Spain) and 5 µl of template DNA (primary reaction) or 2.5 µl PCR product (secondary reaction). In the first reaction, templates were subjected to an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 7 min. For the secondary reaction, an initial denaturation at 94°C for 5 min was followed by 35 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 7 min. Markers used were G7: 5'-AAGCCCGACGACCTCACCCGCAGTGC-3' and G759: 5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3' in the first step and BG-For: 5'-GAACGAGATCGAGGTCCG-3' and BG-Rev: 5'-CTCGACGAGCTTCGTGTT-3' in the second one. Positive and negative controls were included for all PCRs. A 5 µl aliquot of the PCR products was examined following electrophoresis in 1% agarose/ethidium bromide gels.

Toxoplasma *detection*

For detection of antibodies against *Toxoplasma gondii*, a commercial kit was used (Toxo-Screen DA, BioMerieux® , France). This commercial kit is based on detection of specific IgG from sera by direct agglutination with sensitized antigen of Sabin Strain Formolated Toxoplasma taquizoites obtained from mice ascitic fluid. The procedure was followed according to the manufacturer's instructions. For initial screening, 1:20 and 1:100 final dilutions of sera were mixed in round bottom well microplates with the antigen suspension and 2-mercaptoethanol to avoid non-specific agglutination. Both positive and negative controls included in the kit were added. Results were read after overnight incubation (18 hours approximately). Samples that showed a smooth mat across the bottom of the well indicating the agglutination of specific antibodies in the serum with the antigen sensitized particles were recorded as positive. The presence of a compact button at the bottom of wells formed by the settling of the non-agglutinated particles, which indicates lack of specific antibody in serum samples, was recorded as negative. The sensitivity and specificity of this commercial kit, reported by the manufacturer, is 96.22% and 98.80% respectively.

Results and discussion

We did not find any positive result in any of the samples studied either for *Cryptosporidium*, *Giardia* or *Toxoplasma gondii*. Upper 95% confidence intervals for infection are as follows: *Cryptosporidium/Giardia* - gentoo penguin (3.6%), *Cryptosporidium/Giardia* - chinstrap penguin (1.5%), *Cryptosporidium/Giardia* - Adélie penguin (8.2%), *Toxoplasma gondii* - gentoo penguin (4.8%), *Toxoplasma gondii* - chinstrap penguin (2.3%), *Toxoplasma gondii* - Adélie penguin (4.8%). According to these results and although it would seem reasonable to assume that pygoscelid penguins are free from these parasites in the studied locations, we are cautious and consider that the prevalence could be as high as the figures shown by the upper confidence intervals (see Bush *et al*. 1997). In the case of *Cryptosporidium* and *Giardia*, detection of these organisms was done by PCR and no specific amplicons were observed which indicates that these parasites might be absent or there was an insufficient amount of the parasites' DNA present. Other possible reasons for the lack of amplification are degradation of the DNA during storage, and/or copurification of inhibitors of the PCR. We believe that degradation of the DNA is unlikely since it has been previously reported that, at least for *Cryptosporidium*, DNA can be successfully isolated from whole faeces by the methods described here after storage at room temperature for six weeks or at 4°C for four years (McLauchlin *et al*. 1999). Copurification of inhibitors in the DNA extraction is also unlikely since unspecific bands were observed in some samples after PCR. In addition, dilutions of positive *Cryptosporidium* and *Giardia* control DNAs seeded into a subset of extracts from penguins resulted in successful amplification of the targets.

There have been three previous surveys looking for *Cryptosporidium* in Antarctic penguins (Fredes *et al*. 2007a, 2007b, 2008). The first one described negative results in 52 gentoo penguin samples from Paradise Bay (64°49'S 62°51'W) using the Ziehl-Neelsen method (Fredes *et al*. 2007b). However, the other two studies, using acid-fast stain, found some parasite structures compatible with *Cryptosporidium* oocysts in Adélie penguins (Fredes *et al*. 2007a) and gentoo penguins (Fredes *et al*. 2008) both in Ardley Island, South Shetland Islands (62°13'S 58°54'W). At the moment, only penguin populations breeding in this island seem to be infected by *Cryptosporidium*, while extensive parts of the South Shetland Islands and Antarctic Peninsula seem to be free or at least with low prevalences of this parasite (Fredes *et al*. 2007a and results from this study). However, a positive record has been found in one elephant seal (*Mirounga leonina* (L.)) in Avian Island (Pedraza-Diaz *et al*. unpublished).

Cryptosporidium is a significant pathogen of humans and other animals (Fayer *et al*. 1997) that is transmitted through contamination with human and domestic animal faecal material (Appelbee *et al*. 2005). Specifically *Cryptosporidium* together with *Giardia* have been detected in marine water samples from areas of treated sewage disposal (Johnson *et al*. 1995a). The only place where *Cryptosporidium* has been detected in Antarctica in penguins is very close to the Fildes Peninsula (King George Island) (Fredes *et al*. 2007a, 2008). In this place extensive scientific, logistic and tourist activities are carried out (Pfeiffer 2005), and sewage water disposal without treatment was carried out in former times (Tin & Roura 2004) therefore increasing the probability of its presence. However, we have not found *Cryptosporidium* in other parts of King George Island, such as the penguin rockery in Stranger Point (62°15'S 58°37'W). This place is the ASPA (Antarctic Special Protected Area) No. 132 and is located 5 km from the nearest research station. Moreover, other places with scientific research stations in the locality such as Paradise Bay have not shown the presence of *Cryptosporidium* (Fredes *et al*. 2007b and the present study). Results from Fredes *et al*. (2007b) should be taken with caution because they used direct microscopic identification of oocyst for detection, which gives a high probability of false negatives and is around 100 to 1000 times less efficient than the use of molecular methods (Atías 1998, but see Ballweber *et al*. 2009 who stated that microscopic and PCR methods are equivalent for *Giardia* detection). In addition, the use of molecular methods allows the identification of the species or genotypes involved in infection and may contribute to understanding the routes of transmission. However, in some cases, inhibitory substances can affect PCR performance leading to false-negative PCR results, therefore reducing its efficacy (Johnson *et al*. 1995b). In our study we can rule out this possibility since unspecific bands were detected after PCR and dilutions of positive *Cryptosporidium* and *Giardia* control DNAs seeded into a subset of extracts from the studied species resulted in successful amplification of the targets (see above). In any case, our results using PCR assays seem to confirm the absence of *Cryptosporidium* in this location obtained previously by means of microscopic techniques.

Giardia has been found in polar environments, specifically in marine mammals in the Arctic and sub-Arctic area (Appelbee *et al*. 2005). As far as we know, this is the first time that the presence of *Giardia* has been studied in Antarctica and we have obtained negative results. *Giardia* and *Cryptosporidium* share many characteristics. Both parasitize similar hosts, have the same route of transmission and produce similar effects on the hosts (Appelbee *et al*. 2005). Therefore, it is not surprising that the same results were found for both protozoan parasites, in this case negative.

In our study an agglutination test was used for the detection of antibodies to *T. gondii*. To our knowledge this is the first time this procedure has been used in penguins although it has been widely used, either as kit or as a modified agglutination test (MAT), in humans and other mammals and in birds (Desmonts & Remington 1980, Dubey & Desmonts 1987, Dubey 2002, Meunier *et al*. 2006, de Camps *et al*. 2008, Dubey & Jones 2008, Waap *et al*. 2008, Zhu *et al*. 2008, Salant *et al*. 2009). It has been reported that amongst different serological tests available, the agglutination test is most useful because it is species independent (does not require species specific conjugates), sensitive, and specific (Desmonts & Remington 1980, Dubey 2002, Negash *et al*. 2004), and no cross-reactivity of *T. gondii* with other possible infective organisms has been reported (Salant *et al*. 2009). Most authors have considered titres of 1:25 as positive, although as low as 1:2 or 1:5 have also been reported (Dubey & Jones 2008), therefore screenings were done at 1:20 and 1:100 final dilutions. We did not detect the presence of antibodies to *T. gondii* in any of the samples analysed. However, *Toxoplasma* has been found in different groups of birds (Dubey 2002) including penguins in captivity (Ratcliffe & Worth 1951, Mason *et al*. 1991).

To our knowledge the study presented here constitutes the first investigation on the presence of *Toxoplasma* in Antarctica, although it was found previously in the Iles Kerguelen in the sub-Antarctic region (Afonso *et al*. 2007) and in the Arctic (Prestrud *et al*. 2007). As domestic cats and other felids are the only definitive hosts for *Toxoplasma* (Frenckel *et al*. 1970), the presence of this parasite in Antarctica is not expected *a priori*. However, a wide range of birds serve as intermediate hosts, being infected by direct ingestion of oocysts and also by vertical transmission (Dubey & Beattie 1988). Therefore, the introduction of *Toxoplasma* in the Antarctic ecosystem by migrating birds could be a risk, although when it happens, the probability of transmission to penguins would be very low. However, scavenger species like the skuas or giant petrels are probably at a higher risk. On the other hand, *Toxoplasma* transmission by marine invertebrates have been suspected (Prestrud *et al*. 2007) and transmission from human activities in the Arctic has not been discarded (Prestrud *et al*. 2007), posing a risk for a similar situation in Antarctica.

We report negative findings, although calculating the upper 95% confidence intervals prevalence could be between 1.53% and 8.22%. Therefore, it would be important to implement long-term monitoring programs to confirm the presence/absence of this kind of organism in different places with different infection susceptibility according to human presence or environmental factors (Barbosa & Palacios 2009). Searching in the future for parasites and diseases in places where they seem to be absent nowadays will allow us to detect new introductions and their causes.

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