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Mounting evidence for the presence of influenza A virus in the avifauna of the Antarctic region

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Abstract: Penguin blood samples collected at Bird Island, sub-Antarctic South Georgia, and faecal samples taken from penguins at several localities along the Antarctic Peninsula were analysed in order to investigate if influenza A virus is present in penguin populations in the South Atlantic Antarctic region. Serology was performed on the blood samples while the faecal samples were screened by a RT-PCR method directed at the matrix protein gene for determining the presence of influenza A virus. All faecal samples were negative by PCR, but the blood samples gave serologic indications that influenza A virus is present amongst these penguin species, confirming previous studies, although the virus has still not been isolated from any bird in the Antarctic region.

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Key words: avian influenza, RT-PCR, serology, sub-Antarctica

Introduction

Influenza A virus has been isolated from at least 105 species of birds representing a span of 26 bird families (Olsen *et al*. 2006). The virus is most frequently found in bird species associated with aquatic environments, and is especially prevalent in dabbling ducks of the Anatidae family. However, the virus has also been detected or isolated in geese (Anseridae), shorebirds (Scolopacidae and Charadriidae) and gulls (Laridae) (Olsen *et al*. 2006). All the known hemagglutinin and neuraminidase subtypes of influenza A strains, including the recently characterized H16 (Fouchier *et al*. 2005), have been isolated from wild birds, and nearly all of them from dabbling ducks. Generally, ducks, geese, shorebirds and gulls are infected with low pathogenic strains of influenza A virus that do not cause disease symptoms, and hence these hosts are considered as the main natural reservoir of influenza A viruses (Hinshaw *et al.* 1980), while infections in other families of birds appear sporadic.

Influenza A virus has so far been isolated in birds on all continents with the exception of Antarctica. To date there is only serological data suggesting the presence of influenza A viruses in the Antarctic avifauna. Three different studies have found elevated antibody titres against influenza A viruses in either penguins or skuas in Antarctica (Morgan & Westbury 1981, Austin & Webster 1993, Baumeister *et al.* 2004). Here we present data from two expeditions to the Antarctic and sub-Antarctic region, that adds to the knowledge on the presence of the virus in the most remote of all continents.

Material and methods

Serological analysis of penguin sera

Sera was collected from 76 blood samples obtained from gentoo penguins (*Pygoscelis papua* Forster) that were sampled on Bird Island off the sub-Antarctic island of South Georgia (54°00'S, 38°03'W) in 1996. Penguin blood samples were collected as whole blood, centrifuged for 10 minutes at 14 000 rpm and serum was separated. The sera were analysed using the commercially available Influenza type A virus antibody ELISA kit (European Veterinary Laboratory, Woerden, The Netherlands) according to the manufacturers' instructions. For the detection of specific anti-NP antibodies, we used horseradish peroxidase-conjugated goat anti-wild bird immunoglobulin (Ig) G (Bethyl Laboratories, Inc., TX, USA) instead of the supplied anti-bird conjugate (Ebel *et al* 2002). Prior to running the ELISA, the recognition of penguin sera by the anti-wild bird conjugate was confirmed by using 96 well high binding EIA plates (Corning, NY, USA) coated with penguin sera. Subsequently, penguin sera samples were

Table I. Number of faecal sampled penguins at the different localities.

	Robert Island	Paulet Island	Brown Bluff	Hannah Point	Necco Harbour	No. sampled birds
Longitude	59.27	55.767	56.917	60.600	62.533	
Latitude	62.28	63.583	63.533	62.650	64.850	
Breeding pairs in colony	no data	60 000	250	1000	250	
Gentoo penguin (*Pygoscelis papua*)			41	41	49	131
Adélie penguin (*Pygoscelis adeliae*)		30				30
Chinstrap penguin (*Pygoscelis antartica*)	29					29
Total	29	30	41	41	49	190

diluted 1:100 in PBS buffer with pH 7.4 (Gibco, Breda, The Netherlands) and were applied to the wells of a NP-coated ELISA plate and were left to incubate at 37ºC for 60 min. The plate was washed four times with PBS. Thereafter, 100 µl of anti-bird conjugate diluted 1:100 in conjugate buffer was applied to each well, followed by incubation at 37ºC for 60 min. After incubation and washing as above, plates were developed with 100 µl TMB substrate for 15 min. The reaction was stopped by applying 50 µl of stop solution (0.5 M sulphuric acid) and the optical density (OD) was read at 450 nm and 620 nm. Blank, positive and negative controls were included on each plate. To compute the positive/negative (P/N) value of each sample, we divided the mean OD of positive antigen-containing wells by the OD of negative antigen-containing wells. Samples with a P/N value ≥ 2 were considered positive.

Analysis of penguin faecal samples

Five penguin colonies along the Antarctic Peninsula (Table I) were visited during a week in February 2002. Penguins of three different species, gentoo penguin (*Pygoscelis papua*), Adélie penguin (*P. adeliae* Hombron & Jacquinot), and chinstrap penguin (*P. antartica* Forster), were caught in their breeding colonies using hand nets. In total 190 penguins were sampled (Table I). Each bird was sampled through the insertion of a sterile cotton wool swab into the cloacae. The swabs were then placed in transport media (Hanks balanced salt solution containing 10% glycerol, 200 U ml^{-1} penicillin, 200 mg ml^{-1} streptomycin, 100 U ml^{-1} polymyxin B sulphate, and 250 mg ml^{-1} gentamycin, (MP biomedicals, Zoetermeer, The Netherlands), and stored in refrigerator temperatures during the remainder of the expedition (four days of travel after completion of fieldwork), and subsequently stored at -70°C until analyses.

Virus detection was performed by RNA isolation, RT-PCR and dotblot detection as previously described (Fouchier *et al.* 2000). In brief, RNA was isolated using a high pure RNA isolation kit (Roche Diagnostics, Almere, The Netherlands) and viral sequences were amplified in a one-step RT-PCR using primers M52C (5'- CTT CTA ACC GAG GTC GAA ACG –3') and M253R (5'- AGG GCA TTT TGG ACA AAG/T CGT CTA -3') targeting the matrix gene segment. RT-PCR products were transferred to dotblots and visualized using ECL detection reagents and exposure to hyperfilm (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) upon hybridization with the biotinylated oligonucleotide Bio-M93C (5'- CCG TCA GGC CCC CTC AAAGCC GA –3').

Results and discussion

Apart from being geographically separated, sample material also differed between studies, and consequentially two analysis methods were used. Of the 76 blood samples from Bird Island, nine (11.8%) gave positive serology results for influenza A virus. Eight of these birds were adult individuals (out of 33 tested adults) and one was juvenile (out of 40 tested juveniles), while the remaining three penguins were not aged. These results contrast with the 190 faecal samples from the Antarctic Peninsula, that were all negative when analysed by RT-PCR. The PCR methodology used is routinely used in other studies, with a generally high detection rate (Fouchier *et al.* 2003, Wallensten *et al.* 2004). Therefore, we see the lack of positive amplicons as evidence that none of the sampled penguins were infected and excreting influenza A virus at the time of sampling.

This pattern with serologic findings, but without isolation of virus is similar to the results from the few previous studies from the region. One of the earlier studies failed to isolate virus from tracheal and cloacal swabs obtained from 550 Adélie penguins in the Eastern Antarctic (66º17'S, 110º32'E), but found serological evidence of influenza A virus subtype H7 in 6 out of 285 blood samples (Morgan & Westbury 1981). Similarly, Austin & Webster (1993) did not isolate virus from Adélie penguins or skuas (*Catharacta* spp.) on Ross Island (77º51'S, 166ºE) either in 1978 or 1986, but found evidence of subtype H10 in 26% (24 out of 91 birds) of serological samples from Adélie penguins as well as elevated titres of serum antibodies in Antarctic skuas (*Stercorarius skua maccormicki* Saunders). Finally, Baumeister *et al.* (2004) reported serological evidence of influenza A virus in gentoo penguins, chinstrap penguins, skuas and giant petrels (*Macronectes giganteus* Gmelin) at Potter Peninsula (62º15'S, 58º39'E) and in samples from Adélie penguins and skuas at Hope Bay (63º24'S, 56º59'E)

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on the Antarctic Peninsula. They also found serological evidence in samples from giant petrels at Harmony Point (63º24'S, 56º59'E) on Nelson Island, South Shetland Islands. Attempts have also been made to detect influenza A virus in penguin guano, using sensitive RT-PCR, but without success (Briggs *et al.* 2003). Hence, our study like the few earlier studies finds serologic indications of influenza A virus in Antarctic birds, but yet there is no successful isolation of the virus.

Negative virus isolation results could be interpreted in different ways. First of all, virus prevalence could peak at other time periods of the year than that of the sampling. Similarly, if the virus is confined to certain penguin colonies, or, if it only infects a low proportion of individuals in each colony, failure to detect virus could be due to chance events when choosing colonies or individuals to sample. Our study is fairly large, 190 samples from five localities and for instance, at Paulet Island we would have expected to find positive samples if the prevalence had been > 5–10%, given our sample size of 30 birds and a population larger than 3000 individuals (Cannon & Roe 1982). The moderate prevalence of seroconverted penguins (2–26%) in earlier serological studies and in our study (11.8%), may together with the negative PCR results indicate that penguins are not a primary host for influenza A virus but rather a species that is occasionally infected by means of spill over from other species. Given the crowded nature of a penguin colony, one would expect most individuals within a colony to contract the disease if a contagious virus was introduced into a large susceptible population, which would result in high seroprevalence. On the other hand, observed low seroprevalences may reflect a lack of yearly epizootics, where low seroprevalence will be found in in-between years when the cohort of juvenile birds has not been exposed.

There is no obvious reason why influenza A viruses should be absent from the Antarctic avifauna. Antarctica is not as remote as it may appear; a large proportion of a variety of bird species migrate from northerly latitudes to breed there, including procellarids, cormorants and terns. The most extreme long-distant migrant, the arctic tern (*Sterna paradisaea* Pontoppidan), migrates from its Arctic breeding grounds to spend part of its non-breeding season in the Antarctic waters during the summer. Interestingly, influenza A virus has been isolated from this species (Lvov 1978, Sinnecker *et al.* 1983). These and other birds could potentially introduce the virus to penguin populations, where transmission would be rapid in the dense colonies. There are also indications that the virus can persist for several months in cold water (Stallknecht *et al.* 1990a, 1990b), and would therefore be well adapted for the temperatures prevailing during the summer. On the other hand climatic factors such as high UV-radiation and the lack of freshwater (Pearce & Wilson 2003) may have a negative impact on the existence of influenza A viruses in Antarctica as well as the absence of dabbling ducks as a primary host species for the virus.

To conclude, no isolations of influenza A virus from Antarctic birds have been made so far, even though more than 1000 birds have been tested at different locations, at different times and by different methods. However, four independent studies report serologic evidence of past infections, indicating that the virus either is endemic to the region in penguins or in an unknown host, or that the virus has been repeatedly introduced from other continents. There is clearly a need for additional sampling and screening of wildlife in the region, especially now when a highly pathogenic influenza A virus of the subtype H5N1 is spreading rapidly in wild bird populations in Eurasia and Africa causing morbidity and mortality among birds (Olsen *et al*. 2006). We therefore believe that publishing data, both positive and negative, is of utmost importance in enabling future expeditions to make better judgements upon choice of timing, species to sample and methods to be used.

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