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SHORT NOTE

Molecular surveillance for avian influenza A virus in king penguins (*Aptenodytes patagonicus*)

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Abstract An investigation of the presence of influenza A virus has been conducted in king penguins (*Aptenodytes patagonicus*) at the Possession Island in the Crozet Archipelago, Antarctica, using a rapid molecular diagnostic method based on real-time polymerase chain reaction. No evidence of outbreak or positive viral infection of influenza A virus was found in this study. We however recommend the implementation of long-term surveillance in seabird populations of polar ecosystems to detect the potential introduction of exotic strains and potential existence of a local epidemiological cycle for avian influenza viruses.

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Introduction

Wild aquatic birds in the orders Anseriformes (e.g. ducks, geese, swans) and Charadriiformes (e.g. gulls, terns, shorebirds) are traditionally considered as natural hosts for most avian influenza viruses (AIV) (Webster et al. 1992). Although AIV have been isolated from other wild bird species (Olsen et al. 2006), less attention has been devoted to species living in polar ecosystems. Arctic ecosystems are hypothesized to be a potential long-term source of strains conserved in cold waters (Ito et al. 1995; Zhang et al. 2006). However, in Antarctic and subantarctic areas, the situation remains largely unknown because of lack of studies. Millions of seabirds, penguins, etc. breed in very dense colonies in a few restricted areas in subantarctic islands and on the coast of the Antarctica continent, providing potentially ideal conditions for exchanges of pathogens (Gauthier-Clerc et al. 1999). However, these birds are not completely isolated from the surrounding continents and may be exposed to their pathogens. For example, brown skuas (*Catharacta lonnbergi*) and southern giant petrels (*Macronectes giganteus*) that are breeding in Antarctica winter near the coasts of Africa, Australia, New Zealand and South America (Shirihai 2002); and Arctic terns (*Sterna paradisaea*) breeding in the Arctic winter near the coasts of Antarctica during the southern summer months, reaching well into the pack ice zone. Each year, waders, such as greenshank (*Tringa nebularia*) or curlew sandpiper (*Calidris ferruginea*) are vagrants to subantarctic islands and may carry pathogens from the Arctic (Gauthier-Clerc

et al. 2002b; Frenot et al. 2005). The natural transhemispheric exchanges of pathogens have already been demonstrated for the Lyme disease spirochetes (Olsen et al. 1995). The introduction of avian influenza A to Antarctica or subantarctic islands from other continents is thus possible. The increases of human activities in these areas (e.g. scientific research, resources exploitation or tourism) are also threats for local wildlife populations through the introduction of pathogens and potential for exotic disease outbreaks (Gardner et al. 1997; Frenot et al. 2005). Antibodies to influenza A viruses have been detected in gentoo penguins (*Pygoscelis papua*) and southern giant petrel (*Macronectes giganteus*) from the Antarctic Peninsula (Baumeister et al. 2004). The origin of these viruses is unknown; either local circulation or introduction from other continents should be considered. In this study, we investigated the presence of avian influenza viruses (AIV) in the king penguin (*Aptenodytes patagonicus*), one of the most numerous species in the Southern Ocean, in a colony located a few hundred metres from a French scientific station.

Materials and methods

Samples

The study was conducted in a king penguin colony which has estimated 25,000 breeding pairs at Possession Island (46°25′S, 51°45′E), on the Crozet Archipelago, during the austral summer 2006/2007. One hundred king penguin chicks were sampled. Cloacal swabs were collected using the Viral Pack kit (Biomedics, S.L.) and kept at −80°C until RNA extraction was performed. Extraction has been performed in France. Time between sample collection and extraction was 2 weeks. This delay was not a problem for virus detection because of the storage at −80°C.

RNA extraction and cDNA synthesis

Avian influenza virus RNA extraction was performed using the automatic BioRobot MDx workstation and QIAamp Virus BioRobot MDX kit (Qiagen) according to the manufacturers' instructions. Extraction was from 120 µl of specimen and the RNA was eluted into a final volume of 60 µl. The cDNA synthesis reaction mixture contained 1× RT buffer, 0.5 mM of each dNTP, 0.5 µM of Uni12 primer (5′-AGC AAA AGC AGG-3′), 20 units of RNAsin, 10 units of RT-AMV reverse transcriptase (Promega), 5 µl of sample RNA and RNase free water up to a final reaction volume of 20 µl. Reverse transcription was performed under the following conditions: 37°C for 45 min, 42°C for 15 min, 95°C for 5 min and the cDNA stored at 4°C.

Diagnostic real-time PCR (qPCR)

For the detection of influenza viruses, a real-time polymerase chain reaction (qPCR) method was used, targeting the conserved matrix gene segment. A SYBR green based qPCR was undertaken with the ABI Prism 7000 Sequence Detection Systems (Applied Biosystems) using 96 well plates (ABgene); reactions were in a total volume of 25 µl of 1× PCR mix (SYBR green I PCR core kit, Applied Biosystems) containing 10 µl of viral cDNA, and oligonucleotides M52C: 5′-CTT CTA ACC GAG GTC GAA ACG-3′ and M253R: 5′-AGG GCA TTT TGG ACA AAK CGT CTA-3′ (Fouchier et al. 2000) at 200 nM each. Amplification conditions were: 50°C for 2 min, 95°C for 10 min and 45 cycles of amplification (95°C for 15 s, 60°C for 1 min). A melting curve analysis was performed after the PCR cycling. All samples were run in duplicate in qPCR and regarded as positive by comparison with the melting curve analysis for A/Chicken/HK/G9/97(H9N2) used as positive control.

Results and discussion

A total of 100 king penguin cloacal samples was analysed for influenza A virus using SYBR green real-time PCR reaction. Real-time PCR methods are broadly used for screening of influenza viruses because of their sensitivity and specificity (Spackman et al. 2002; Payungporn et al. 2006; Senne et al. 2006; Hoffmann et al. 2007; Karlsson et al. 2007).

None of the cloacal specimens analysed in this study was found positive. Although we did not find any evidence for influenza A virus infection in the king penguin samples obtained and examined in this study, we recommend the implementation of long-term surveillance in this, and related species of Southern Ocean seabirds. Until now, there has been no report of detection of either influenza A virus or influenza antibodies in king penguins (Gauthier-Clerc et al. 2002a), although antibodies specific for influenza A virus have been detected in a related species, Adélie penguin (*Pygoscelis adeliae*) in East Antarctica (Morgan and Westbury 1981; Austin and Webster 1993).

The highly pathogenic AIV subtypes have been increasingly responsible for outbreaks associated with high mortality in poultry over recent decades (Alexander 2000). Previously, the outbreaks generally remained spatially constrained in extent. However, recently more widespread outbreaks were observed as for instance in Mexico in 1994, or in Pakistan in 1995 (Gauthier-Clerc et al. 2007). Since late 2003, the highly pathogenic H5N1 viruses caused devastating outbreaks in poultry in South-East Asia and spread to reach the Middle-East, Africa and Europe in 2005 and 2006

(Horimoto and Kawaoka 2005; Malik-Peiris et al. 2007). These highly pathogenic H5N1 viruses were also responsible for high mortalities of the threatened bar-headed geese (*Anser indicus*) in the centre of China (Chen et al. 2005) and may challenge the conservation of specific bird populations (Gauthier-Clerc et al. 2007).

Long-term surveillance in Antarctica and the peri-Antarctic islands in the Southern Ocean has two complementary interests in the context of an increased presence of human populations in these areas. First, a public health interest, as most emerging infectious diseases are zoonotic (Taylor et al. 2001); the resident wildlife constituting a large and unknown reservoir from which new pathogens may ultimately be introduced in the human population, as for example for SARS and Ebola viruses (Chomel et al. 2007). Second, an interest in terms of biological conservation. Indeed, the Antarctic area is presently subjected to increasing human activities, and both human and wildlife might be under threat of new emerging infections diseases. Therefore, surveillance will increase the likelihood of detecting the introduction of exotic pathogens and potential outbreaks. Surveillance may also allow the detection of endemic or currently unknown pathogens in the Antarctic region that might be a threat to human public health.

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