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ANIMAL SCIENCE

Active surveillance for influenza virus and coronavirus infection in Antarctic birds and mammals in environmental fecal samples, South Shetland Islands

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Abstract: Numerous Antarctic species are recognized as reservoirs for various pathogens, and their migratory behavior allows them to reach the Brazilian coast, potentially contributing to the emergence and circulation of new infectious diseases. To address the potential zoonotic risks, we conducted surveillance of influenza A virus (IAV) and coronaviruses (CoVs) in the Antarctic Peninsula, specifically focusing on different bird and mammal species in the region. During the summer of 2021/2022, as part of the Brazilian Antarctic Expedition, we collected and examined a total of 315 fecal samples to target these respiratory viruses. Although we did not detect the viruses of interest during this particular expedition, previous research conducted by our team has shown the presence of the H11N2 subtype of influenza A virus in penguin fecal samples from the same region. Given the continuous emergence of new viral strains worldwide, it is crucial to maintain active surveillance in the area, contributing to strengthening integrated One Health surveillance efforts.

Key words: Active surveillance, Antarctic wildlife, viral infections, zoonotic risks, environmental fecal samples.

INTRODUCTION

To prevent the emergence of new diseases and epidemics and effectively address the impacts of infectious agents on ecosystems and society, it is essential to enhance surveillance systems capable of monitoring circulating pathogens in humans, animals (both domestic and wild), and the environment. This approach aligns with the principles of One Health. According to the World Organization for Animal Health (WOAH), it is estimated that 75% of emerging diseases have a zoonotic origin, underscoring the crucial need for close collaboration between public health and animal surveillance authorities (Keesing et al. 2010, Tazerji et al. 2022).

The emergence of zoonotic diseases typically occurs as a result of various factors, including: (i) Anthropogenic actions, such as urbanization, agricultural expansion, deforestation, globalization, socioeconomic development, the use of agrochemicals, and the application of antimicrobial treatments, as well as other behaviors such as meat consumption, animal production, and animal-human interaction, (ii) environmental factors, including temperature, drought, wind, and climate change, (iii) biological factors, such as genetic drift and rearrangement (Tazerji et al. 2022, WHO 2006).

Climate and environmental changes have significant effects on various regions of the planet, but the Antarctic ecosystem holds particular importance in this context. The native biota of Antarctica has undergone adaptations to the extreme conditions of the region over millions of years, making it a unique and fragile ecosystem that is now facing the challenges of environmental changes and the direct impacts of human activities (Convey & Peck 2019). The expansion of national research programs and tourism activities in Antarctica brings increased risks of introducing infectious diseases to wildlife (Barbosa et al. 2021). Potential impacts of tourism on wildlife include disturbances caused by frequent visits, the introduction of diseases and non-native species, as well as pollution stemming from ship and aircraft operations and sewage disposal (Woehler et al. 2014). The emergence of the COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has further emphasized the urgent need to strengthen surveillance and monitoring systems for viruses with zoonotic potential, even in the most extreme environments like Antarctica (Barbosa et al. 2021). The rapid global spread of the virus has underscored the necessity to intensify our efforts in this regard.

Coronaviruses (CoVs) form a diverse group of highly infectious viruses that pose challenges for control measures due to their extensive genetic diversity, rapid replication time, and high mutation rate. These viruses infect a wide range of animals (Cui et al. 2019, Jordan et al. 2015). CoVs belong to the family Coronaviridae, within the order Nidovirales. They are enveloped, positive-sense RNA viruses known to infect four out of the seven classes of vertebrates: mammals and birds (subfamily: Orthocoronavirinae), amphibians (subfamily: Letovirinae), and bony fish (subfamily: Pitovirinae) (ICTV, https://ictv.global/report/chapter/coronaviridae/coronaviridae). Within the Orthocoronavirinae subfamily, there are four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus, based on their phylogenetic relationships and genomic structures (Cui et al. 2019, Woo et al. 2023). Alphacoronaviruses and betacoronaviruses primarily infect mammals, while gammacoronaviruses and deltacoronaviruses mainly infect birds, although some of them can also infect mammals (Cui et al. 2019). Coronaviruses have a global distribution and can be found in various species of wild animals, serving as potential reservoirs and hosts for genetic mutations and recombination events that can lead to the emergence of new serotypes or genera (Cui et al. 2019, Jordan et al. 2015).

Influenza viruses are enveloped, negative-sense single-stranded RNA viruses with a segmented genome (Javanian et al. 2021, Krammer et al. 2018, Webster et al. 1992). Within the Orthomyxoviridae family, there are four types of influenza viruses (A–D), with three types (A, B, and C) capable of infecting and causing disease in humans (Uyeki et al. 2022). Influenza A viruses (IAVs) not only circulate in humans but also in domestic animals such as pigs, horses, and poultry, as well as in wild migratory birds, where more than 100 species of ducks, geese, swans, gulls, waders, and wild aquatic birds are considered natural reservoirs (Krammer et al. 2018, Webster et al. 1992). Influenza B viruses primarily infect humans, while influenza C viruses can infect humans, pigs, and dogs, and influenza D viruses primarily infect cattle with occasional spillover to other animals (Uyeki et al. 2022).

The IAVs are classified into subtypes based on the types of surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA) they possess (Krammer et al. 2018, Uyeki et al.

2022). To date, 18 HA and 11 NA subtypes have been identified, with 16 HA (H1–16) and nine NA (N1–9) subtypes being enzootic in avian species, particularly in wild waterfowl (Olsen et al. 2006, Tong et al. 2012, 2013, Webster et al. 1992). These viruses can periodically infect other animals like poultry and pigs, establishing enzootic and endemic lineages. The high error rate of the RNA-dependent RNA polymerase (*RdRp*) and the reassortment of RNA segments during co-infections provide IAVs with evolutionary flexibility, enabling them to circulate among new hosts (Uyeki et al. 2022). This characteristic plays a central role in the emergence of novel influenza A subtypes, often through zoonotic transmission (Krammer et al. 2018, Uyeki et al. 2022). For instance, in 2009, the first influenza virus pandemic of the 21st century was caused by a novel H1N1 influenza A virus reassortant that was previously circulating in pigs (Krammer et al. 2018).

Numerous studies have investigated the occurrence of IAVs in the avifauna of Antarctica, consistently showing that the local fauna is constantly exposed to IAVs infections (Abad et al. 2013, Austin & Webster 1993, Baumeister et al. 2004, de Seixas et al. 2022, de Souza Petersen et al. 2017, Hurt et al. 2014, 2016, Morgan & Westbury 1981, Ogrzewalska et al. 2022, Wallensten et al. 2006).

In the Antarctic environment, the proximity and coexistence of different animal species, which form large colonies along the shores during the summer, create favorable conditions for the emergence of new viruses. This is due to the potential for co-infections between viruses found in different species of birds and other animals (Smeele et al. 2018a). Furthermore, human-wildlife interactions in Antarctica occur regularly, mainly during research activities, tourist visits, and unexpected encounters associated with operations, logistics, or fishing activities (Barbosa et al. 2021, Woehler et al. 2014).

Although previous studies have demonstrated the presence of a variety of viruses in Antarctic animals, including avian avulaviruses, birnaviruses, herpesviruses, caliciviruses, picornaviruses, flaviviruses, as well as numerous new and unclassified viruses (Smeele et al. 2018a, b, Wang et al. 2022, Wille et al. 2019, 2020, Zamora et al. 2023), our current study specifically focused on monitoring viruses with potential zoonotic risks, such as CoVs and IAVs. To conduct this surveillance, we collected fresh feces from wild birds and mammals in different locations within the South Shetland Islands of the Antarctic Peninsula during the XL Brazilian Antarctic Expedition.

MATERIALS AND METHODS

Ethical aspects

Permissions to collect samples were granted by the Environmental Assessment Group of the Brazilian Antarctic Program (GAAm-PROANTAR XL) for specific locations (Antarctic Specially Protected Areas, ASPAs) within the Antarctic region. These locations include the Western Shore of Admiralty Bay (ASPA 128), Lions Rump (ASPA 151), Potter Peninsula (ASPA 132), Ardley Island (ASPA 150), Byers Peninsula (ASPA 126), Harmony Point (ASPA 133), and Deception Island (ASPA 140). No permission was required for sampling in other areas as they did not have any access restrictions or specific regulations in place.

Sample collection

Fecal samples were collected during field expeditions conducted in the South Shetland Islands, near the Antarctic Peninsula, throughout the breeding season of the 2021-2022 Antarctica summer season, specifically from October 2021

to March 2022. The sampling efforts covered six islands and 14 different localities (Table I, Fig. 1).

During the sampling process, both individual fresh samples from monitored animals and feces from penguins' nesting sites were collected. Fecal material was collected using sterile Dacron swabs, which were promptly placed into tubes containing 2 mL of viral transport medium, following the method described previously (Ogrzewalska et al. 2022). The samples were then refrigerated for up to 4 hours before being frozen at -80°C for further analysis.

Viral RNA extraction

Clarified fecal suspensions (20%, wt/vol) were prepared with 1 phosphate-buffered saline (PBS) by vortex mixing, followed by centrifugation at 3,000 x g for 20 min, and 140 mL of the supernatant was used for viral RNA extraction. Viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen, CA, USA) and a QIAcube automated system (Qiagen), according to the manufacturer's instructions. Viral RNA was eluted in 60 mL of the elution buffer. The isolated RNA was immediately stored at -80°C until molecular analysis. For each extraction procedure, RNase/DNase-free water was used as a negative control.

Influenza A virus (IAV) screening by quantitative one-step real-time RT-PCR

The IAVs were screened using TaqMan based quantitative one-step real-time RT-PCR. All reactions were performed using the SuperScript III Platinum one-step quantitative RT-PCR (qRT-PCR) kit (Thermo Fisher Scientific, Invitrogen Division, Carlsbad, CA, USA) according to the protocol established by the Collaborative Influenza Center, Centers for Disease Control and Prevention, Atlanta, GA (Shu et al. 2011). Samples that crossed the threshold line below a threshold cycle (CT) value of 38 and showed a characteristic sigmoid curve were regarded as positive.

Coronavirus (CoVs) screening by conventional pancoronavirus RT-PCR

All the samples were also subjected also to pancoronavirus PCR targeting the RNA-dependent RNA polymerase (*RdRp*) gene as described previously (Chu et al. 2011). Briefly, RNA was amplified in a first-round PCR (RdRp S1 5´-GGKTGGGAYTAYCCKAARTG -3', RdRp R1 5'-TGYTGTSWRCARAAYTCRTG-3') using One-Step RT-PCR Enzyme MixKit (Qiagen) with the total expected size of 620 base pairs (bp). All reactions were conducted in Verit Thermo Cycler (Applied Biosystems) with the following conditions:

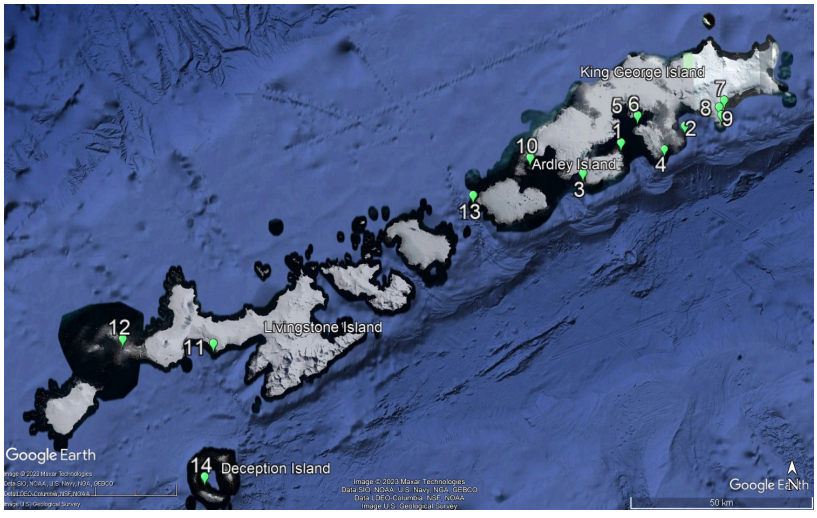

Figure 1. Localization of the collection sites in the present study, South Shetland Islands, October 2021 - March 2022. 1 - Western Shore of Admiralty Bay (ASPA 128), 2 - Lions Rump (ASPA 151), 3 - Potter Peninsula (ASPA 132), 4 - Martins Head, 5 - Keller Peninsula, 6 - Point Hannequin, 7- Three Sister´s Point, 8 - Turret Point, 9 - Penguin Island, 10 - Ardley Island (ASPA 150), 11 - Hannah Point, 12 - Byers Peninsula, 13 - Harmony Point, 14 - Deception Island.

reverse transcription (50˚C, 30 min), reverse transcriptase inactivation, and DNA polymerase activation (95˚C, 15 min), followed by 40 cycles of DNA denaturation (94˚C, 45 s) and annealing (52˚C, 45 s) and extension (72˚C, 45 s) and one cycle of the final extension step (72˚C, 10 min). Following, the second PCR using Phusion RT-PCR Enzyme Mix kit (Sigma-Aldrich), primers Bat1F 5'-GGTTGGGACTATCCTAAGTGTGA -3' and Bat1R 5'-CCATCATCAGATAGAATCATCAT-3' and 1 uL of the amplified product as a template were used in the following conditions: denaturation (98˚C, 30 s), followed by 35 cycles of DNA denaturation (98˚C, 15 s) and annealing (52˚C, 15 s), extension (72˚C, 30 s) and one cycle of the final extension step (72˚C, 5 min). Amplicons (~440 bp) were visualized on 1.5% agarose gels with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific).

RESULTS AND DISCUSSION

The Antarctic region is characterized by its unique and diverse fauna, including various species of seabirds such as Adélie (*Pygoscelis adeliae*), chinstrap (*P. antarcticus*), gentoo (*P. papua*), and macaroni (*Eudyptes chrysolophus*) penguins, Antarctic terns (*Sterna vittata*), skuas (*Stercorarius* spp) and gulls (*Larus dominicanus*). Additionally, the region is inhabited by several species of pagophilic true seals, including the crabeater (*Lobodon carcinophaga*), leopard (*Hydrurga leptonyx*), Ross (*Ommatophoca rossii*), and Weddell (*Leptonychotes weddellii*) seals,

Table I. Geographical Localization of Collection Sites: South Shetland Islands (October 2021 - March 2022) with ASPA (Antarctic Specially Protected Area) and IBA (Important Bird Area) Designations.

No.	Island	Study site	Latitude (S)	Longitude (W)	Observation
1	King George Island	Western Shore of Admiralty Bay	62°12'	58°28'	Colony of *Pygoscelis papua* and *Pygoscelis adeliae*, IBA 46, ASPA 128
2		Lions Rump	62°08'	58°08'	Colony of *P. papua* and *P. adeliae*, ASPA 151
3		Potter Peninsula	58°39'	58°39'	Colony of *P. papua,* IBA 47, ASPA 132
4		Martins Head	62°11'	58°14'	Rocky shore, without penguin colony
5		Keller Peninsula	62°05'	58°23'	Rocky shore, without penguin colony
6		Point Hennequin	62°06'	58°22'	Rocky shore, *Stercorarius maccormicki* colony, IBA 45
7		Three Sister's Point	62°04'	57°53'	Rocky shore, without penguin colony
8		Turret Point	62°05'	57°55'	Colony of *P. papua*
9	Penguin Island		62°06'	57°55'	Colony of *P. antarcticus*
10	Ardley Island		62°13'	58°56'	Colony of *P. papua*, IBA 48, ASPA 150
11	Livingston Island	Hannah Point	62°39'	60°37'	Colony of *P. papua*
12		Byers Peninsula	62°38'	61°05'	Colony of *P. papua*, IBA 54, ASPA 126
13	Nelson Island	Harmony Point	62°18'	59°12'	Colony of *P. antarcticus*, IBA 49, ASPA 133
14	Deception Island	Fumarole Bay, Whaler`s bay	62°58'	60°40'	Rocky shore, without penguin colony, IBA 55 and 56, ASPA 140

as well as the Antarctic fur seal (*Arctocephalus gazella*) (Shirihai, 2008). Many of these species have been observed in the South Shetland Islands. During the Antarctica Expedition in 2021/2022 fresh fecal samples were collected from these animals, resulting in a total of 254 samples of seabird feces and 61 samples of mammalian feces (Tables II and III). Out of the total 315 fecal samples collected and tested for coronaviruses and influenza A viruses, none of them tested positive.

In a previous expedition to Antarctica conducted by your research group in 2019/2020, the presence of IA virus was detected in environmental fecal samples collected in the same study areas (Ogrzewalska et al. 2022). A total of 95 fecal samples were collected from bird colonies and screened for IA. Among the seven samples collected on Penguin Island, five tested positive for IA. Upon analyzing the genomes obtained from four samples, the subtype H11N2 was identified in fecal samples from *P. adeliae* and a colony of *P. antarcticus*. This subtype had previously been observed in *P. adeliae* colonies on King George Island (Hurt et al. 2016), in snowy sheathbill (*Chionis albus*) on Kopaitik Island, Antarctic Peninsula (Hurt et al. 2014), and in *P. antarcticus* in Cape Shirreff, Livingston Island (Shu & McCauley 2017). Bayesian phylogeographic analysis revealed that all currently available H11N2 samples from Antarctica's avifauna cluster together in a single group that emerged in the early 2010s, indicating its continued circulation on the continent. Other subtypes, such as H6N8 (de Seixas et al. 2022), H4N7 (de Souza Petersen et al. 2017), and H5N5 (Hurt et al. 2016, Wille et al. 2019), have also been detected in Antarctic birds. Additionally, previous serological studies have demonstrated the presence of antibodies against IAVs in various Antarctic seabirds, including *P. adeliae, P. antarcticus, P. papua, C. maccormicki*, and *C.*

Table II. Bird Fecal Sample Collection on South Shetland Islands: October 2021-March 2022.

Study site	Species	No of samples
1	*Pygoscelis adeliae*	8
	Pygoscelis papua	6
	Pygoscelis spp	3
	Stercorarius skua	2
	Sterna spp	3
	not indentified	1
2	*P. adeliae*	8
	P. papua	24
	Pygoscelis sp	1
3	*Pygoscelis antarcticus*	1
	P. papua	4
	Pygoscelis spp	10
4	*Py. antarcticus*	1
	Pygoscelis spp	3
	Phalacrocorax bransfieldensis	1
	Larus dominicanus	5
	Pygoscelis spp	13
5	*P. papua*	12
	Pygoscelis sp	1
6	*P. papua*	2
	not indentified	1
7	*P. adeliae*	1
	Pygoscelis spp	4
8	*P. papua*	3
9	*P. antarcticus*	27
10	*P. papua*	37
11	*P. papua*	11
	Pygoscelis spp	7
	P.bransfieldensis	1
12	*P. antarcticus*	3
	P. papua	23
	Pygoscelis sp	1
13	*P. papua*	7
	P. papua	6
14	*P. antarcticus*	1
	P. papua	10
	Pygoscelis spp	2
	TOTAL	254

antarctica lonnbergi (Austin & Webster 1993. Miller et al. 2008, Morgan & Westbury 1981, 1988) However, the previous studies that have assessed the circulation of IAVs in Antarctic seabirds by PCR methods (Barriga et al. 2016, de Seixas et al. 2022, de Souza Petersen et al. 2017, Hurt et al. 2016, Hurt et al. 2014) have consistently observed a low prevalence of infection, typically below 5%. Therefore, the absence of virus detection in the collected environmental samples during your expedition could be attributed to the low occurrence of IAV in the sampled seabird populations during that specific period. It highlights the importance of continuous surveillance and monitoring efforts to understand the dynamics of IAV circulation in Antarctic wildlife.

Table III. Mammal Fecal Sample Collection on South Shetland Islands: October 2021-March 2022.

Study site	Species	No of samples
1	*Leptonychotes weddellii*	1
2	*Mirounga leonina*	1
	Arctophoca gazella	4
3	*M. leonina*	1
	A. gazella	3
4	*A. gazella*	10
5	*L. weddellii*	2
7	*M. leonina*	3
	A. gazella	1
8	*M. leonina*	3
	A. gazella	16
9	*A. gazella*	2
10	*A. gazella*	4
11	*M. leonina*	4
12	*M. leonina*	4
13	*M. leonina*	2
Total		61

Regarding coronavirus surveillance, to the best of our knowledge, there have been no studies conducted on coronaviruses in Antarctic mammals, however, marine mammals, like other animals, can be susceptible to infectious diseases. Previous studies have already reported the presence of *Gammacoronavirus* in marine mammal, such a deceased beluga whale (*Delphinapterus leucas*) (Mihindukulasuriya et al. 2008) and in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) (Woo et al. 2014) that are associated with respiratory diseases in these animals. The transmission of viruses between humans and marine mammals is a possibility as well, particularly in cases where there is close contact or potential exposure. It is believed that the transmission of SARS-CoV-2 to marine mammals could occur through respiratory droplets or contact with contaminated surfaces. Given the limited information available, it is essential for researchers and experts to continue monitoring the situation and studying the potential impacts of COVID-19 on marine mammals (Audino et al. 2021, Barbosa et al. 2021).

According to a recent review, avian coronaviruses have been detected in 15 orders, comprising 30 families, and across 108 species of wild birds (Wille & Holmes 2020). In Antarctica, some avian coronaviruses have been more recently identified through next-generation sequencing (NGS). Using this approach, researchers detected novel *Deltacoronavirus* in *P. papua* from Kopaitik Island, Antarctic Peninsula (Wille et al. 2020) as well as in environmental samples from *C. albus* from Nelson Island and around Isabel Riquelme Islet (Zamora et al. 2023).

Therefore, there is still much to be explored regarding the ecology of IAs and CoVs in the Antarctic ecosystems, as the current knowledge remains incomplete. The primary concern now

lies in the surveillance of the Highly Pathogenic Avian Influenza Virus (HPAIV) caused by subtype H5N1. Recent outbreaks of the highly virulent HPAI H5N1 influenza virus in South America indicate a significant risk of its introduction to the Antarctic Peninsula and South Shetland Islands. This risk is heightened due to the presence of several species that are currently affected in the Northern Hemisphere, including Antarctic migrants such as skuas, terns, and other migratory bird species that interact with them. The implications of this subtype not only endanger penguins and marine mammals but also pose a risk to the safety of researchers and tourists visiting colonies.

We also recognize the limitations of our study, particularly the omission of collecting other tissues, such as serum, which might have yielded valuable insights into the presence of antibodies against influenza and coronavirus in Antarctica's animals. Unfortunately, the harsh and remote Antarctic environment presented logistical challenges, making the collection of additional sample types impossible during this expedition. Yet, we are committed to addressing this limitation in future collections, aiming to include diverse tissue samples to improve the comprehensiveness of our research. Additionally, we acknowledge another limitation of our study, namely the small sample size. At the outset, logistical and environmental challenges in Antarctica constrained our ability to collect a larger number of samples. However, it is essential to emphasize that despite the modest sample size, our study makes a substantial contribution to enhancing our understanding of virus circulation within the Antarctic ecosystem.

In a changing world, characterized by the emergence of zoonotic diseases, the surveillance of pathogenic viruses in birds and mammals inhabiting the Antarctic ecosystem is an integral part of health monitoring strategies, with a specific focus on the One Health perspective. Moreover, it will be crucial to integrate data elements ranging from the micro level (genes) to the macro level (social, political, climate, and global migration routes) to anticipate, assess risks, and adequately prepare for potential epidemics.

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Conception of the study: MO, MS, WD. Sample collection: LM, MM, HM, RV, MO, MB. Methodology, data analysis and interpretation: MO, MS, FG, TP, MB. The initial draft of the manuscript was written by FG, TP, and MO. Contributed with manuscript revision: RV, MB, MS, WD. All authors read and approved the final manuscript.

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