

Molecular detection of highly pathogenic H5N1 Avian Influenza in seabirds from Lima, Peru in 2022

Detección molecular del virus de influenza aviar H5N1 de alta patogenicidad en aves marinas en Lima, Perú (2022)

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Abstract

The ongoing global epidemic of highly pathogenic avian influenza A (H5N1) has caused unprecedented mortality in wild birds and poultry since 2021, with sustained transmission across multiple continents. In late 2022, the virus was first detected in wild seabirds along the Peruvian coast. In this study, we report the detection of H5N1 avian influenza virus by RT-qPCR in deceased free-living seabirds collected from a beach in Lima, Peru. Viral RNA was identified using validated RT-qPCR assays targeting influenza A, and the H5 and N1 subtypes. Positive results were obtained from one adult Peruvian pelican (*Pelecanus thagus*) and one juvenile Inca tern (*Larosterna inca*), representing the first confirmed detection of H5N1 in *L. inca* in Peru. These findings provided early evidence of the virus's southward spread and highlight the role of migratory and coastal seabirds in dissemination. Continued surveillance integrating wildlife monitoring, genomic analysis, and a One Health approach is essential to assess viral evolution and mitigate risks to wildlife and human health.

Keywords: H5N1, highly pathogenic avian influenza, *Pelecanus thagus*, *Larosterna inca*, Peru

Resumen

La actual epidemia global de influenza A aviar altamente patógena (H5N1) ha causado una mortalidad sin precedentes en aves silvestres y de corral desde 2021, con transmisión sostenida en múltiples continentes. A finales de 2022, el virus fue detectado por primera vez en aves marinas silvestres a lo largo de la costa peruana. En este estudio, reportamos la detección del virus de influenza aviar H5N1 mediante RT-qPCR en aves marinas libres encontradas muertas en una playa de Lima, Perú. El ARN viral se identificó utilizando ensayos validados de RT-qPCR dirigidos a influenza A y a los subtipos H5 y N1. Se obtuvieron resultados positivos en un pelicano peruano adulto (*Pelecanus thagus*) y en un gaviotín inca juvenil (*Larosterna inca*), representando la primera detección confirmada de H5N1 en *L. inca* en Perú. Estos hallazgos proporcionan evidencia temprana de la propagación del virus hacia el sur y resaltan el papel de las aves marinas migratorias y costeras en su diseminación. La vigilancia continua que integre monitoreo de fauna silvestre, análisis genómico y un enfoque One Health es esencial para evaluar la evolución viral y mitigar los riesgos para la vida silvestre y la salud humana.

Palabras clave: H5N1, Influenza A altamente patogénica, *Pelecanus thagus*, *Larosterna inca*, Peru

INTRODUCTION

Influenza A viruses (AIVs) continue to pose a global health challenge for both animals and humans due to their zoonotic potential and broad ecological circulation in wild birds (Jiménez-Bluhm *et al.*, 2022). The emergence of highly pathogenic avian influenza (HPAI) associated with the A/goose/Guangdong/1/96 (H5N1) lineage marked a shift in AIV dynamics, and since 2014, viruses belonging to clade 2.3.4.4—particularly subclade 2.3.4.4b—have driven intercontinental outbreaks and caused massive mortality events in avian populations (González Reiche and Pérez, 2012; Williams, 2012).

In 2021, H5N1 clade 2.3.4.4b was introduced into North America by migratory birds via transatlantic flyways, subsequently spreading across the continent and facilitating reassortment events and host range expansion (Xu *et al.*, 2025; Ramey *et al.*, 2022). In South America, the introduction of H5N1 was confirmed in late 2022 (Kuiken *et al.*, 2025); in Chile, near the border with Peru, clade 2.3.4.4b was detected in wild birds and showed close genetic links to contemporaneous Peruvian strains, with a wave-like southward spread documented during 2022–2023 (Sánchez Rojas *et al.*, 2025; Brunó *et al.*, 2025; Muñoz *et al.*, 2024). In Peru, H5N1 (clade 2.3.4.4b) was first identified in November 2022, triggering massive mortality events in seabirds, particularly affecting the Peruvian booby (*Sula variegata*), the Guanay cormorant (*Leucocarbo bougainvillii*), and the Peruvian pelican (*Pelecanus thagus*) (Gamarrá-Toledo *et al.*, 2023). In addition, interspecies transmission to marine mammals was reported, resulting in exceptional mortality events among South American sea lions (*Otaria flavescens*) (Gamarrá-Toledo *et al.*, 2023).

Within the context of the ongoing outbreak in Peru, oropharyngeal swabs were collected from fresh carcasses of two Peruvian boobies (*Sula variegata*), two Peruvian pelicans (*Pelecanus thagus*), and one Inca tern (*Larosterna inca*) on a beach in Lima, in order to confirm H5N1 infection by RT-qPCR.

MATERIALS AND METHODS

Oropharyngeal swabs from five fresh bird carcasses were taken on Venecia Beach - Villa El Salvador, Lima, Peru (coordinates -12.23048 degrees, -76.97560 degrees) - in December 2022, stored in UTM transport medium (Copan Diagnostics Inc., Brescia, Italy), and kept refrigerated at -80 °C until use. The carcasses were identified as two adult Peruvian boobies (*Sula variegata*), two adult Peruvian pelicans (*Pelecanus thagus*) and one juvenile Inca tern (*Larosterna inca*).

RNA extraction was done using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, Irvine, USA), with an elution volume of 50 µL. For cDNA synthesis, the First Strand cDNA Synthesis Kit (Canvax, Valladolid, Spain) was employed using 5 µL of RNA per sample, according to the manufacturer's instructions. A negative control using nuclease-free water was included. Reaction conditions were 25°C for 10 minutes

and 50°C for 50 minutes in a Benchmark TC 9639 Gradient Thermal Cycler (Benchmark Scientific, Sayreville, USA).

RT-qPCR was performed in a LineGene K plus thermocycler (Bioer, Hangzhou, China). The primers and 5' FAM - 3' BHQ1 labeled hydrolysis probes (Macrogen, Seoul, Korea) used were taken from a validated detection panel of avian influenza viruses (Hassan *et al.*, 2022), which included assays for avian influenza A viruses in general, and those specifically for H5 and N1 subtypes. Primer-probe mixes were prepared as specified therein. Reagent composition per single reaction (12.5 µL) was as follows: 1.25 µL 10x Buffer, 1.2 µL MgCl₂ (25mM), 1.2 µL dNTP mix (2mM each), 1 µL primer-probe mix, 0.12 µL HotBegan Taq DNA polymerase (Canvax, Valladolid, Spain), 5.23 µL nuclease-free water and 2.5 µL of sample cDNA. Cycling conditions were 95 °C for 10 minutes, 45 cycles of 95°C for 15 s, 56.5 °C for 20 s and 72°C for 30 s. Data were analysed using the LineGene 4800 Software v. 1.0.13 for the amplification curves, with automatic threshold setting. RT-qPCR included nuclease free water instead of sample as Non-Template Control.

This study was approved by the institutional ethics committee of the Universidad Científica del Sur.

RESULTS

Consistent amplification curves for avian influenza in general (Figures 1-3), H5 and N1 subtypes were found in two out of the five samples (40%), one from a Peruvian pelican and the other from the juvenile Inca Tern. Cycle Threshold (Ct) values for general avian influenza qPCR were 31.16 and 34.41, for H5 subtype were 27.37 and 28.23, and for N1 subtype were 28.98 and 27.5 respectively.

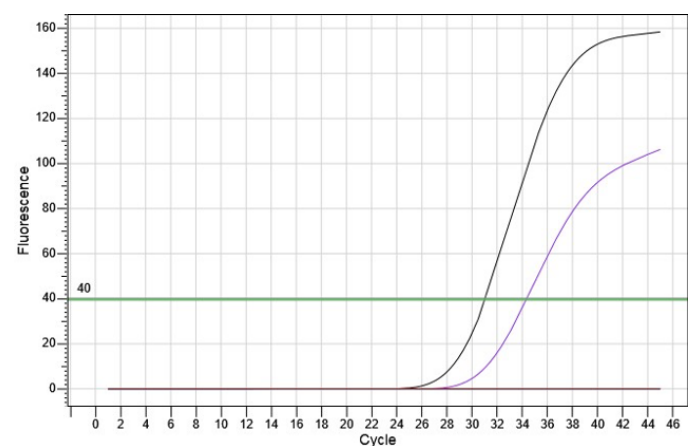


Figure 1. Amplification plot for the general avian influenza assay. Black and purple curves correspond to Peruvian pelican (*Pelecanus thagus*) and Inca Tern (*Larosterna inca*) respectively.

Notwithstanding, amplification was detected for one of the Peruvian booby samples in the H5 and N1 subtype assays (Figures 2 and 3), albeit with non-sigmoidal curves.

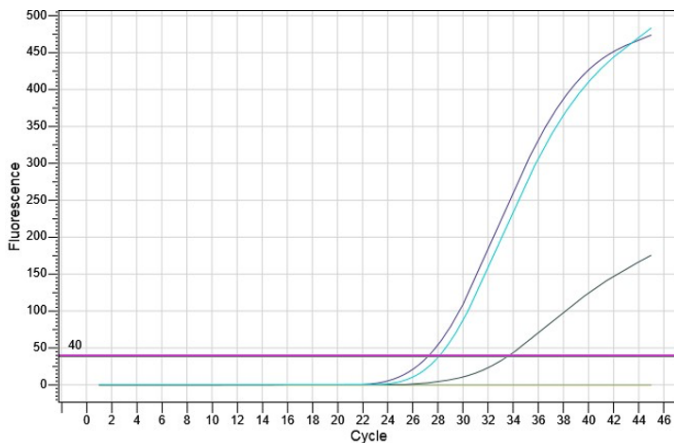


Figure 2. Amplification plot for H5 subtype assay. Blue and light blue sigmoidal curves correspond to Peruvian pelican (*Pelecanus thagus*) and Inca Tern (*Larosterna inca*) respectively, while black curve correspond to Peruvian booby (*Sula variegata*).

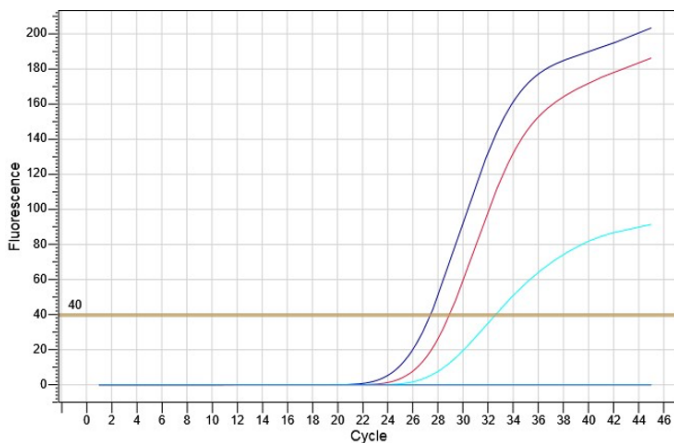


Figure 3. Amplification plot for the N1 subtype assay. Red and blue sigmoidal curves correspond to Peruvian pelican (*Pelecanus thagus*) and Inca Tern (*Larosterna inca*) respectively, while light blue curve correspond to Peruvian booby (*Sula variegata*).

DISCUSSION

This report confirms the presence of the H5N1 virus in at least two species of seabirds off the coast of Peru in December 2022. This finding is consistent with the detection of highly pathogenic avian influenza H5N1 clade 2.3.4.4b in Peru between November 2022 and February 2023, with evidence of widespread viral circulation among marine wildlife species, including *Larosterna inca* and *Pelecanus thagus* (Kuiken *et al.*, 2025; Leguia *et al.*, 2023; Cruz *et al.*, 2023). The carcasses of at least 55,000 of dead seabirds from different species on beaches and islands of the Peruvian coast between November 2022 and January 2023 (Gamarratoledo *et al.*, 2023; SERNANP, 2023) confirm one of the major avian influenza outbreaks on record in Peru. In particular, seabirds have been identified as particularly vulnerable to AIVs due to nesting in dense colonies (Stokstad, 2022). Since seabirds in Peru are of both ecological and economical importance, accurate detection of H5N1 avian influenza would assist the national authorities in the development of mitigation strategies and epidemiological surveillance.

The highly pathogenic avian influenza A (H5N1) outbreak that began in 2022 has caused unprecedented mortality among wild and domestic birds across the Americas (Kuiken *et al.*, 2025). By the end of 2022, the virus had already been detected in numerous countries throughout the continent, affecting both wild birds and poultry (Pan American Health Organization, 2024, 2025). Between 2023 and 2025, the situation intensified, with thousands of outbreaks reported in 19 countries and territories, along with multiple spillover events to mammals, including mass die-offs in marine mammals, infections in terrestrial wildlife, domestic cats, and the emerging detection in dairy cattle in the United States—underscoring the critical importance of the One Health approach (U.S. Department of Agriculture, Animal and Plant Health Inspection Service, 2025).

Before 2022, the subtypes of avian influenza previously reported in Peru were H3N8, H4N5, H10N9, H13N2, H7N3, H2N9, H1N1, H1N9, H3N1, H3N2, H10N2, H10N7, H11N9, H12N5, H6N8, H13N6, H6N2, H2N6 (Castro-Sanguinetti *et al.*, 2022; Ghersi *et al.*, 2011; Ghersi *et al.*, 2019; Nelson *et al.*, 2016).

The origin of the H5N1 strain in Peru remains under debate, with hypotheses pointing to birds of the family *Laridae* (gulls and terns) as well as *Anatidae* (ducks and geese) as potential sources of virus introduction. While *Anatidae* are considered the classical reservoirs of avian influenza viruses, *Laridae* play a relevant—though less studied—role in the intercontinental dispersal of the virus (Arnal *et al.*, 2015; Williams *et al.*, 2012). Genomic evidence from the Peruvian strain of clade 2.3.4.4b, closely related to those detected in North America and Chile, supports the hypothesis of a single introduction of H5N1 through migratory birds arriving from the Northern Hemisphere between October and November 2022, likely mediated by species from both families (Kandeil *et al.*, 2023).

The main limitations of the present study were non-probability sampling and a very small sample size. Moreover, the presence of the virus in the other species tested, the Peruvian booby, is not ruled out due to the detection limit of the technique or possible sample degradation, since the time from death until sampling is unknown. The overall prevalence of H5N1 avian influenza in the zone is quite underestimated since at the moment of sampling several deceased birds were present at the referred location, with the majority of carcasses being Peruvian pelicans, consistent with the reports from the national sanitary authorities.

More recently, H5N1 (clade 2.3.4.4b) has expanded its host range in an unprecedented manner: infection has been reported in dairy cattle in the United States (2024), detection of the virus in raw milk, and human cases linked to occupational exposure, reinforcing the urgency of a One Health approach in the region (Gamarratoledo *et al.*, 2023). Additionally, the arrival of H5N1 in Antarctica and sub-antarctic islands was confirmed in 2025, with genomes closely related to South American strains and an unusual 17-amino acid deletion in the neuraminidase stalk region, highlighting biogeographic connectivity and the ongoing evolution of the virus (Xu *et al.*, 2025; Kuiken *et al.*, 2025; Leguia *et al.*, 2023; Bruno *et al.*, 2025; Clessin *et al.* 2025).

This report confirms the presence of H5N1 avian influenza by molecular methods in at least two species of seabirds from Peru. Further continuous studies should focus on H5N1 incidence determinations, viral strain sequencing for epidemiological surveillance and evaluation of population decline of the aforementioned wild bird species.

AUTHOR CONTRIBUTIONS

LPK, MP, CZ conceived the study. LPK and MP collected the samples and performed RNA extraction. LPK performed cDNA synthesis, RT-qPCR and data analysis. CZ identified the seabird species and gave recommendations for sampling. LPK, MP, CZ wrote the manuscript. All authors made contributions to the draft and gave final approval for publication.

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COMPETING INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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