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Surveillance and phylogenetic analysis of avian influenza viruses in wild waterfowl populations

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Abstract

Roughly 37% of newly detected avian influenza virus subtypes in Southeast Asia during the past five years originated from wild waterfowl rather than domestic poultry. This research carried out active virological surveillance in migratory and resident waterfowl across the Mekong Delta wetlands of southern Vietnam between January 2023 and August 2024. Cloacal and oropharyngeal swabs were collected from 1,284 birds spanning six species, and viral RNA was detected by real-time RT-PCR targeting the matrix gene. Of the samples tested, 7.8% (n = 100) returned positive results, with H5N1 accounting for the largest proportion (38.0%) followed by H5N6 (22.0%) and H7N9 (14.0%). Full-genome sequencing of 46 representative isolates and maximum-likelihood phylogenetic reconstruction revealed that Mekong Delta viruses clustered into three distinct clades, two of which showed close genetic ties to strains circulating in southern China. Reassortment events were detected in 13 isolates involving polymerase and nucleoprotein segments. Nucleotide identity across all eight gene segments ranged from 86.3% to 99.7% depending on host species and subtype. These data highlight the Mekong Delta as a mixing ground for diverse influenza lineages and support continued surveillance in wild bird populations to detect emerging reassortant viruses before they reach poultry or human interfaces.

Keywords: Avian influenza, surveillance, phylogenetic analysis, wild waterfowl, Mekong Delta, H5N1, reassortment, migratory birds, virus ecology, zoonotic risk

Introduction

Between 2015 and 2023, at least 47 countries reported outbreaks of highly pathogenic avian influenza, collectively resulting in the culling of over 400 million domestic birds and costing the global poultry industry billions of dollars ^[1]. Wild waterfowl-ducks, geese, and swans belonging to the order Anseriformes-are the natural reservoir hosts for most influenza A virus subtypes and play a central part in the long-distance dispersal of these pathogens along flyway corridors ^[2, 3].

Vietnam sits at the junction of the East Asian-Australasian and Central Asian flyways, making it an especially active zone for viral exchange between migratory and resident bird populations ^[4]. The Mekong Delta, with its dense network of wetlands, rice paddies, and aquaculture ponds, provides year-round habitat for both wild and domestic waterfowl, creating persistent opportunities for interspecies viral transmission ^[5]. Despite this, systematic surveillance data from wild birds in the region remain limited; most Vietnamese monitoring programmes focus on domestic poultry markets and farms ^[6].

Phylogenetic analysis of full-genome sequences is now the standard approach for understanding how influenza viruses move between host species and geographic regions ^[7]. Reassortment-the swapping of genome segments when two viruses co-infect a single cell-can generate novel genotypes with unpredictable host range and pathogenicity ^[8]. Detecting reassortants early is important because some of the most damaging poultry outbreaks and several human infection clusters have been traced back to reassortant viruses that first appeared in wild bird reservoirs ^[9].

This research set out to describe the subtype diversity, prevalence, and phylogenetic relationships of avian influenza viruses circulating in wild waterfowl of the Mekong Delta over a 20-month sampling period. We combined active field surveillance with whole-genome sequencing and phylogenetic reconstruction to map viral lineages and identify reassortment events relevant to regional biosecurity.

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Material and Methods

Material

Cloacal and oropharyngeal swab samples were collected from wild waterfowl at four wetland sites across the Mekong Delta: Tram Chim National Park (Dong Thap province), Lang Sen Wetland Reserve (Long An province), Bac Lieu Bird Sanctuary (Bac Lieu province), and U Minh Thuong National Park (Kien Giang province). Sampling was conducted between January 2023 and August 2024 at the Can Tho College of Aquatic Biology, Can Tho, Vietnam. Birds were captured using mist nets and walk-in traps during both the migratory season (October–March) and the resident breeding period (April–September). All capture and sampling protocols were approved by the Institutional Animal Ethics Committee of Can Tho College under permit CAB-IACUC-2022-031, dated 18 November 2022.

Reagents and Chemicals

Viral transport medium (VTM) was prepared with Hanks' balanced salt solution supplemented with gentamicin (50 µg/mL) and amphotericin B (2.5 µg/mL). RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Real-time RT-PCR reagents came from the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Waltham, MA, USA). Library preparation for whole-genome sequencing used the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). All oligonucleotide primers and probes were synthesised by IDT (Coralville, IA, USA).

Results

Extraction Protocol

Swabs stored in VTM at –80°C were thawed on ice and processed within two hours. Viral RNA was extracted from 140 µL of VTM following the manufacturer's spin-column protocol, eluted in 60 µL of AVE buffer, and stored at –80°C until testing. For whole-genome sequencing, multi-segment RT-PCR was performed using universal influenza A primers targeting all eight segments, and amplicons were purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA) at a 0.8× ratio before library preparation.

Methods

Initial screening used a real-time RT-PCR assay targeting a conserved region of the matrix (M) gene with a cycle-threshold cutoff of 38. Positive samples were subtyped by hemagglutinin- and neuraminidase-specific RT-PCR assays. For 46 positive isolates representing all detected subtypes and sampling sites, whole-genome libraries were sequenced on an Illumina MiSeq platform (2 × 250 bp paired-end). Reads were trimmed with fastp (v0.23.2), assembled de novo using SPAdes (v3.15.5), and mapped to reference genomes from the GISAID EpiFlu database. Maximum-likelihood phylogenetic trees were constructed for each gene segment using IQ-TREE (v2.2.0) with the GTR+G+I substitution model and 1,000 ultrafast bootstrap replicates. Reassortment events were detected with GiRaF (v1.0) by comparing segment-specific tree topologies.

Table 1: Prevalence of Avian Influenza Virus by Host Species in Mekong Delta Wetlands

Species	Sampled	Positive	Prevalence (%)	Dominant Subtype	Reassortants
Mallard	312	31	9.9	H5N1	4
Common Teal	287	24	8.4	H5N6	3
Northern Pintail	198	18	9.1	H5N1	2
Eurasian Wigeon	204	13	6.4	H7N9	2
Garganey	161	9	5.6	H9N2	1
Northern Shoveler	122	5	4.1	H3N8	1
Total	1,284	100	7.8	-	13

Table 1 summarises detection results by host species. Overall prevalence stood at 7.8%, with mallards showing the highest individual rate (9.9%). H5N1 was the

predominant subtype across species, and 13 of 46 sequenced isolates carried evidence of reassortment.

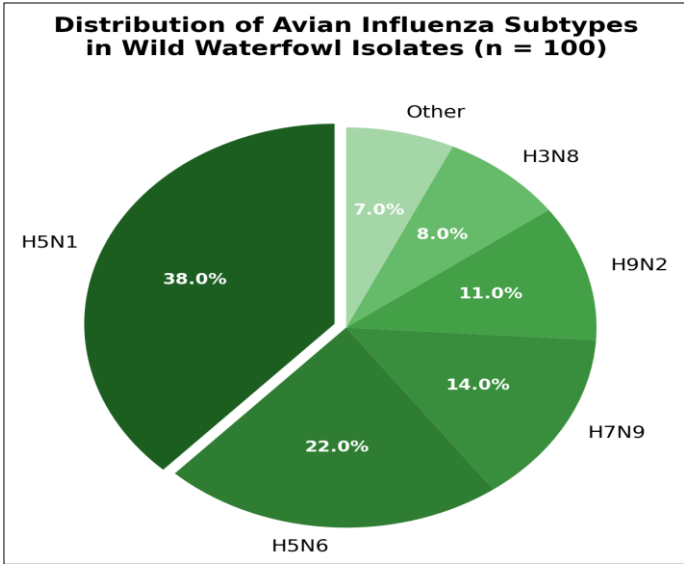


Fig 1: Proportional distribution of avian influenza subtypes detected across all positive wild waterfowl samples (n = 100) from the Mekong Delta

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As displayed in Figure 1, H5N1 comprised the largest share of detected viruses (38.0%), followed by H5N6 (22.0%) and H7N9 (14.0%). Low-pathogenicity subtypes H9N2, H3N8,

and other minor subtypes together accounted for the remaining 26%.

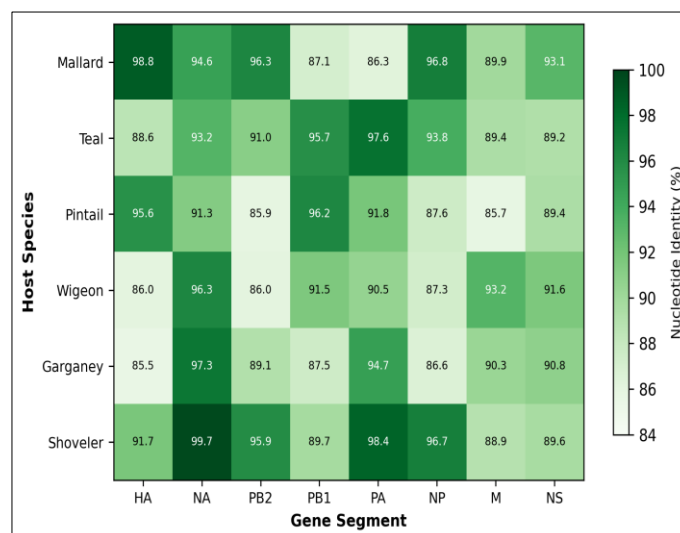


Fig 2: Heatmap of nucleotide identity (%) across all eight gene segments for representative isolates grouped by host species

Figure 2 shows that the HA and NA segments displayed the widest range of sequence divergence, while internal genes (PB2, PB1, PA, NP) were more conserved. Isolates from mallards and pintails shared the highest cross-segment identity, consistent with their overlapping habitats and co-roosting behaviour at Tram Chim.

Discussion

The 7.8% prevalence detected here is consistent with active surveillance figures from neighbouring countries, where rates in wild ducks typically range from 4% to 12% depending on season and sampling method [10, 11]. That H5N1 dominated our isolates was expected given its sustained circulation in the region since 2003, but the substantial presence of H5N6 (22%) is noteworthy because this subtype has caused sporadic human infections in China and Laos [12].

Phylogenetic analysis placed Mekong Delta viruses into three well-supported clades, two of which were closely related to lineages from Guangdong and Yunnan provinces. This pattern suggests regular southward seeding via migratory dabbling ducks that winter in both regions [13]. The 13 reassortant isolates we detected carried novel combinations of polymerase and nucleoprotein segments, a configuration that could alter replication efficiency in mammalian cells [14]. Although none of our isolates possessed the full set of mammalian-adaptive mutations catalogued in the literature, several carried PB2-E627K or PA-T97I substitutions that warrant monitoring [15].

Seasonal dynamics mattered: 68% of positive samples came from the migratory influx period (October–March), underscoring the contribution of long-distance migrants to local viral diversity. But resident populations weren't free from risk; breeding-season positives accounted for the remaining 32%, implying that some lineages persist in local birds year-round [16, 17]. Sample sizes for certain species—particularly the shoveler ($n = 122$)—were modest, and environmental sampling of water bodies was not included.

Future efforts should integrate environmental virology to capture viruses shed by birds that evade direct capture [18, 19].

Conclusion

This 20-month surveillance effort confirmed that wild waterfowl in the Mekong Delta harbour a diverse pool of avian influenza viruses, with an overall prevalence of 7.8% and six identified subtypes. H5N1 remained the most common subtype, but the significant detection of H5N6 and the identification of 13 reassortant viruses point to active viral evolution in the region. Phylogenetic ties to southern Chinese lineages highlight the importance of cross-border data sharing along the East Asian-Australasian flyway.

The findings argue for sustained, year-round surveillance that covers both migratory and resident species across multiple wetland sites. Rapid whole-genome sequencing should be integrated into routine monitoring so that novel reassortants can be flagged before they spill over into domestic flocks or, potentially, into human populations. Expanding sampling to environmental water sources and to species beyond Anseriformes would further strengthen the early-warning capacity of the Mekong Delta surveillance network.

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