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Virology

Genetic and antigenic analyses of H5N8 and H5N1 subtypes high pathogenicity avian influenza viruses isolated from wild birds and poultry farms in Japan in the winter of 2021–2022

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ABSTRACT. In the winter of 2021–2022, multiple subtypes (H5N8 and H5N1) of high pathogenicity avian influenza viruses (HPAIVs) were confirmed to be circulating simultaneously in Japan. Here, we phylogenetically and antigenically analyzed HPAIVs that were isolated from infected wild birds, an epidemiological investigation of affected poultry farms, and our own active surveillance study. H5 subtype hemagglutinin (HA) genes of 32 representative HPAIV isolates were classified into clade 2.3.4.4b lineage and subsequently divided into three groups (G2a, G2b, and G2d). All H5N8 HPAIVs were isolated in early winter and had HA genes belonging to the G2a group. H5N1 HPAIVs belong to the G2b and G2d groups. Although G2b viruses were widespread throughout the season, G2d viruses endemically circulated in Northeast Japan after January 2022. Deep sequence analysis showed that the four HPAIVs isolated at the beginning of winter had both N8 and N1 subtypes of neuraminidase genes. Environmental water-derived G2a HPAIV, A/water/Tottori/NK1201-2/2021 (H5N8), has unique polymerase basic protein 1 and nucleoprotein genes, similar to those of low pathogenicity avian influenza viruses (LPAIVs). These results indicate that multiple H5 HPAIVs and LPAIVs disseminated to Japan via transboundary winter migration of wild birds, and HPAIVs with novel gene constellations could emerge in these populations. Cross-neutralization test revealed that G2a H5N8 HPAIVs were antigenically distinct from a G2b H5N1 HPAIV, suggesting that antibody pressure in wild birds was involved in the transition of the HPAIV groups during the season.

KEYWORDS: avian viral disease, antigenicity, epidemiology, influenza virus, phylogeny

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After the high pathogenicity avian influenza (HPAI) outbreak caused by an H5N1 subtype influenza virus on a goose farm in China in 1996, its virus progenies (Gs/GD-like viruses) have evolved both in phylogenetic and antigenic senses [36]. Initially, HPAI outbreaks largely occurred in poultry populations; however, after more than a thousand migratory birds died from HPAIV infection in 2005 in Lake Qinghai, China [5, 18], HPAI viruses (HPAIVs) has been repeatedly reported globally, suggesting the involvement of infected migratory birds. Gs/GD-like viruses are classified into 10 clades, ranging from 0 to 9, according to the phylogenetic lineage of the H5 subtype hemagglutinin (HA) [35]. More recently, clade 2.3.4.4 H5Nx HPAIVs have become common worldwide and are subdivided into eight groups: a–h [17].

Since October 2020, clade 2.3.4.4b H5 subtype HPAIVs have become disseminated worldwide, causing outbreaks in wild birds and poultry populations [1, 8]. In Europe, H5N8 HPAIVs were predominantly circulating in the winter of 2020–2021, and the subtype of circulating viruses shifted to H5N1 in the following winter [1]. Recently, H5N1 HPAIVs have been classified into at least 16 genotypes (G1–G16) based on their gene constellations [8]. According to this classification, the recent European H5N1 HPAIVs mostly belong to genotype G1; such viruses have also been observed in African and North American countries. While the number of reported HPAI outbreaks in Europe and Africa has decreased after the winter of 2021–2022, Canada and the United States have

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not passed the peak of HPAI cases as of June 2022 [1]. Similarly, a shift in circulating HPAIVs subtypes has been observed in East Asian countries. In the winter of 2020–2021, H5N8 HPAIVs caused outbreaks in China, South Korea, and Japan [3, 21, 38]. At least seven gene constellations, genotypes E1–E7, have been found in causal H5N8 HPAIVs [3]. Subsequently, in late 2021, novel H5N1 HPAIVs bearing HA genes similar to those of previous European H5N8 HPAIVs were isolated from a captured Mandarin duck and a dead quail in South Korea [25]. Four genotypes of H5N1 HPAIVs (G1, G7, G9, and G10) were isolated from a wild duck and poultry in China between September 2021 and March 2022 [8]. In Japan, HPAI caused by clade 2.3.4.4b H5N8 and H5N1 HPAIVs were coincidentally observed in November 2021 [13, 23]. This was the first time that HPAIVs of multiple subtypes were confirmed in one winter in Japan. Eventually, HPAIVs were confirmed in 25 poultry farms and 107 wild birds or their environmental samples in the winter of 2021–2022. In the present study, we genetically and antigenically analyzed Japanese HPAIVs from this season that were isolated by our institutes in (I) the definitive diagnosis of infected wild birds performed in collaboration with the Ministry of Environment, Japan [22], (II) an epidemiological investigation in affected poultry farms with the Ministry of Agriculture, Japan, and (III) our own active surveillance to complement the initial reports [13, 23] and to understand the overall HPAI circumstances.

MATERIALS AND METHODS

Isolation and identification of viruses

In total, we isolated 62 H5 subtype HPAIVs from wild birds, poultry, and their environmental samples in the winter of 2021–2022 (listed as "Present study" in the reference columns of Supplementary Tables 1 and 2). For virus isolation, tracheal or cloacal swabs of dead birds or environmental samples (swabs of floor, fans, doors, walls, cages, work boots and gloves, etc.) from farms were collected and placed in 2 mL of phosphate-buffered saline. The samples were passed through a 0.45-µm pored filter (Sartorius, Göttingen, Germany) and inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs (Aoki Breeder Farm, Nasu, Japan). After incubation for 48 hr at 35°C, the allantoic fluids were harvested and their HA activities were examined [12]. The HA and neuraminidase (NA) subtypes of the influenza virus isolates were identified by the hemagglutinin inhibition (HI) [26] and neuraminidase inhibition (NI) tests [2] using reference antisera of avian influenza viruses. The deduced amino acid sequences at the HA cleavage sites were obtained by direct sequencing, as described previously [30]. The viruses were stored as seed virus stocks and were used in subsequent experiments.

Next-generation sequencing

Eleven representative HPAIV isolates from wild birds or their environments were selected based on the reasons as described in the result section. These viruses and 21 poultry-origin HPAIVs were used for next-generation sequencing. Briefly, viral RNA was extracted using the Quick RNA Viral Kit (Zymo Research, Irvine, CA, USA). Libraries were prepared using the MGIEasy RNA Directional Library Prep Set (MGI Tech, Shenzhen, China). Single-stranded circular DNA was prepared based on the library using an MGIEasy Circularization Kit (MGI Tech). A DNA nanoball was prepared using a DNBSEQ-G400RS high-throughput sequencing kit (MGI Tech), which was sequenced using DNBSEQ-G400 (MGI Tech). The sequences matched to chicken-related genes were excluded using HISAT 2 (version 2.2.1). The remaining FASTQ format data were analyzed using the Automatic Influenza virus Genome Assembly and subtyping System (FluGAS) (World Fusion, Tokyo, Japan).

Phylogenetic analysis

Whole nucleotide sequences in coding regions of polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), H5 HA, nucleoprotein (NP), N8 or N1 NA, matrix protein (M), and nonstructural protein (NS) genes of animal-derived influenza A virus isolates from October 2020 to June 2022 were obtained from the Global Initiative on Sharing Avian Influenza Data website (https://www.gisaid.org/). The data were subsequently selected based on the profiles of their origin viruses (subtypes, sampling months, and countries) to include the viruses at different times and locations as much as possible. In total, the nucleotide sequences of 1,230 PB2, 1,191 PB1, 1,247 PA, 1,059 H5HA, 1,214 NP, 471 N8 NA, 589 N1 NA, 1,191 M, and 1,242 NS were eventually used for phylogenetic analyses. Each gene segment was aligned using the MUSCLE program [9], and the best-fit substitution models were all identified as GTR+G+I model by MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms [16]. Maximum likelihood trees were constructed using MEGA X with a resampling process of 1,000 replicates.

Antigenic analysis

The Japanese stockpile vaccine strains and HPAIVs shown in Supplementary Table 3 were used to prepare a panel of chicken polyclonal antisera. Briefly, each virus was propagated in embryonated chicken eggs, inactivated with formalin (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan; final concentration, 0.1%), and purified by sucrose density-gradient ultracentrifugation [15]. Chickens were hatched from fertilized eggs (influenza A virus-free White Leghorn, Aoki Breeder Farm) and reared exclusively in our laboratory. These were immunized at four weeks of age with purified viruses and Complete Freund's Adjuvant (FUJIFILM Wako Pure Chemical Corp.). Additional immunization was conducted at six weeks of age in chickens with low antibody responses. Two weeks after the last immunization, whole blood was sampled via cardiac puncture under isoflurane anesthesia. Serum samples were retrieved and stored as polyclonal antibodies for subsequent cross-HI and neutralization tests. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tottori University (approval number: 22-T-2). For the neutralization assay, polyclonal chicken antisera were serially diluted two-fold and a $10^{2.0}$ 50% tissue culture infectious dose of the virus was mixed and incubated for 1 hr at room temperature. This mixture was inoculated into MDCK cells and overlaid with

Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing 0.7% Bacto-agar (BD, Franklin Lakes, NJ, USA). After 72 hr of incubation at 35°C, cells were fixed and stained with formalin and crystal violet. The neutralizing titer was expressed as the reciprocal of the highest serum dilution resulting in a ≥50% reduction in the number of plaques.

RESULTS

Isolation and identification of H5N8 and H5N1 HPAIVs in Japan in the winter of 2021–2022

In the present study, 62 H5 subtype influenza viruses were isolated from wild birds, poultry, and their environmental samples during the winter of 2021–2022 (Supplementary Tables 1 and 2). The subtypes of the viruses were identified as H5N8 (three strains) or H5N1 (59 strains) by HI and NI testing using reference antisera (data not shown). H5N8 viruses were isolated only at the beginning of winter (November 10th to December 1st). All viruses had multiple basic amino acid motifs (KRRKR or RRRKR) at their cleavage sites, and were identified as HPAIVs.

Phylogenetic analysis of the Japanese H5N8 and H5N1 HPAIV isolates in 2021–2022

Eleven representative HPAIV isolates from wild birds or their environments were selected. A/teal/Miyazaki/211109-32/2021 (H5N1) [Miya32], A/water/Tottori/NK1201-2/2021 (H5N8) [NK1201], A/buzzard/Kyoto/2601B013/2022 (H5N1), and A/jungle crow/Akita/0504F001/2022 (H5N1) were the only HPAIV isolates from wild birds and their environments in each prefecture (case numbers: #3, #9, #24, and #100, Supplementary Table 1). A/whooper swan/Iwate/0302I001T/2022 (H5N1), A/jungle crow/Iwate/0303I003/2022 (H5N1), and A/jungle crow/Iwate/0304I001/2022 (H5N1) were selected as the initial/midterm/last phase candidates among the series of HPAI outbreaks at the same place in Kuji City, Iwate Prefecture (case numbers: #31, #60, and #85, Supplementary Table 1). A/white-fronted goose/Iwate/TU16-74/2022 (H5N1) was isolated from a dead goose (case number #48, Supplementary Table 1) found near the HPAI-affected poultry farm (case number #16, Supplementary Table 2). A/whooper swan/Iwate/0303B006/2022 (H5N1) was the only isolate in Hachimantai City, Iwate Prefecture, apart from Kuji City (case number #69, Supplementary Table 1). A/jungle crow/Hokkaido/0104B085/2022 (H5N1) and A/jungle crow/Hokkaido/0104B087/2022 (H5N1) were selected as initial/last phase candidates in the series of HPAI outbreaks in Sapporo City, Hokkaido Prefecture (case numbers: #91 and #110, Supplementary Table 1), where HPAI cases in a fox (*Vulpes vulpes schrencki*) and racoon dog (*Nyctereutes procyonoides*) were also confirmed (no case number was officially provided for these mammalian infection cases, Supplementary Table 1). The complete genome sequences of these viruses and all 21 poultry-related HPAIVs were determined by next-generation sequencing and used for phylogenic analyses. The HPAIVs were classified into clade 2.3.4.4b and further divided into three groups based on the phylogenetic tree of H5 HA genes (Fig. 1A). These were designated as G2a, G2b, and G2d groups based on the classification by Baek *et al*. [3] and the consensus of Japanese researchers in all institutes conducting definitive HPAI diagnosis. The G2a group comprises Japanese H5N8 HPAIVs isolated from November to December 2021. The G2b group was comprised of East Asian H5N1 or H5N6 HPAIVs isolated from October 2021 to May 2022, including 13 H5N1 strains in the present study. Clusters of European H5N8 HPAIVs isolated from 2020 to 2021 were located next to the G2b group in the phylogenetic tree (Fig. 1A). The G2d group was comprised of Japanese and European H5N1 HPAIVs isolated after January 2022 and from 2021 to 2022, respectively. These groups were also found in other phylogenetic trees based on the PB2, PB1, PA, NP, N8 NA, N1 NA, M, and NS gene segments (Fig. 1B and Supplementary Fig. 1). Among the Japanese HPAIVs, four strains isolated from Ehime and Tottori Prefectures had unique PB1 and/or NP genes (Fig. 1B and Supplementary Fig. 1C). The genes of the Tottori strain, G2a group NK1201, were similar to those of A/duck/Bangladesh/19D1742/2021 (H6N1). Notably, consensus sequences of multiple subtypes of NA genes were detected by next-generation sequencing in four HPAIV isolates at the beginning of the winter (November 9–15th, 2021) (Table 1). The leads of N1 NA genes were detected as minor populations (i.e., low next generation sequencing coverage) in the H5N8 HPAIVs, and *vice versa*. The following minor consensus sequences were closest to those of the Japanese HPAIVs in the early winter of the 2021–2022: G2a group A/chicken/Kagoshima/B3T/2021 (H5N8) and NK1201, and G2b group A/chicken/Kagoshima/21A6T/2021 (H5N1). Furthermore, two PB2 consensus sequences were obtained from one of the HPAIVs with multiple NA genes, G2b group Miya32, and two deduced amino acid differences at positions 286 (serine/glycine) and 292 (threonine/isoleucine) were found between these sequences (data not shown).

Epidemiology

The locations and times of HPAI outbreaks in wild birds and poultry in Japan from 2021 to 2022 are shown in Fig. 2. G2a group H5N8 virus outbreaks have been observed in the Akita, Tottori, and Kagoshima Prefectures. G2b group H5N1 HPAIVs were mainly isolated in the central to western regions and also caused sporadic outbreaks in the northernmost region, Aomori and Hokkaido Prefectures. After January 2022, the northeastern region became the epicenter of outbreaks caused by G2d group HPAIVs. HPAIVs in the same groups were concurrently isolated from both wild birds and poultry in some prefectures: G2a HPAIVs in Kagoshima and G2d HPAIVs in Akita, Iwate, and Hokkaido Prefectures.

Antigenic relationships among Japanese H5 subtype influenza viruses

Based on the phylogenic analyses, we selected three isolates from the present study as reference strains: NK1201, Miya32, and G2d group A/jungle crow/Hokkaido/0104B085/2022 (H5N1). These viruses, together with the Japanese stockpile vaccine strains and HPAIVs shown in Supplementary Table 3, were used to prepare a panel of chicken polyclonal antisera, and their antigenic relationships were evaluated by cross-HI and neutralization tests (Tables 2, 3 and Supplementary Table 4). The cross-HI test revealed that clade 2.3.4.4b HPAIVs isolated in the winters of 2017–2018, 2020–2021, and 2021–2022 were antigenically different from previous

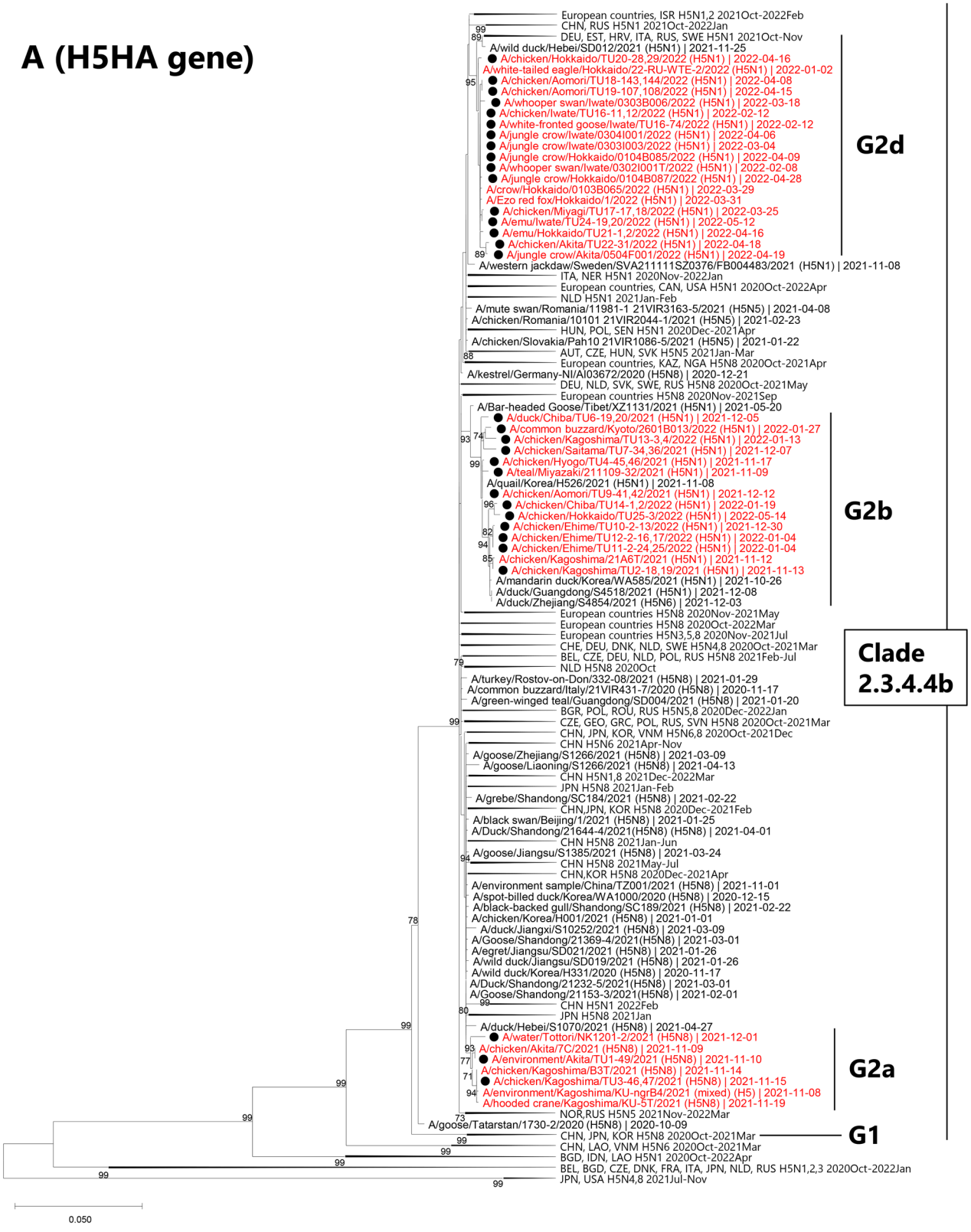

Fig. 1. Phylogenetic trees of H5 hemagglutinin (**A**) and polymerase basic protein 1 (**B**) gene segments of animal-derived influenza A viruses isolated from October 2020 to June 2022. Sequence data of high pathogenicity avian influenza virus (HPAIV) isolates in the present study and other viruses obtained from the Global Initiative on Sharing Avian Influenza Database were applied to construct the phylogenetic trees. Sequences were analyzed with a maximum-likelihood method using MEGA X software. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The numbers at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. Bootstrap values of ≥70% are shown at each branch. Japanese HPAIVs are shown in red, and the present isolates are with filled circles. Taxa are compressed as much as possible and described by their subtypes, regions/country codes (ISO 3166-1 alpha-3), and isolation month durations.

Fig. 1. Continued.

clades 2.3.2.1, 2.3.4.4c, and 2.3.4.4e HPAIVs (Table 2). The antigenicity of clade 2.3.4.4b HPAIVs was conserved; the differences in reactivity among these viruses were fewer than fourfold. However, in the cross-neutralization test, Miya32 showed eightfold reduced reactivity with antisera raised against some H5N8 HPAIVs, namely: G1 group A/mallard/Miyazaki/4501C605/2021 (H5N8) and NK1201 (Table 3). In the HI test, antisera against Japanese stockpile vaccine viruses showed more than eightfold reduced reactivity to almost all HPAIVs (Table 2). A specific high reactivity between one of the vaccine strains, A/duck/Hokkaido/Vac-3/2007 (H5N1), and Miya32 was detected in the HI test (Table 2), but not in the neutralization test (Supplementary Table 4).

Table 1. Profiles of multiple consensus sequences of neuraminidase gene segments detected via next generation sequencing

Sampling day	Strain name	NA subtype	NGS coverage*	Closest genetic relative** [HA group]	Identity*** (%)
November, 2021					
9th	A/teal/Miyazaki/211109-32/2021 (H5N1)	N1	7,428.396	A/chicken/Kagoshima/21A6T/2021 (H5N1) [G2b]	99.72
		N8	28.309	A/water/Tottori/NK1201-2/2021 (H5N8) [G2a]	100.00
10th	A/environment/Akita/TU1-49/2021 (H5N8)	N8	148,695.800	A/chicken/Kagoshima/B3T/2021 (H5N8) [G2a]	99.59
		N1	3.496	A/chicken/Kagoshima/21A6T/2021 (H5N1) [G2b]	100.00
13th	A/chicken/Kagoshima/TU2-18,19/2021 (H5N1)	N1	47,512.260	A/chicken/Kagoshima/21A6T/2021 (H5N1) [G2b]	100.00
		N8	39.990	A/chicken/Kagoshima/B3T/2021 (H5N8) [G2a]	99.58
15th	A/chicken/Kagoshima/TU3-46,47/2021 (H5N8)	N8	75,080.790	A/chicken/Kagoshima/B3T/2021 (H5N8) [G2a]	100.00
		N1	9.555	A/chicken/Kagoshima/21A6T/2021 (H5N1) [G2b]	99.77

* Calculated as follows: the total number of sequenced bases / reference genome size (bp). ** Viruses with highest nucleotide homology retrieved from GISAID platform (https://gisaid.org/on) on September 15, 2022. *** Gaps between the compared nucleotide sequences were excluded for calculation. NA, neuraminidase; NGS, next generation sequencing; HA, hemagglutinin.

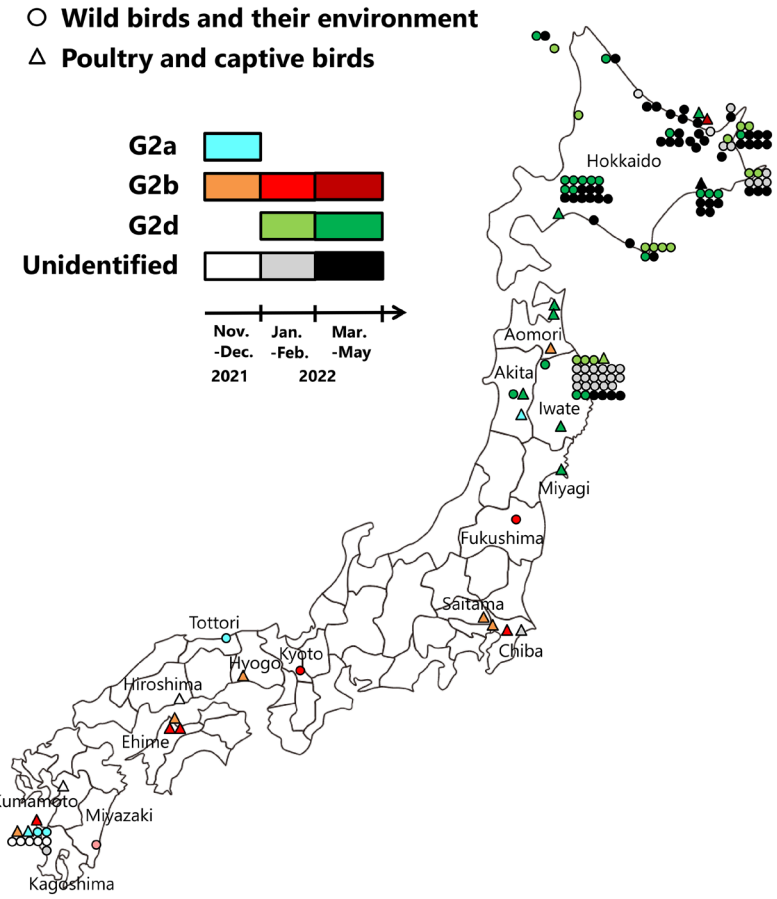

Fig. 2. Map of H5N8 and H5N1 virus infections in wild birds and their environments (circles) and poultry (triangles) in the winter of 2021–2022 in Japan. Each case is represented by a different color according to the times of outbreak and genetic hemagglutinin (HA) groups of the causal viruses. HA groups of 32 causal high pathogenicity avian influenza viruses (HPAIVs) (11 cases in wild birds/their environments and 21 cases in poultry farms) were identified by the present analyses. In addition, HA groups of 28 HPAIVs in wild animals and their environments were shown based on disclosed genetic data from the previous reports [13, 23].

Table 2. Cross-reactivity of Japanese vaccine strains and H5 high pathogenicity avian influenza virus isolates in hemagglutination inhibition test

Strain	HA clade	HA group*	Chicken anti-serum against											
			Vaccine strain		HPAIV									
					2010–2011	2014–2015	2016–2017	2017–2018	2020–2021			2021–2022		
			Vac-1	Vac-3					2.3.4.4b			2.3.4.4b		
					2.3.2.1	2.3.4.4c	2.3.4.4e	2.3.4.4b	G1 (T1-7)	G1 (NK1209)	G2a	G2a	G2b	G2d
Stockpile vaccine strains in Japan														
A/duck/Hokkaido/Vac-1/2004 (H5N1)			**2,048****	1,024***	64***	8***	64***	64***	64***	64	32***	8***	256***	4***
A/duck/Hokkaido/Vac-3/2007 (H5N1)			2,048	**8,192**	512	<2***	64***	64***	64***	64	64	32***	512	32***
2010–2011 HPAIV														
A/Mandarin duck/Miyazaki/22M807-1/2011 (H5N1)	2.3.2.1		64***	64***	**1,024**	16***	8***	8***	64***	32***	16***	32***	512	16***
2014–2015 HPAIV														
A/Mandarin duck/Gifu/2112D001/2014 (H5N8)	2.3.4.4c		16***	16***	128***	**512**	16***	8***	256	64	64	256	512	256
2016–2017 HPAIV														
A/teal/Tottori/1/2016 (H5N6)	2.3.4.4e		32***	64***	64***	16***	**512**	16***	128***	32***	128	128***	512	128***
2017–2018 HPAIV														
A/mute swan/Shimane/3211A001/2017 (H5N6)	2.3.4.4b		32***	128***	64***	128	256	**1,024**	2,048	256	512	2,048	4,096	4,096
2020–2021 HPAIV														
A/environment/Kagawa/T1-7/2020 (H5N8)	2.3.4.4b	G1	32***	32***	32***	128	128	512	**1,024**	256	256	1,024	1.024	512
A/water/Tottori/NK1209/2020 (H5N8)	2.3.4.4b	G1	16***	32***	32***	128	128	512	1,024	**256**	256	512	512	512
A/mallard/Miyazaki/4501C605/2021 (H5N8)	2.3.4.4b	G2a	16***	64***	32***	64***	128	512	512	128	**256**	1,024	1,024	1,024
2021–2022 HPAIV														
A/water/Tottori/NK1201-2/2021 (H5N8)	2.3.4.4b	G2a	256***	64***	16***	64***	256	512	2,048	128	256	**1,024**	1,024	1,024
A/teal/Miyazaki/211109-32/2021 (H5N1)	2.3.4.4b	G2b	128***	2,048	128***	16***	256	256	256	128	256	256	**2,048**	512
A/jungle crow/Hokkaido/0104B085/2022 (H5N1)	2.3.4.4b	G2d	64***	128***	16***	128	256	1,024	1,024	128	512	512	1,024	**1,024**

* HA groups of 2020–2022 HPAIVs are based on the classification by Baek *et al*. [2] and the consensus of Japanese researchers in all institutes conducting definitive HPAI diagnosis. ** Homologous titer. *** The titer eight times and less lower than homologous titer. HPAIV, high pathogenicity avian influenza virus; HA, hemagglutinin; HPAI, high pathogenicity avian influenza.

Table 3. Cross-reactivity among the most recent clade 2.3.4.4b H5 high pathogenicity avian influenza virus isolates in neutralization test

Strain	HA group*	Chicken anti-serum against					
		2020–2021			2021–2022		
		G1 (T1-7)	G1 (NK1209)	G2a	G2a	G2b	G2d
2020–2021 HPAIV							
A/environment/Kagawa/T1-7/2020 (H5N8)	G1	**1,280****	320	320	320	1,280	160
A/water/Tottori/NK1209/2020 (H5N8)	G1	1,280	**320**	320	640	640	320
A/mallard/Miyazaki/4501C605/2021 (H5N8)	G2a	1,280	80	**320**	320	2,560	1,280
2021–2022 HPAIV							
A/water/Tottori/NK1201-2/2021 (H5N8)	G2a	640	80	320	**320**	2,560	320
A/teal/Miyazaki/211109-32/2021 (H5N1)	G2b	320	80	40***	40***	**2,560**	160
A/jungle crow/Hokkaido/0104B085/2022 (H5N1)	G2d	640	160	640	1,280	2,560	**640**

* HA groups are based on the classification by Baek *et al*. [2] and the consensus of Japanese researchers in all institutes conducting definitive HPAI diagnosis. ** Homologous titer. *** The titer eight times and less lower than homologous titer. HA, hemagglutinin; HPAIV, high pathogenicity avian influenza virus; HPAI, high pathogenicity avian influenza.

DISCUSSION

During the winter of 2021–2022, Japan experienced HPAI outbreaks caused by multiple subtypes of H5 HPAIVs. While the HPAIV isolates in the present study reacted to specific reference antisera in NI testing and their NA subtypes were clearly determined, four isolates in the early winter had both N1 and N8 NA genes: three isolates from the affected poultry farms in Akita and Kagoshima Prefectures and one isolate, Miya32, from the feces of a migratory teal in Miyazaki Prefecture. H5N8 and H5N1 HPAIVs were also reportedly found in a very limited area of Kagoshima Prefecture including poultry farms and wintering sites of migratory birds in November 2021 [23]. These prefectures have major wintering sites for waterbirds [28, 37], suggesting that G2b H5N1 and G2a H5N8 HPAIVs were brought simultaneously via their migrations in the early winter, contaminating the environments. We previously found a feral mallard infected with multiple H5N8 HPAIVs in Miyazaki Prefecture in the winter of 2020–2021 [32]. From the mallard, HPAIVs with several gene constellations were cloned by plaque assay. The results support the notion that wintering birds can be infected with multiple HPAIVs, and the mixture of HPAIVs or their genetic reassorted viruses can cause outbreak in nearby poultry farms. Miya32 also had multiple PB2 motifs at positions 286 (serine/glycine) and 292 (threonine/isoleucine). The S286G and V292I mutations have already been observed in some H5N1 and H7N9 influenza viruses in China [20, 39]. These mutations were likely maintained as quasispecies in some G2b H5N1 HPAIVs that perpetuated in wild bird populations in China, and subsequently disseminated to Japan. Previous studies have shown that amino acids at these positions contribute to the adaptation of the influenza A virus to the mammalian host [24, 39]. The S286G mutation was positively selected in a viral passaging study in mice [24]. Human-origin H7N9 viruses with a V292I mutation in PB2 have become more prevalent. This mutation enhances the effects of the E627K mutation in PB2, which is associated with high viral replication in human embryonic kidney 293T cells [39]. Whether diversity in these amino acids is maintained in avian hosts should be further investigated to estimate the public health risk.

One of the HPAIVs in the present study, NK1201, was isolated from the environmental water of a wintering site in Tottori Prefecture [6] and had similar PB1 and NP gene segments with an H6 subtype low pathogenicity avian influenza virus (LPAIV) strain. To discuss when and where such genetic reassortment events between HPAIVs and LPAIVs occurred, the profiles of LPAIV isolates in the same season should be further elucidated.

Between 2020 and 2022, subtypes of circulating HPAIVs dynamically shifted from H5N8 to H5N1 in European and Asian countries [1, 8]. Similarly, during the winter of 2021–2022 in Japan, G2a group H5N8 HPAIVs immediately disappeared and were replaced with G2b and G2d group H5N1 HPAIVs. One hypothesis that may explain these shifts is that antibody pressure in wild bird populations previously infected with H5N8 HPAIVs contributed to the selection of H5N1 HPAIVs as novel circulating strains. This hypothesis was supported by the results of the cross-neutralization test: G2b group Miya32 was antigenically distinct from the G2a group H5N8 HPAIV strains. In the winter of 2020–2021, H5N8 HPAIVs caused outbreaks in East Asian countries [3, 21, 38], suggesting that a considerable number of migratory bird populations contracted to HPAIVs and contributed to the selection of the most recent H5N1 HPAIVs as circulating strains. The history of previous H5N8 HPAIV infections might reflect the overall HPAI circumstances in Japan in the winter of 2021–2022; HPAIV infection cases were predominantly observed in sedentary raptors and crows compared with migratory waterbirds (see Supplementary Table 1). This notion is also supported by a previous report showing that captive mallards and swans retained detectable serum HI antibodies against HPAIV approximately half a year after infection [33]. NA proteins of influenza A viruses are immunogenic, although viral antigenicity is mostly dependent on their HA proteins [7]. The immunological memory of the N8 NA protein acquired by H5N8 HPAIV infection in the last winter might be involved in the selection of H5N1 HPAIVs as major circulating strains. Another hypothesis is that the novel clade 2.3.4.4 H5N1 HPAIVs have adapted to wild birds, including sedentary raptors and crows; therefore, these HPAIVs can be more easily replicated and transmitted in the above-mentioned birds when compared to the previous H5N8 HPAIVs. The pathogenicity of clade 2.3.4.4 HPAIVs in European wigeons reportedly differed according to their genetic groups [4].

A previous report suggested that recent H5N1 HPAIVs could be classified into 16 genotypes, G1–G16 [8]. The G2b and G2d

groups in the present study, defined by consensus of Japanese researchers dealing with HPAI diagnoses, corresponded to the G7 and G1 genotypes, respectively. G2b H5N1 HPAIVs have been endemically observed in East Asia since October 2021 [8], suggesting that these viruses emerged in northern nesting areas or southern wintering sites in Eastern Eurasia, and multiple migrating bird flocks on the East Asian flyway [19] subsequently disseminated the viruses to wide areas in Japan during the winter of 2021–2022. Meanwhile, G2d H5N1 HPAIVs caused outbreaks in Europe and Africa in the winter of 2020–2021, and then disseminated to East Asian and North American regions in the following winter. These HPAIVs were likely brought by European migratory waterbirds to northern nesting areas in the summer of 2021, before the viruses were shared with bird populations using other migratory flyways, leading to intercontinental viral disseminations. G2d HPAIVs were mainly isolated from migratory whooper swans (*Cygnus cygnus*) and sedentary jungle crows (*Corvus macrorhynchos*) in Northeast Japan in the winter of 2021–2022. Whooper swans are well known to use Northeast Japan as a stopover and establish wintering sites there [28], suggesting that G2d HPAIVs were brought by their specific flocks and subsequently disseminated into sedentary birds such as crows. Disseminations of specific groups of HPAIVs in Northeast Japan were also observed in the previous outbreaks in the winters of 2010–2011 [29] and 2016–2017 [30]. Migration routes of specific bird species have likely been conserved at least for a decade, while being involved in some dissemination patterns of HPAIVs.

In Japan, HPAI outbreaks in poultry are controlled using a stamping-out strategy [10]. Inactivated vaccines based on H5N1 LPAIVs, A/duck/Hokkaido/Vac-1/2004, and A/duck/Hokkaido/Vac-3/2007 [Vac-3] [31], have been stockpiled only for emergent use to contain causal viruses in limited regions. The potency of the vaccines against clades 1, 2.2, 2.3.2.1, and 2.5 HPAIVs has been confirmed by experimental infection studies [14, 27]; however, differences in antigenicity between the vaccine viruses and recent clade 2.3.4.4 HPAIVs have been of concern [11]. Here, antibodies raised against the vaccine viruses weakly reacted to clade 2.3.4.4b HPAIVs in HI and neutralization tests, and these titers were comparable to those in the previous clade 2.3.2.1 HPAIV, A/Mandarin duck/Miyazaki/22M807-1/2011 (H5N1). Together with previous findings, the current vaccines may confer protective immunity against not only clade 2.3.2.1 HPAIVs but also the recent clade 2.3.4.4b HPAIVs. Notably, antiserum against Vac-3 showed a high reactivity to Miya32 in the HI test. This cross-reactivity was not observed in the neutralization test. Antibodies against N1 NA likely interfered with hemagglutination through the N1 subtype virus. This cross-reactivity could not be explained by the nucleotide and amino acid homologies between NAs of Vac-3 and Miya32 (94% and 90%, respectively, not particularly higher than the other antigen-antibody combinations). Specific amino acid similarities at the known N1 NA epitopes involved in viral antigenicity were not found between them [34]. The antigenic relationships among the N1 NA of both HPAIVs and LPAIVs should be further characterized.

In the winter of 2021–2022, concurrent HPAI outbreaks in both poultry and wild birds occurred in some prefectures in Japan. For example, an G2d group virus caused an outbreak at a chicken farm in Iwate Prefecture on February 12th, and a dead goose found in the vicinity of the farm on the same day also harbored the same virus, A/white-fronted goose/Iwate/TU16-74/2022 (H5N1). In the winter of 2020–2021, the invasion of HPAIV in Miyazaki Prefecture was first recognized following an outbreak in a poultry farm (December 1st), and nine consecutive farm cases were reported within the month [21]. However, there were no HPAI cases in Miyazaki Prefecture after the isolation of Miya32 in 2021–2022. These contrasting consequences suggest that poultry farmers, veterinarians, and regional offices of the livestock industry should be on strict alert against HPAI to reduce possible economic losses, even when HPAIVs are only detected during an active virus surveillance in the field; this is because the impact of HPAI outbreaks on neighboring farms is significantly greater.

POTENTIAL CONFLICTS OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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