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H5N8 avian influenza virus acquires enhanced pathogenicity after a single passage in chicken

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ABSTRACT

The H5N8 highly pathogenic avian influenza viruses (HPAIVs) isolated in Japan during the 2014–2015 winter differed in their pathogenicity in chickens. In the present study, we examined the possibility that a comparatively less pathogenic strain was first brought into the country by migratory birds, and then acquired enhanced pathogenicity by infecting chicken flocks. We showed that the A/tundra swan/Tottori/C6nk/2014 (H5N8) (Tottori P0) strain required 10 days to kill all chickens via the intranasal route. However, Tottori P1-B, a strain recovered from the brain of a chicken infected with parental Tottori P0, showed enhanced pathogenicity; Tottori P1-B replicated significantly in the lung and liver, and killed all infected birds within 6 days, which was comparable to a chicken farm isolate obtained in the same season, A/environment/Miyazaki/11/2014 (H5N8). Tottori P1-B showed more marked proliferation in MDCK and chicken fibroblast cells, especially during the early phase of infection. Sequence analysis revealed a single mutation, M374 V, in nucleoprotein (NP) of the passaged virus, and this substitution was conserved after a further inoculation study. Position 374 in NP is located in the functional domain interacting with polymerase protein, PB2, indicating that viral polymerase activity was involved in the rapid growth of Tottori P1-B *in vitro* and *in vivo*. These results suggest that HPAIV, which originally had comparatively low pathogenicity to chickens, can increase its pathogenicity through the infection from migratory birds to domestic chickens.

1. Introduction

The influenza virus is a negative-sense, single-stranded, segmented RNA virus belonging to the *Orthomyxoviridae* family, and its genome is divided into 7–8 segments. Furthermore, it is categorized into 4 types—A, B, C, and D by their antigenicity (Alexander, 1982; Asha and Kumar, 2019; Hause et al., 2014). Influenza A virus is classified by the antigenicity and/or genome sequences of its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), into HA subtypes H1—H18 and NA subtypes N1—N11 (Fouchier et al., 2005; Webster et al., 1992; Wu et al., 2014b). To date, H1—H16 and N1—N9 subtypes of the virus have been isolated from ducks, swans, and other wild waterfowl, which are thought to be the natural hosts of the virus (Alexander, 1982). However, the influenza A virus that is maintained in waterfowl populations does not exhibit pathogenicity to chickens (OIE, 2018). Yet, these viruses can pass through quail, turkey, and other terrestrial poultry to infect chicken populations. Among them, the H5 and H7 subtype viruses have repeatedly infected chickens and acquired pathogenicity (Alexander, 1982; Webster et al., 1992). Viruses that meet certain criteria set forth by the World Organization for Animal Health (OIE) and exhibit high pathogenicity to chickens are termed highly pathogenic avian influenza viruses (HPAIVs) (OIE, 2018).

Following the discovery and report in 1996 of highly pathogenic avian influenza (HPAI) caused by the H5N1 HPAIV found in a goose farm in Guangdong Province, China, the progeny viruses of that strain have spread through Asia, the Middle East, Europe, and Africa—60 countries in all—while undergoing antigenic change and genetic reassortment. These viruses have caused vast damage to the poultry industry (Xu et al., 1999). Phylogenetically, based on the HA genes, these progeny viruses has been organized into 10 clades, numbered clades 0–9 (WHO/OIE/FAO-Evolution-Working-Group, 2008).

At the end of 2013, clade 2.3.4.4 H5N8 HPAIV was first isolated from a duck at a live poultry market in Zhejiang province, China (Wu et al., 2014a). Thereafter, the same H5N8 HPAIV was isolated from wildfowl in Korea, Japan, Russia, and other Eurasian as well as North American countries (Ku et al., 2014; Lee et al., 2015; Marchenko et al., 2015; Usui et al., 2017; Verhagen et al., 2015). Additionally, this virus also caused HPAI in poultry populations. In Japan, an outbreak was

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mainly reported in the 2014–2015 winter; in that season, clade 2.3.4.4 H5N8 HPAIV was isolated from bird remains and environmental water sources in Shimane, Chiba, Tottori, and Gifu prefectures (Ozawa et al., 2015; Tanikawa et al., 2016; Usui et al., 2017). In addition, HPAI outbreaks caused by this virus occurred in poultry farms in Miyazaki, Yamaguchi, Okayama, and Saga prefectures, resulting in disposal of 3.6 million chickens (Saito et al., 2015; Tanikawa et al., 2016; Usui et al., 2017).

Several reports on the pathogenicity of the H5N8 HPAIV strain—isolated in Japan—in chickens have been made. Intravenous inoculation of 4- to 5-week-old chickens with $10^{6.0}$ 50% egg infectious dose (EID_{50}) of the A/chicken/Miyazaki/7/2014 (H5N8) strain isolated from the farm in Miyazaki prefecture where an HPAI outbreak occurred, caused 80% of the infected chickens to die within 3–5 days of inoculation, and all birds died within 10 days (Tanikawa et al., 2016). Additionally, all chickens given intravenous inoculations of A/mandarin duck/Gifu/2112D001/2014 (H5N8), isolated from wildfowl (mandarin ducks) in Gifu prefecture, died within 3 days (Usui et al., 2017). Conversely, A/tundra swan/Tottori/C6nk/2014 (H5N8) (Tottori strain), isolated from tundra swan feces in Tottori prefecture, exhibited comparatively lower pathogenicity in chickens than did other strains isolated in the same season. Furthermore, when given as intravenous inoculations at the same titer, chickens died after 5–10 days.

Because H5N8 HPAIV strains isolated in Japan within the same season exhibited differences in pathogenicity, and because a comparatively less-pathogenic strain among them was isolated from the feces of a migrant bird (the tundra swan), it is quite possible that this variant of HPAIV was first brought into the country by migratory birds. It then likely invaded poultry populations, repeatedly infected them, and increased in pathogenicity. At present, in 2019, clade 2.3.4.4 H5N8 HPAIV is still an epidemic in worldwide (OIE, 2019), and there are concerns about its re-entry into Japan. In order to gain understanding of the increased pathogenicity of the virus in domestic birds, we passaged the aforementioned Tottori strain in chickens and examined the changes in its pathogenicity, with a view to preventing future epidemics. In addition, we sought to elucidate the molecular mechanism through which H5N8 HPAIV acquired pathogenicity in chickens.

2. Materials and methods

2.1. Virus specimens

Two HPAIVs were used in this study: A/tundra swan/Tottori/C6nk/2014 (H5N8) (Tottori strain) and A/environment/Miyazaki/11/2014 (H5N8) (Miyazaki strain). The former strain was isolated on November 18, 2014, from tundra swan (*Cygnus columbianus bewickii*) feces collected at Nikko Pond, Tottori, Tottori prefecture, as part of the independent virus surveillance efforts undertaken by Tottori University (Usui et al., 2017). The latter strain was isolated from an environmental sample obtained on December 16, 2014, from the chicken farm at Nobeoka, Miyazaki prefecture (location of the HPAI outbreak). Viruses were inoculated into 10-day-old embryonated eggs (Aoki breeder farm, Tochigi, Japan). After incubation, allantoic fluid from these eggs was collected and stored at −80 °C as the seed virus stock and was used in the subsequent experiments. For the Tottori strain, the prepared virus stock was referred to as the Tottori P0 strain.

2.2. Measurement of 50% egg infectious dose

Samples were diluted 10-fold in phosphate-buffered saline (PBS, Takara, Shiga, Japan) supplemented with 1000 U of penicillin G potassium (Meiji Seika, Tokyo, Japan) and streptomycin sulfate (Meiji Seika) (PS-PBS), and were inoculated at a volume of 100 µL into 10-day-old embryonated eggs, with 3 eggs per sample. Eggs were incubated at 35 °C for 48 h. A hemagglutination test (Sever, 1962) was performed using allantoic fluid, and the EID_{50} was calculated using the Reed and Muench method (Reed and Muench, 1938).

2.3. Sequencing

Virus RNA was extracted from 140 µL of allantoic fluids of inoculated eggs by using a QIAamp® Viral Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and was reverse transcribed with the Uni12 primer (Hoffmann et al., 2001) and PrimeScript RTase (Takara). Full-length cDNAs of the 8 viral gene segments were amplified by polymerase chain reaction with the KOD Dash DNA polymerase (Toyobo, Osaka, Japan) and gene-specific primer sets (Hoffmann et al., 2001). Each gene segment was directly sequenced with 3130 Genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.4. Analysis of the 3D structure model of nucleoprotein (NP)

Using Discovery Studio Visualizer (VIOBIA, France), the 374th amino acid in the NP 3D structure model was analyzed. The 3D structure model used was PBD ID:3ZDP, A/WSN/1933 (H1N1) NP (Chenavas et al., 2013).

2.5. Animal infection experiments

2.5.1. Test animals

Fertilized eggs of chickens (white leghorns, Aoki breeder farm) and ducks (Cherry Valley var., Takahashi artificial hatchery, Osaka, Japan) were purchased, hatched, and reared in the Tottori University Animal Experimentation Building until the age of 4 weeks for chickens and 1 week for ducks. The day before viral inoculation, blood samples were collected from each chicken. We selected 1 duck from the flock, placed it under isoflurane (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) anesthesia, and obtained a blood sample via cardiac puncture, because blood collection via vessels from 1-week-old ducklings is technically difficult. The blood sample was incubated at 37 °C for 2 h, allowed to stand at 4 °C for 12 h and then centrifuged to collect the supernatant. Using inactivated antigens of the Tottori and Miyazaki strains, the hemagglutinin inhibition test (Sever, 1962) was carried out to confirm that serum antibodies to each challenge virus strain were within the limits of detectability. In the present study, if the selected duck was seronegative, all the ducks in the same flock were also considered as naive. To prevent bias, animals were separated into flocks according to their weights and subjected to the following virus inoculation experiments. All animal infection experiments were carried out in self-contained isolator units (CLEA, Tokyo, Japan) at a biosafety level 3 facility at the Avian Zoonosis Research Center, Tottori University, Japan. The experiments were performed according to the guidelines of the institutional animal care and use committee of Tottori University (approved number: 15-T-34). Throughout the present study, any birds unable to feed or drink were euthanized, and recorded as dead at the following day's observation time.

2.5.2. Chicken infection experiments

To assess the intravenous pathogenicity of each H5N8 virus, the modified intravenous pathogenicity index (IVPI) test, which can reduce the number of birds used for the experiment, was applied. We inoculated 3–4 chickens, not 10 as per the OIE manual, intravenously with $10^{6.0}$ EID_{50}/0.2 mL of each virus. Each bird was observed for disease manifestation at intervals of 24 h over a 10-day period and scored 0 if normal, 1 if sick, 2 if severely sick, and 3 if dead. The IVPI was presumed as the mean score per bird per observation over the 10-day period.

To test the pathogenicity of the viruses via the natural route of infection, 3–7 chickens were intranasally inoculated with $10^{6.0}$ EID_{50}/0.1 mL of each virus. Their clinical symptoms were monitored for a maximum 14 days. Animals that died or were euthanized according to the above-mentioned endpoint during the experimental period were

dissected, and their brain, trachea, lungs, liver, kidneys, and colon were aseptically sampled. After adding PS-PBS at 10:1 wt ratio, the organ was homogenized using either a pestle and mortar or a Multi-Bead Shocker (Micro Smash™ MS-100R, Tomy Seiko, Tokyo, Japan, 3000 rpm, 30 s), to create a 10% organ emulsion. The viral titer of each sample was measured using embryonated eggs.

2.5.3. Domestic duck infection experiments

Four domestic ducks were housed per isolator. Each virus sample was intranasally inoculated into ducks at a titer of $10^{6.0}$ EID_{50}/0.1 mL, and their clinical symptoms were monitored for 14 days. At 0, 1, 2, 4, 6, 8, 10, and 14 days post-inoculation (DPI), the oral cavity and cloaca of each duck were swabbed, and the viral titer of each swab was measured. Any ducks that died during the duration of the experiment were subjected to the same procedure discussed in the previous section to measure the organ viral titers.

2.6. Virus growth kinetics

2.6.1. Virus inoculation into cells

We used Madin—Darby canine kidney (MDCK) cells, chicken embryo fibroblast (CEF) cells, and domestic duck embryo fibroblast (DEF) cells. For culture media, Eagle's minimum essential medium (E-MEM, FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (FBS, GE Healthcare UK Ltd., Amersham, UK), penicillin, and streptomycin sulfate were used. Cell culture was carried out at 37 °C and 5% CO_2. Cells were grown on 24-well plates until they formed a full monolayer. Subsequently, they were inoculated with virus samples to a multiplicity of infection of 0.01. After allowing viral adherence to the cells for 1 h, the culture was washed with PBS, supplemented with antibiotic-added E-MEM, and cultured at 37 °C and 5% CO_2. At 0, 8, 24, 48, and 72-h post-inoculation (HPI), cell supernatant was collected from every third well, centrifuged at 1,400 × g for 5 min, and stored as a sample.

2.6.2. Measurement of viral titer

MDCK cells were cultured in a 24-well plate until forming a confluent monolayer and were then washed once with PBS, and samples were inoculated at 100 µL/well. Following incubation for 1 h at 37 °C, supernatant was removed, and wells were washed once with PBS. Further, 500 µL of a culture solution prepared by mixing equal amounts of 2× antibiotic-added E-MEM and 1.4% Bacto Agar (FUJIFILM Wako Pure Chemical Corporation) was added to all wells. After coagulation, plates were turned upside down, and were placed in a CO_2 incubator set at 37 °C for 48 h. Crystal violet in formalin (FUJIFILM Wako Pure Chemical Corporation) was used to fix and stain cells. One day later, plaque numbers were counted, and the plaque-forming unit (PFU) was calculated.

Because the Tottori strain did not form clear plaques in MDCK cells, we used the following method to measure viral titer. As described in Section 2.6.1, samples were prepared and inoculated into MDCK cells. Briefly, 500 µL of 1× antibiotic-added E-MEM was added to each well, and plates were incubated in a CO_2 incubator set to 37 °C for 72 h. The presence or absence of a cytopathic effect was judged using a microscope, and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed and Muench method (Reed and Muench, 1938). According to the Poisson distribution, a virus with a $TCID_{50}$ of 1 was equivalent to a virus with 0.69 PFU. Based on this, the theoretical values of the PFU for Tottori and its related strains were calculated by multiplying the $TCID_{50}$ by 0.69.

Table 1
Disease scores of the chickens intravenously inoculated with each H5N8 virus strain.

DPI		Tottori P0			Tottori P1-B				Miyazaki		
	ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
0		0	0	0	0	0	0	0	0	0	0
1		0	0	0	3	3	3	3	0	0	0
2		2	1	0	3	3	3	3	2	2	2
3		2	2	2	3	3	3	3	3	3	3
4		2	2	2	3	3	3	3	3	3	3
5		2	2	2	3	3	3	3	3	3	3
6		3	3	3	3	3	3	3	3	3	3
7		3	3	3	3	3	3	3	3	3	3
8		3	3	3	3	3	3	3	3	3	3
9		3	3	3	3	3	3	3	3	3	3
10		3	3	3	3	3	3	3	3	3	3
Total		23	22	21	30	30	30	30	26	26	26
Presumed IVPI		2.20			3.00				2.60		

Each bird (#1—10) was observed for disease manifestation at intervals of 24 h over a 10-day period and scored 0 if normal, 1 if sick, 2 if severely sick, and 3 if dead. DPI, days post-infection; IVPI, intravenous pathogenicity index.

3. Results

3.1. The Tottori P0 strain shows lower pathogenicity in chickens than the Miyazaki Strain

To confirm the pathogenicity of the Tottori P0 and Miyazaki strains in chickens, we inoculated each virus intravenously or intranasally into chickens and tracked their survival rates and clinical symptoms. Additionally, for the intranasally inoculated group, we collected their organs at 3 DPI and/or at their death and measured the intraorgan viral titer. After intravenously inoculating three 4-week-old chickens with the Tottori P0 strain at $10^{6.0}$ EID_{50}/0.1 mL, all birds (#1—3) were observed to have clinical symptoms of either sluggishness or ruffled feathers at 2–3 DPI (Table 1). At 5 DPI, all chickens were extremely debilitated, judged to be beyond the point of recovery, and therefore euthanized. After scoring their symptoms in accordance with the outline set for intravenous inoculation testing by the OIE (2018), the IVPI for this strain was presumed as 2.20. Upon inoculating the Miyazaki strain under the same conditions, similar clinical symptoms were observed at 2 DPI, and by the next day, all birds (#8—10) had died (IVPI = 2.60).

After nasal inoculation of four 4-week-old chickens (#14—17) with Tottori P0 strain at a titer of $10^{6.0}$ EID_{50}/0.1 mL, dead birds were observed at 5 DPI (Fig. 1). By 10 DPI, all birds had died. Chickens inoculated with the Miyazaki strain under the same conditions (#26 and #28—30) had all died by 5 DPI. No significant differences were observed in the viral titer of organ emulsions prepared from birds inoculated intranasally with each strain who died or were euthanized (#11—13 and #26—28) by 3 DPI (Table 2). However, when comparing birds that had died (#14—17, #26, and #28—30), the viral titers in the livers ($p < 0.05$) and colons ($p < 0.01$) of the Miyazaki strain were significantly higher than that of the Tottori P0 strain.

3.2. The Tottori P0 strain acquires higher pathogenicity by a single passage in chickens

To examine whether a passage in chickens would increase the pathogenicity of the Tottori P0 strain, we inoculated new chickens with organ emulsions obtained from chickens that had been inoculated with the P0 strain. For inoculant material, we used the brain (Tottori P1-B strain) and trachea (Tottori P1-T strain) of bird #17, which were found to have high intraorgan viral titers (Table 2). Chickens (#4—7) inoculated intravenously with the Tottori P1-B strain at $10^{6.0}$ EID_{50}/0.1 mL titer had all died by 1 DPI (IVPI = 3.00, Table 1). Chickens

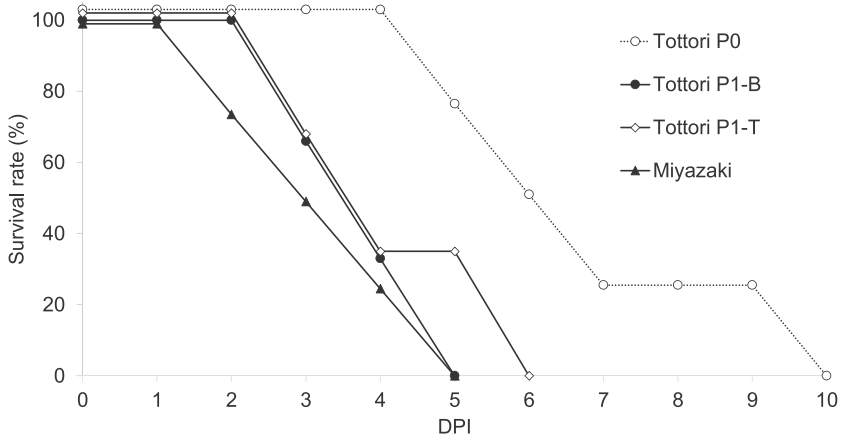

Fig. 1. Survival rate in chickens after intranasal challenge with each H5N8 strain. Three to four 4-week-old chickens were intranasally inoculated with $10^{5.5-6.0}$ EID_{50} of the Tottori P0 (circles, dashed line), P1-B (filled circles), P1-T (diamonds), or Miyazaki (filled triangles) strains and were then observed for clinical signs.

Table 2
Viral titers in tissues of the chikens intranasally inoculated with each H5N8 virus strain.

Strains	ID	DPI	Viral titers (log EID_{50}/ g)					
			Brain	Trachea	Lung	Liver	Kidney	Colon
Tottori P0	#11	3[1]	4.5	3.3	5.5	3.5	4.3	3.8
	#12	3[1]	3.3	3.5	5.3	3.0	4.8	3.8
	#13	3[1]	4.3	4.3	6.5	3.8	6.3	5.6
	#14	5	8.8	6.8	5.8	3.5	5.5	4.8
	#15	6	6.5	6.0	6.8	4.8	8.0	5.8
	#16	7	7.5	6.8	4.5	≤2.5	4.8	4.5
	#17	10	8.8[a]	7.5[a]	6.5	3.4	7.7	6.3
Tottori P1-B	#18	3[1]	4.8	3.8	6.3	2.8	4.8	4.7
	#19	3[1]	5.5	5.0	6.3	4.5	5.5	4.8
	#20	3	6.8	6.8	7.5**	6.5*	8.3	6.8
	#21	4	6.8	7.3	7.5**	7.4*	6.8	6.3
	#22	5	8.5[b]	8.8	7.8**	7.3*	6.8	7.3
Tottori P1-T	#23	3	6.3	5.5	6.8	4.8	6.8	6.8*
	#24	4	7.5	7.8[b]	7.5	4.8	6.0	6.7*
	#25	6	9.0[b]	7.3	6.0	≤2.5	6.5	5.8*
Miyazaki	#26	2	7.8	6.3	7.5	6.3*	7.5	7.5**
	#27	3[1]	6.2	4.5	6.5	5.0	6.3	5.8
	#28	3	7.5	8.5	8.8	7.5*	7.5	6.8**
	#29	4	7.3	7.3	7.8	7.5*	7.8	7.5**
	#30	5	8.8	7.5	8.3	6.8*	7.8	7.3**

*, ** Showed significantly higher titer than that in the birds that died after inoculation with the Tottori P0 strain (#14—17) (*$p < 0.05$; **$p < 0.01$).
[1] Euthanized to evaluate the viral titers in the acute phase of infection.
[a] Recovered viruses were applied for the next passage experiments.
[b] ecovered viruses were sequenced.

(#20—22) inoculated intranasally with this strain at the same titer had all died by 3–5 DPI (Fig. 1 and Table 2). Chickens (#23—25) inoculated with the Tottori P1-T strain at $10^{5.5}$ EID_{50}/0.1 mL (stock concentration) all died at 3–6 DPI.

In chickens inoculated with the Tottori P1-B strain that died or were euthanized by 3 DPI (#18—20), the organ emulsion viral titer was not significantly different from that in the group inoculated with the Tottori P0 strain (#11—13) (Table 2). However, when comparing the chickens that had died (#14—17 and #20—22), the P1-B inoculated group had a significantly higher viral titer in their lungs ($p < 0.01$) and livers ($p < 0.05$). Additionally, chickens inoculated with the Tottori P1-T strain that later died (#23—25) had a significantly higher viral titer in their colons than did chickens inoculated with the Tottori P0 strain ($p < 0.05$).

3.3. The passaged virus has a substitution at the 374th position of the NP

We compared the genes and amino acid sequences of H5N8 HPAIVs with differing levels of pathogenicity in chickens. Forty-two amino acid differences were found in major proteins (excepting M1 and M2) of the Tottori P0 and Miyazaki strains (Table 3). In the passaged strains, Tottori P1-B and P1-T, a substitution of valine for methionine at the 374th position of the NP was observed (Table 4). We inoculated these strains intranasally into chickens again, then excised their organs (brains of #22 and #25, trachea of #24), and recovered the viruses (Tottori P2B-B, P2T-B, and P2T-T strains) (Table 2). We found that this amino acid mutation had been preserved in these strains (Table 4). On the other hand, the 374th amino acid of the NP of the Miyazaki strain was methionine, similar to the Tottori P0 strain (Table 3). In the Tottori P2T-B and P2T-T strains, a synonymous mutation at the 663rd position

Table 3
Deduced amino acid differences between Tottori P0 and Miyazaki strains.

Amino acid in Tottori P0 (Miyazaki)											
PB2	PB1	PB1-F2	PA	PA-X	HA	NP	NA	M1	M2	NS1	NS2
I 57 (L) K 249 (E) I 366 (V)	V 191 (I) I 219 (V) R 571 (K) S 627 (P)	V 50 (D) S 63 (F) K 73 (R) R 85 (K) I 89 (T)	L 226 (I) S 287 (A) V 379 (M) S 560 (P)	A 192 (V) Q 215 (R) A 218 (V) D 240 (A) Q 250 (P)	I 11 (V) N 112 (K) R 135 (K) F 160 (Y) K 177 (E) S 239 (R) V 265 (A) M 285 (V) G 339 (R) I 512 (G)	I 217 (L) V 286 (A) R 490 (G) S 492 (I)	S 39 (G) G 126 (E) V 201 (I) A 230 (T) K 265 (T)	–	–	V 180 (I)	T 89 (I)

Methionine encoded by the AUG start codon is defined as position 1.

Table 4
Nucleotide and amino acid mutations in passaged Tottori P0-based strains.

Strains	Gene segments							
	PB2	PB1	PA	HA	NP	NA	M	NS
Tottori P1-B	–	–	–	–	A1150G[a] (M374 V)[b]	–	–	–
Tottori P1-T	–	–	–	–	A1150G (M374 V)	–	–	–
Tottori P2B-B	–	–	–	–	A1150G (M374 V)	–	–	–
Tottori P2T-B	–	–	G663A (Silent)	–	A1150G (M374 V)	–	–	–
Tottori P2T-T	–	–	G663A (Silent)	–	A1150G (M374 V)	–	–	–

Methionine encoded by the AUG start codon is defined as position 1. [a,b] Nucleotide and amino acid mutations, respectively.

of the PA gene was also observed (Table 4).

3.4. Residue 374 in the NP is located in the body domain of the protein and protrudes from the protein surface

We compared residue 374 of the NP in 3826 other H5 avian influenza virus (AIV) strains listed in the Influenza Research Database (NCBI) by June 2019 and found NP residue 374 was a methionine in 98.2%. In the remaining strains (1.8%), isoleucine (64 strains), leucine (2 strains), or valine (3 strains) occupied this position. In strains that had amino acids other than methionine at position 374 in the NP, 4.2% were of chicken origin (1174 strains), while 0.8% originated in other bird species.

To examine the 3D location of residue 374 in the NP, we used a previously known 3D structure model of the NP (Chenavas et al., 2013) (Fig. 2A). The 3D structure of the NP was divided into 3 domains (head, body, and tail). The amino acid at the 374th position was located in the body domain, and protruded from the NP surface. Based on studies of the functional domains of NP, the 374th amino acid appears to be involved in the interaction between NP and PB2 (Fig. 2B) (Neumann et al., 1997; Ng et al., 2009; Weber et al., 1998). Additionally, this residue is in the vicinity of 4 residues known to be highly variable among the NPs of influenza viruses (hypervariable residues) (Ng et al., 2009).

3.5. The passaged virus rapidly replicates, particularly in chicken cells

We compared the growth of each H5N8 HPAIV strain in various cell types (Fig. 3). Upon inoculation of MDCK cells with each virus strain, at 8-h post-inoculation (HPI), cells inoculated with the Tottori P1-B strain had higher viral titers than cells inoculated with the Tottori P0 strain ($p < 0.05$). Additionally, by 24 HPI, all viruses reached a plateau of around $10^{4.0-5.5}$ PFU/mL, and no significant difference was observed between strains. Upon inoculating CEF cells with the different strains, at 8 HPI, we found that viral titers were higher in those inoculated with the Tottori P1-B strain and the Miyazaki strain than those inoculated with the Tottori P0 strain ($p < 0.05$). On the other hand, no significant difference in viral titers was observed between strains in DEF cells.

3.6. Passaged viruses do not show exacerbated pathogenicity in domestic ducks

To examine the pathogenicity of the passaged virus strain to other poultry, we performed infection experiments using domestic ducks (Fig. 4). Upon nasal inoculation of each virus strain into four 1-week-old ducks, at 5 DPI, 1 duck inoculated with the Miyazaki strain had died. Virus was recovered at $10^{5.3-8.0}$ EID_{50}/g from that specimen's organs (data not shown). The other ducks survived for 14 days, and serum from all ducks was found to have 32- to 128-fold increased HI antibodies (data not shown). Excretion of the virus was detected in the oral cavity and cloacal swabs collected from ducks inoculated with the Miyazaki strain at 1–7 DPI (Fig. 4). Virus was detected in oral swabs collected at 2–3 DPI from some ducks inoculated with the Tottori P0 strain. Furthermore, virus was detected in the oral cavity and cloacal swabs collected at 2–3 DPI from some ducks inoculated with the Tottori P1-B strain.

4. Discussion

The H5N8 HPAIV strains isolated in Japan during 2014–2015 were reported to exhibit high pathogenicity to chickens (Tanikawa et al., 2016). However, the Tottori P0 strain had comparatively lower pathogenicity than other isolated strains in the same season (Usui et al., 2017). In order to gain insight into this phenomenon, we passaged the Tottori P0 strain through chickens, which verified the phenomenon (Fig. 1), and identified that an amino acid substitution at residue 374 of the NP, located in a hypervariable region, may be responsible for this increased pathogenicity.

Variations of the Tottori strain yielded by intravenous and

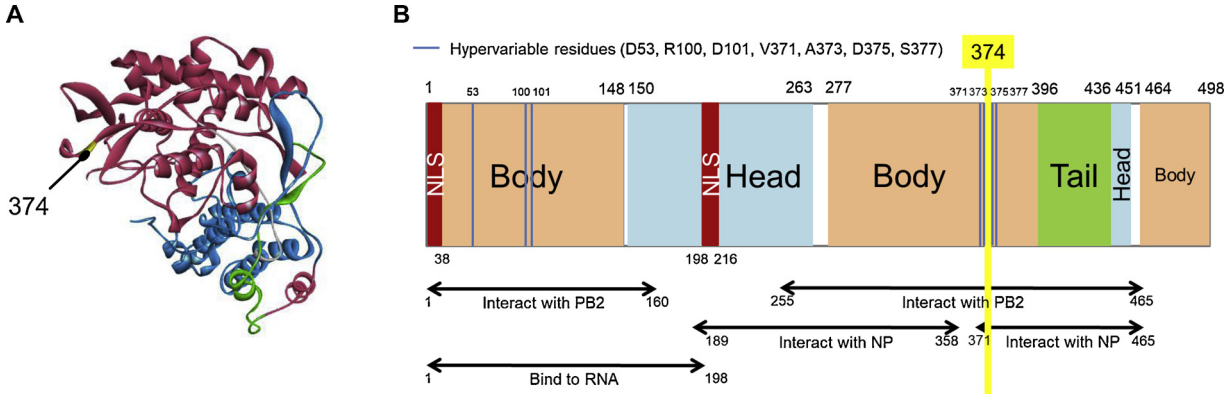

Fig. 2. Location of amino acid 374 of nucleoprotein (NP) in a 3D structure model and functional domain map. (A) Structure of the NP was analyzed using Discovery Studio Visualizer (VIOBIA, France), based on PBD ID:3ZDP, A/WSN/1933 (H1N1) NP (Chenavas et al., 2013). Body, head, and tail domains are shown in red, blue, and green, respectively. Amino acid 374, shown in yellow, is located in the body domain, and protrudes from the NP surface. (B) Functional domains (black arrows) and hypervariable residues (blue lines) are summarized according to previous reports (Neumann et al., 1997; Ng et al., 2009; Weber et al., 1998). Methionine encoded by the AUG start codon is defined as position 1. Amino acid 374, shown in yellow, is involved in the interaction between NP and PB2, and is located in an area containing hypervariable residues (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

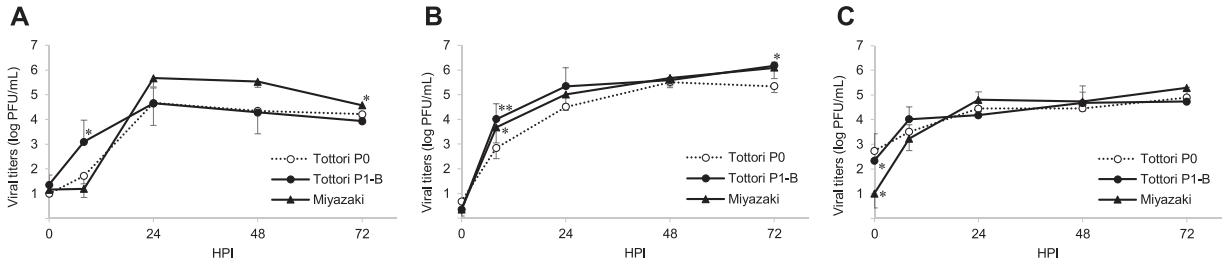

Fig. 3. Growth kinetics of the Tottori P0, P1-B, and Miyazaki strains in MDCK (A), CEF (B), and DEF (C) cells. Each virus was infected at a multiplicity of infection (MOI) of 0.01. After adsorption for 1 h, the cells were washed and overlaid with medium without trypsin. At the indicated time points, culture supernatants were collected for virus titration by the plaque or $TCID_{50}$ methods in MDCK cells. Data represent the mean ± SD of triplicate wells. The viral titer differences of strains compared to the Tottori P0 strain (white circles with dashed lines) were compared using Student's *t*-test (*p < 0.05; **p < 0.01).

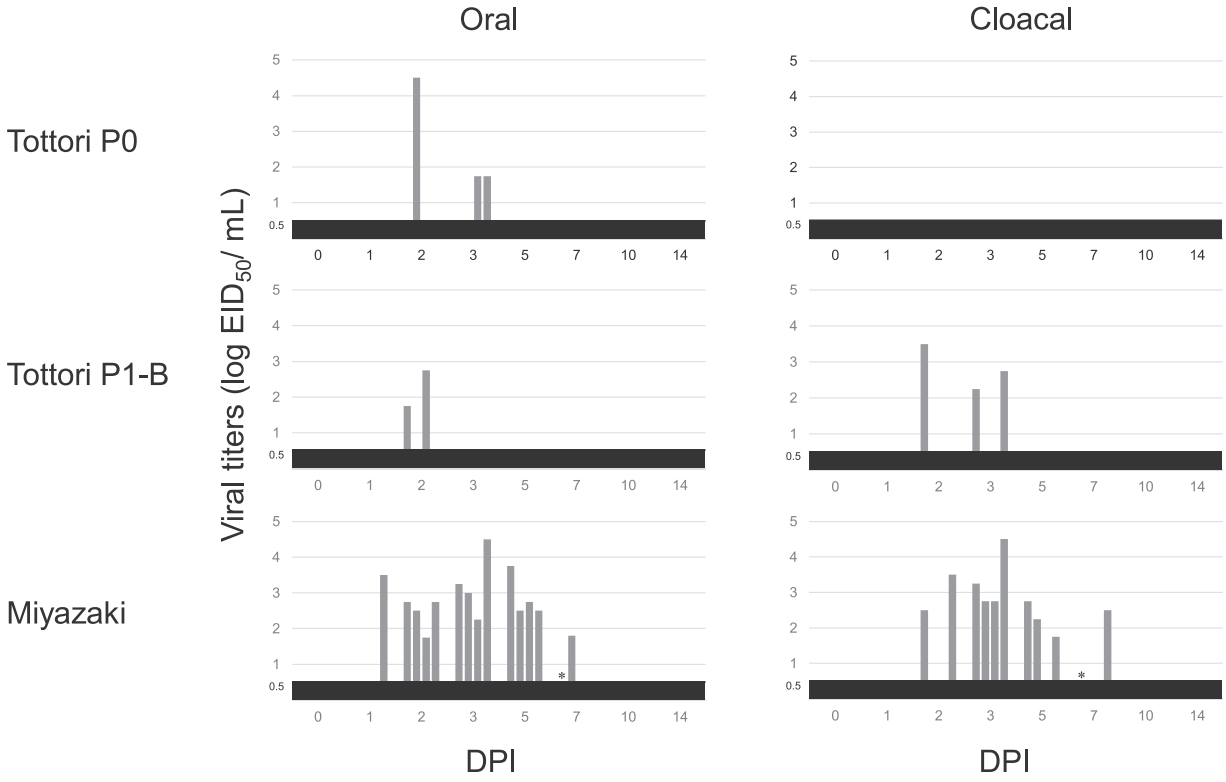

Fig. 4. Virus shedding of the domestic ducks inoculated with each H5N8 virus strain. For each strain, 10^6 EID_{50} of virus was intranasally inoculated into four 1-week-old ducks, and their viral titers were determined from oral (left side) and cloacal (right side) swabs; the limit of detection was $10^{0.5}$ EID_{50}/mL. One duck (*) with Miyazaki strain died at 5 days post-inoculation (DPI).

intranasal inoculation of the P0 strain into chickens (P1-B and P1-T) had higher pathogenicity than their parent strain (Table 1 and Fig. 1). Additionally, significantly higher titers of virus were recovered from some organs of chickens that died after intranasal inoculation with each passaged strain than with the Tottori P0 strain (Table 2). Finally, we inoculated CEF cells with each strain and found that, at 8 HPI, the passaged Tottori strain had significantly higher viral titers than the parent strain (Fig. 3). From the above results, we found that, compared to the Tottori P0 strain, the strains passaged through chicken cells replicated more rapidly and exhibited higher pathogenicity.

When we compared the amino acid sequences of the P0 and passaged Tottori strains, we found an amino acid substitution from methionine to valine at residue 374 of the NP (Table 4). Because this mutation was preserved in the second generation of passaged strains, we can surmise that it provided some advantage to virus replication. Of the H5 AIV strains isolated from chickens, deposited in the NCBI Influenza Research Database as of June 2019, 4.2% had an amino acid other than methionine in this position (Section *3.4*). Furthermore, 0.6% of these strains originated in other avian species. Consequently, it is likely that the amino acid at this position in the H5 AIV genome is involved in determining the replicative ability of the virus. While the Miyazaki strain had 42 amino acid loci differing from the Tottori P0 strain (Table 3), the amino acid at NP 374 was a methionine, as in the P0 strain. This implies that the Miyazaki strain acquired greater pathogenicity to chickens via a mechanism other than that used by the passaged Tottori strains (Fig. 1, Tables 1 and 2).

NP, along with viral RNA and a polymerase trimer, forms the vRNP complex (Pflug et al., 2017). NP is known to interact with itself and with PB2, a component of the aforementioned trimer (Biswas et al., 1998). It is possible that, by binding to PB2, NP controls viral polymerase activity. The 3D structure of NP is divided into head, body, and tail domains (Chenavas et al., 2013). The 374^{th} amino acid of this protein—the site of the substitution that occurred through serial passage identified in the present study—is located in the body domain of the protein and protrudes from the protein surface (Fig. 2A). We propose that a mutation at position 374 in the NP affects the activity of the Tottori P0 strain, and ultimately leads to the increased replication exhibited by mutated strains in chickens and cultured cells (Table 2 and

Fig. 3). However, the proliferation of the passaged strains in ducks and DEF cells was similar to that of the P0 strain (Figs. 3 and 4). Thus, the increase in pathogenicity of the H5N8 HPAIV caused by amino acid substitution at NP 374 is a host-specific effect. Similarly, it has previously been reported that H5 HPAIV originating from a domestic duck acquired higher pathogenicity by a single amino acid mutation at position 109 of the NP during replication in chicken brains (Tada et al., 2011); the emerged virus showed strong proliferation in CEF cells, but not in DEF cells, and the mutation conferred enhanced polymerase activity only in chicken-origin DF-1 cells. In future, we plan to examine whether NP residue also contributes to viral polymerase activity and RNA synthesis, and thereby clarify the molecular basis by which pathogenicity is modulated in chickens.

In conclusion, in this study, we have shown that the clade 2.3.4.4 H5N8 HPAIV strain, which originally had comparatively low pathogenicity in chickens, increased its pathogenicity after a single passage in chickens. The HA genes of clade 2.3.4.4 H5Nx HPAIV strains have been categorized into 4 groups (A–D) based on comprehensive phylogenetic analyses (Lee et al., 2016). Group B is primarily composed of H5N8 viruses isolated in China and South Korea during 2013–2014 (Antigua et al., 2019), and included the Tottori P0 strain (Usui et al., 2017). Moreover, the recent H5N6 virus isolates obtained from South Korea during 2017–2018 and H5N5/N8 from Germany during 2016–2017 also belonged to this group. It may be possible that H5N8 HPAIVs with comparatively low pathogenicity, such as the Tottori P0 strain, continue to be maintained in wildfowl populations. Infections with viruses similar to the strain examined in this study appear to remain subclinical for a long duration, during which period they can spread through and out of farms and hatcheries. When observing poultry populations, it is thus vital not only to examine death rates, but also clinical symptoms in detail. Additionally, in seasons during which migratory birds from epidemic regions are present, it is crucial that quarantine measures be adopted, including preventing entry into poultry farms.

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