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Genetics and pathogenicity of H5N6 highly pathogenic avian influenza viruses isolated from wild birds and a chicken in Japan during winter 2017–2018

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ABSTRACT

An H5N6 highly pathogenic avian influenza virus (HPAIV) outbreak occurred in poultry in Japan during January 2018, and H5N6 HPAIVs killed several wild birds in 3 prefectures during Winter 2017–2018. Time-measured phylogenetic analyses demonstrated that the Hemagglutinin (HA) and internal genes of these isolates were genetically similar to clade 2.3.4.4.B H5N8 HPAIVs in Europe during Winter 2016–2017, and Neuraminidase (NA) genes of the poultry and wild bird isolates were gained through distinct reassortments with AIVs that were estimated to have circulated possibly in Siberia during Summer 2017 and Summer 2016, respectively. Lethal infectious dose to chickens was similar between the poultry and wild-bird isolates. H5N6 HPAIVs during Winter 2017–2018 in Japan had higher 50% chicken lethal doses and lower transmission efficiency than the H5Nx HPAIVs that caused previous outbreaks in Japan, thus explaining in part why cases during the 2017–2018 outbreak were sporadic.

1. Introduction

The highly pathogenic avian influenza viruses (HPAIVs) of the Goose/Guangdong (Gs/Gd) lineage arose from the H5N1 HPAIV that caused the first outbreaks among domestic geese in Guangdong province, China, during 1996 (Xu et al., 1999) and have now been circulating for more than 20 years. In Japan, outbreaks due to H5N1 HPAIVs of the Gs/Gd lineage were first reported in 2004 (Mase et al., 2005). In 2007, there were outbreaks of H5N1 HPAIVs related to the 2005 Qinghai strain belonging to the clade 2.2 (Shivakoti et al., 2010); these were followed by the reintroduction of other H5N1 HPAIVs in clade 2.3.2 during Winter 2010–2011. These massive outbreaks yielded 64 recorded cases in wild birds and 24 outbreaks in poultry farms throughout 21 prefectures in Japan (Sakoda et al., 2012; Uchida et al., 2012). In April 2014, H5N8 HPAIVs belonging to clade 2.3.4.4 caused an outbreak at a chicken farm. During Winter 2014–2015, reintroduction of clade 2.3.4.4 but phylogenetically distinct H5N8 HPAIVs from the isolate in April caused outbreaks in 5 other poultry farms and 13 cases in wild birds (Saito et al., 2015; Tanikawa et al., 2016). Among the evolution of 2.3.4.4 HPAIVs in Asia, they have been classified to Group A, B, C, and D (Lee et al., 2014, 2015; Si et al., 2017). During Winter 2016–2017, H5N6 HPAIVs of clade 2.3.4.4.C accounted for 218 cases in wild birds and 12 outbreaks in poultry farms (Takemae et al., 2017; Tsunekuni et al., 2017; Ozawa et al., 2018).

During Winter 2017–2018, there were outbreaks of infection in both Asia and Europe caused by H5N6 HPAIVs with the H5 Hemagglutinin (HA) gene of clade 2.3.4.4.B (World Organization for Animal Health). In Europe, the first report of the H5N6 HPAIV outbreak occurred at a Dutch duck farm in December 2017 (Beerens et al., 2018), after which H5N6 and H5N8 HPAIVs were isolated from both domestic poultry and wild birds. On the other side of the Eurasian continent, Korea experienced an outbreak due to H5N6 HPAIVs on a duck farm in North Jeolla

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province during November 2017. This was followed by outbreaks in poultry farms from Gyeonggi province in the north to South Jeolla province in the south and concurrent isolation of H5N6 HPAIVs from wild birds from Gyeonggi province in the north to Jeju province in the south (Lee et al., 2018a, b; OIE). The H5 HA gene of the HPAIVs isolated from ducks in Korea during November 2017 were genetically similar to those of the December 2017 isolates in the Netherlands, and the N6 Neuraminidase (NA) gene was phylogenetically related with an H3N6 AIV isolated from a barnacle goose in the Netherlands during 2014 (Kim et al., 2018; Lee et al., 2018a). In addition, H5N6 HPAIVs affected a chicken farm as well as wild birds in Japan, including several species of wild birds in Shimane during November 2017, a northern goshawk in Tokyo on 5 January 2018, a broiler chicken farm in Kagawa on 10 January 2018, and jungle crows in Hyogo during March 2018. Furthermore, the Shimane strains carried surface genes related to both the Netherlands and Korean strains (Kim et al., 2018).

In the current study, we phylogenetically compared the H5N6 HPAIVs that affected Japan during Winter 2017–2018 with gene sequences deposited in the GISAID database and our repositories to investigate the origins of these H5N6 HPAIVs. In addition, we characterized the H5N6 HPAIV isolates from a chicken, a northern goshawk, and a jungle crow to assess whether pathogenicity to chickens differed between poultry and wild bird isolates.

2. Materials and methods

2.1. Virus isolation and whole-genome sequencing

Tracheal or cloacal swabs collected from chickens on a farm in Kagawa prefecture with suspected HPAIV infections were inoculated into embryonated chicken eggs for virus isolation at the diagnostic laboratories of the livestock health center in Kagawa prefecture. Allantoic fluid that showed HA activity against chicken red blood cells was submitted to the National Institute of Animal Health of Japan for diagnosis, as were swab samples from dead wild birds in Tokyo and Hyogo prefectures. The whole genomes of the 43 isolated viruses were obtained by using next-generation sequencing (Miseq, Illumina, San Diego, CA, USA). RNA was extracted from isolated viruses by using RNeasy Mini kits (Qiagen, Hilden, Germany). cDNA libraries for next-generation sequencing were prepared by using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). In total,10 pM of synthesized cDNA libraries was mixed with 10 pM of the PhiX control (Illumina) and sequenced by using a Miseq Reagent Kit version 2 (Illumina). Consensus sequences were generated by using Workbench software (version 9.5.3, Qiagen, Hilden, Germany) or FluGAS software (version 1.0.0, World Fusion, Tokyo, Japan). The sequences of the viruses isolated during this study are deposited in the GISAID databases (http://platform.gisaid.org); the accession number of each virus is listed in Table S1.

2.2. Phylogenetic analysis

For phylogenetic analysis, we downloaded sequences of all AIVs from the GISAID database in 25 December 2018; the number of sequences downloaded for each gene was: PB2, 69,552 sequences; PB1, 51,793; PA, 71,568; H5, 10,685; NP, 69,775; N6, 3,693; MP, 87,318; and NS, 72,176. BioEdit (Hall, 1999) and MAFFT (Katoh et al., 2016) were used to align the GISAID sequences with those of AIVs at the National Institute of Animal Health, Japan, and at the Federal Research Center of Fundamental and Translational Medicine, Russia. After alignment, sequences with ambiguous nucleotide bases were removed, after which MAFFT was used to align the remaining sequences in order of decreasing identity with A/chicken/Kagawa/1C-1/2018. Sequences that were most highly related to the Kagawa strain were phylogenetically analyzed by using MEGA-CC with 1000 bootstrap replicates according to the maximum likelihood (ML) method in a general time-reversible model (Kumar et al., 2012).

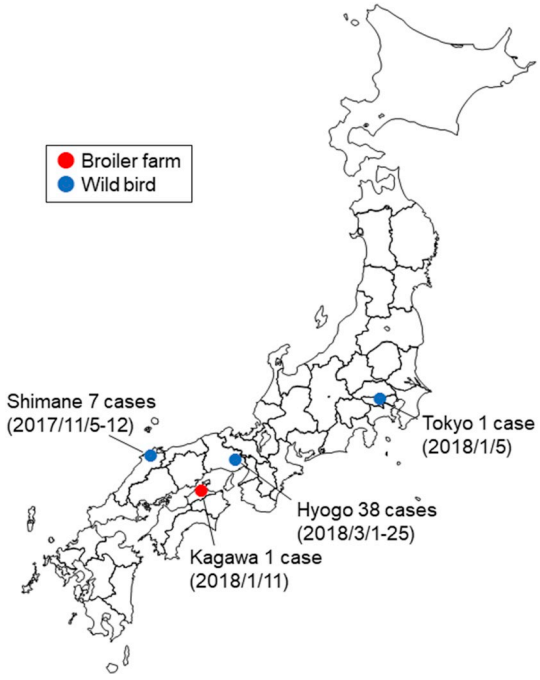

Fig. 1. Geographic locations of the H5N6 HPAI outbreaks affecting 1 poultry farm and 46 wild birds in Japan during the 2017–2018 season. Numbers of reported cases and sample collection dates are shown in parentheses.

Tanglegrams were constructed from pairs of the above-mentioned ML trees by using Dendroscope 3 (Huson and Scornavacca, 2012). Taxa in adjacent trees were connected with those in the tree of H5 genes when the taxa in the two trees corresponded with each other.

We constructed maximum clade credibility trees to calculate molecular estimates of divergence times from the ancestral AIVs for the PB2, PB1, PA, NP, N6, MP, and NS genes of the H5N6 HPAIVs isolated in Europe and Asia during Winter 2017–2018. Maximum clade credibility trees were constructed by using the Bayesian Evolutionary Analysis by Sampling Tree (BEAST) package version 1.8.2 (Drummond et al., 2012). Aligned sequences of the N6 NA genes were formatted by using the Bayesian Evolutionary Analysis Utility (BEAUti) at default settings, except for clock rate: the initial clock rate was set as 1.0×10^{-5} to fit the substitution rate of influenza viruses for constructing trees. Each calculation was set as 1×10^{8} to 1×10^{9} steps in length, where the number of steps was determined as that needed to obtain an effective sample size of more than 200.

2.3. Animal experiments

A/chicken/Kagawa/1T-1/2018 (Kagawa strain; isolated through 2-egg passage from a tracheal swab), A/Northern Goshawk/Tokyo/1301B003T/2018 (Tokyo strain; isolated through 2-egg passage from a tracheal swab), and A/Jungle crow/Hyogo/2803E023C/2018 (Hyogo strain; isolated through 1-egg passage from a cloacal swab) were used for animal experiments. White leghorn chickens (L-M-6 strain; specific pathogen free; age, 4 weeks) were obtained from Nisseiken (Tokyo, Japan). Animal experiments were conducted in Biosafety Level 3 facilities at the National Institute of Animal Health, Japan, and were approved by the institutional committee for Ethics of Animal Experiments. For survival analysis, virus doses of 10^2, 10^4, 10^5, and 10^6 fifty percent egg infectious dose (EID_{50})/100 mL were inoculated intranasally into groups of 4–6 chickens. The chickens were observed for 14 days after inoculation. For virus titration, tracheal and cloacal swabs were collected at 1, 2, 3, 5, 7, 10, and 14 days after inoculation or at death. The swabs were diluted into MEM containing 0.5% BSA, 25 mg/

mL amphotericin B, 1000 units/mL penicillin, 1000 mg/mL streptomycin, 0.01 M HEPES, and 8.8 mg/mL NaHCO3; removed from the medium; and then stored at −80 °C until titration. To investigate tissue dissemination of the virus in chickens, 3 chickens were inoculated intranasally with 10^6 EID_{50}/100 mL of Kagawa, Tokyo, and Hyogo strains. Three chickens were euthanized at 3 days post-inoculation and pancreas, spleen, pectoral major muscle, liver, trachea, lung, kidney, heart, brain, duodenum, rectum, blood, trachea swab, cloaca swab, and conjunctiva swab were collected. Tissues were minced using a Precellys homogenizer (Bertin Technologies, Ile-de-France, France) to prepare a 10% (w/v) emulsion, and the emulsion supernatant was stored at −80 °C until titration by egg inoculation. Frozen samples were thawed and centrifuged in 3,000 g at 4 °C; the supernatant underwent viral titration by EID_{50}. Student's *t*-test was applied for statistical comparison and *P* value of less than 0.05 was considered as significant.

For the transmission study, 1 or 3 chickens were inoculated with 10^6

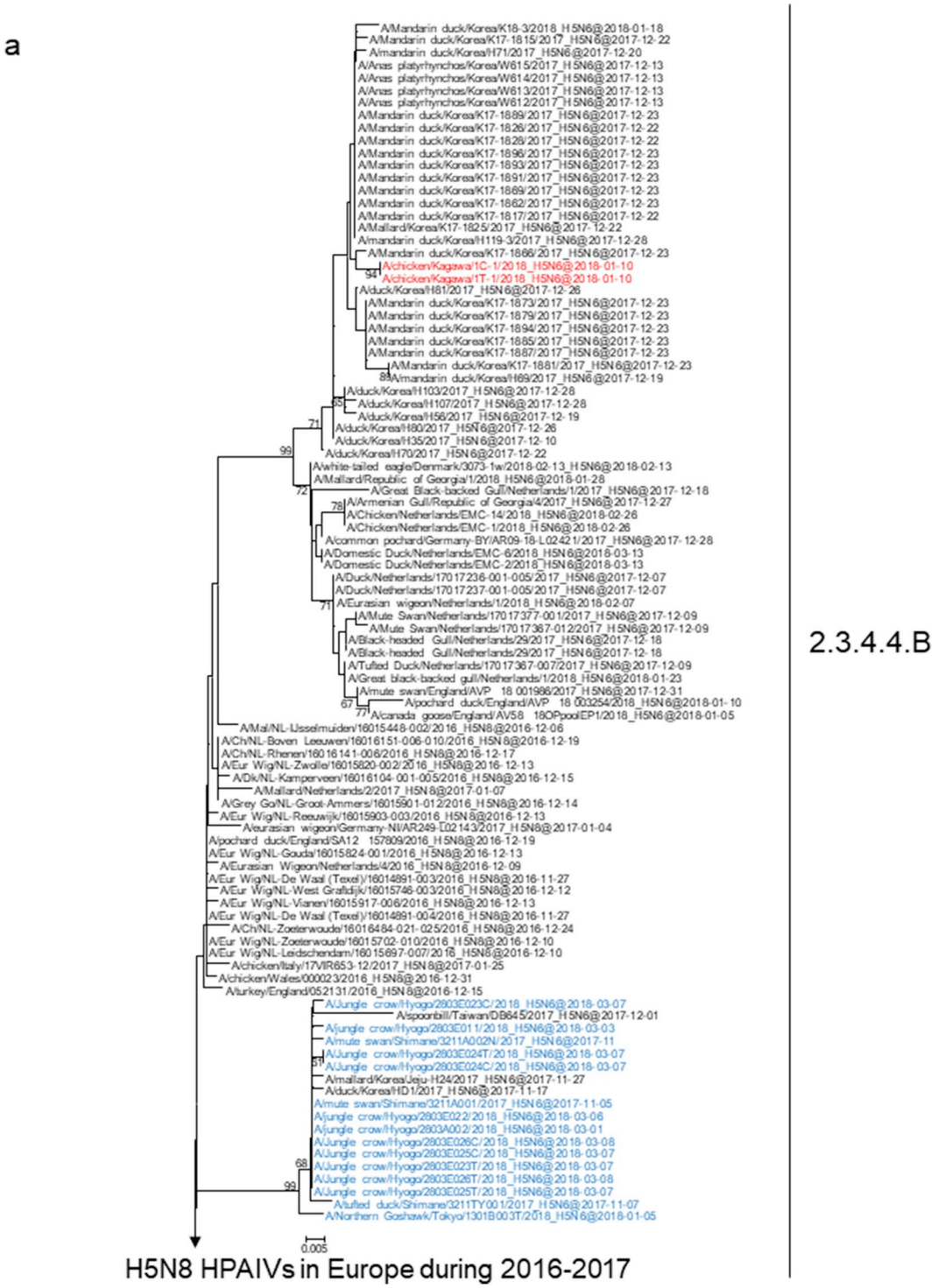

Fig. 2. Details of ML phylogenetic trees based on the (a) H5 HA and (b) N6 NA genes. The Kagawa strain is shown in red, and strains isolated from wild birds are blue. Bootstrap values of 60 or higher are shown.

Fig. 2. (*continued*)

EID_{50}/100 mL of Kagawa strain; beginning at 18 h after inoculation, 6 or 4 chickens, respectively, were cohoused with the inoculated chicken (s) and then observed for 14 days thereafter. Tracheal and cloacal swabs for virus titration were collected at 1, 3, 5, 7, 10, and 14 days after inoculation or at death. To verify viral infection in surviving chickens, blood samples were collected at the end of the observation period, and antibodies against influenza A virus were detected by using an influenza A virus antibody test kit (IDEXX Laboratories, Westbrook, ME, USA).

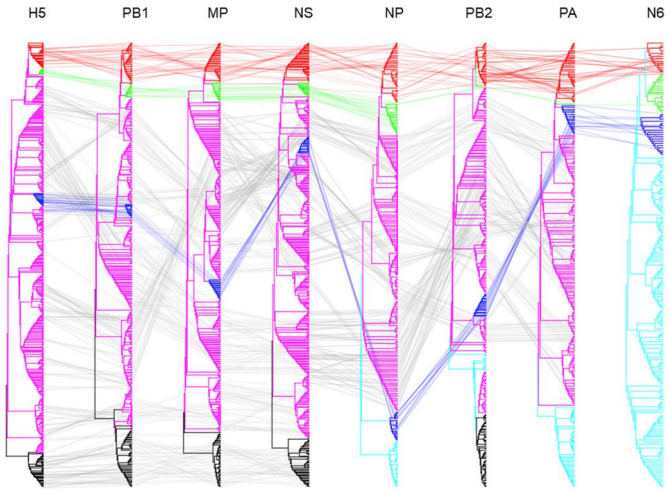

Fig. 3. Tanglegrams constructed from the ML trees of strains containing the individual gene most related to that of the Kagawa strain. Corresponding taxa in adjacent trees are connected by lines. Colors of taxa and lines are as follows: black, isolates of clade 2.3.4.4.
B H5N8 HPAIVs isolated in China and Russia during May 2016; purple, H5N8 HPAIVs isolated during the 2016–2017 season; red, Kagawa strain and Korean strains of December 2017; green, H5N6 HPAIVs isolated in Europe during the 2017–2018 season; blue, H5N6 HPAIVs isolated from wild birds in Japan and Korean HPAIVs during November 2017 and the Taiwanese HPAIV of December 2017; light blue, not HPAIVs.

3. Results

3.1. Outbreaks due to H5N6 strains in Japan during winter 2017–2018

In Japan, 47 reports (1 outbreak in poultry farm and 46 cases in wild birds) due to H5N6 HPAIVs were reported across 4 prefectures between 5 November 2017 and 25 March 2018 (Fig. 1). The first case involved a dead mute swan in Shimane prefecture; this was followed by cases in 5 tufted ducks and 1 black-headed gull. On January 5, a dead northern goshawk was collected in Tokyo prefecture and an H5N6 HPAIV was isolated. Six days after the report of the northern goshawk, an outbreak occurred on a broiler farm in Kagawa prefecture and approximately 90,000 broilers were euthanized to prevent disease spread. Subsequently, H5N6 HPAIVs killed multiple jungle crows in Hyogo prefecture during 1 March through 25 March 2018.

3.2. Phylogenetic analysis of the Japanese isolates

The ML phylogenetic trees constructed for the H5 HA gene revealed that the HA genes of the Japanese H5N6 HPAIVs during Winter 2017–2018 belonged to clade 2.3.4.4.B and thus were related to the H5N8 HPAIVs in Europe during Winter 2016–2017 (Fig. 2a). The Kagawa strain formed a cluster with the Korean HPAIVs of December 2017, sharing a common ancestor with the H5N6 HPAIVs that caused outbreaks in Europe during the same season. However, the isolates from wild birds in Japan formed another cluster with the Korean HPAIVs of November 2017 and the Taiwanese HPAIV from December 2017. The nucleotide identity between the Japanese wild bird isolates and the Kagawa strain was 98.2%–98.3%.

The ML tree of the N6 NA genes demonstrated that NA genes of Japanese H5N6 HPAIVs during Winter 2017–2018 were related to AIVs isolated in European countries, including Croatia, Georgia, and the Netherlands, and in Moscow and Novosibirsk, Russia (Fig. 2b). The Kagawa strain showed low identity (94.5%–94.7%) with the isolates from wild birds in Japan and formed a cluster with the Korean HPAIVs from December 2017, which differed from the cluster containing isolates from wild birds in Japan, Korean HPAIVs isolated in November 2017, and the Taiwanese HPAIV of December 2017. All of the internal genes of the Japanese H5N6 HPAIVs were derived from clade 2.3.4.4.B H5N8 HPAIVs isolated in Europe during Winter 2016–2017, and most of them were most related to isolates in Eastern Europe (Fig. 3, S1). As with the surface genes, the internal genes of the Kagawa strain and the wild bird isolates in Japan formed different clusters and identities between Kagawa strain and wild bird isolates were as follows: PB2, 98.9%–99.0%; PB1, 98.2%–98.3%; PA, 98.5%–98.7%; NP, 98.8%–99.0%; MP, 97.9%–98.1%; and NS, 97.6%–98.0% (Fig. 3).

According to the maximum clade credibility tree that we constructed by using N6 sequence data (Fig. 4), the cluster including the Kagawa strain was estimated to have diverged from that containing the wild bird isolates on 15 September 2016 (95% highest posterior density interval [HPD]: 17 August to 26 October 2016). Furthermore, the subcluster composed of the Kagawa and Korean strains was estimated to have diverged from the subcluster of European H5N6 HPAIVs on 14 August 2017 (95% HPD: 10 June to 21 September 2017). These results suggest that the H5N6 HPAIVs in the Kagawa–Korean cluster shared a common ancestor during Summer 2017 and had been disseminated to both Asia and Europe by December 2017. The cluster including the wild bird isolates in Japan was estimated to have diverged from Greek strains on 30 October 2016 (95% HPD: 16 October to 14 December 2016), thus indicating that the strains diverged from a common ancestor during Autumn 2016. In contrast, Taiwanese and Japanese strains were estimated to have diverged from each other during Summer 2017 (16 August 2017; 95% HPD: 16 May to 5 October 2017).

We then estimated the dates on which various genes of the H5N6 HPAIVs in Japan during Winter 2017–2018 had diverged from an ancestral (non-HPAIV) AIV (Table 1). The divergence times of the PB1 and MP genes of the Japanese H5N6 HPAIVs of Winter 2017–2018, which shared deduced ancestors with the H5N8 HPAIVs in Europe during Winter 2016–2017 that were related to H5N8 HPAIVs in China and Russia during May 2016 (Fig. 3, S1d and g), were 3 October 2015 (95% HPD: 14 September 2015 to 29 January 2016) and 13 October 2015 (95% HPD: 27 April to 14 December 2015), respectively, thus indicating that these viruses diverged during Summer 2015. The H5 and NS genes of Japanese H5N6 HPAIVs during Winter 2017–2018 shared an ancestor with H5N8 HPAIVs in Europe during 2016–2017 that were related to A/duck/Eastern China/S1109/2014 (H5N8) as well as the H5N8 HPAIVs in China and Russia in May 2016 (Fig. 3, S1a and h); the divergence times from AIVs (excluding HPAIVs) were 1 October 2014 (95% HPD: 30 July to 7 November 2014) and 28 July 2014 (95% HPD: 15 June to 7 November 2014), respectively. The PB2, PA, and NP genes, the origins of which were considered to be H5N8 HPAIVs in Europe during Winter 2016–2017 that were different from the H5N8 HPAIVs in China and Russia during May 2016 (Fig. 3, S1b, c, e and f), were estimated to have diverged on 22 April 2016 (95% HPD: 2 April to 14 June 2016), 19 May 2016 (95% HPD: 11 January to 10 June 2016), and 24 June 2016 (95% HPD: 13 April to 4 August 2016), respectively, indicating that they all diverged from an ancestral AIV during Summer 2016. Except for A/Black-headed Gull/Netherlands/29/2017, the PB2 and PA genes of H5N6 HPAIVs in Europe during Winter 2017–2018 were not related to the H5N6 HPAIVs in Asia during 2017–2018 but instead were descended from AIVs in Europe, with estimated divergence dates of 19 May 2017 (95% HPD: 29 December 2016 to 11 September 2017) and 30 September 2016 (95% HPD: 5 May to 10 October 2016), respectively.

3.3. Pathogenicity of poultry and wild bird strains in chickens

To investigate potential differences in pathogenicity among the H5N6 HPAIVs, chickens were intranasally inoculated with several doses of the Kagawa, Tokyo, and Hyogo strains and then observed for 14 days (Fig. 5). All of the chickens inoculated with 10^6 EID_{50} of Kagawa strain died within 3 days, and 4 of the 5 chickens inoculated with 10^5 EID_{50} of this isolate died within 4 days, whereas no chickens inoculated with 10^4

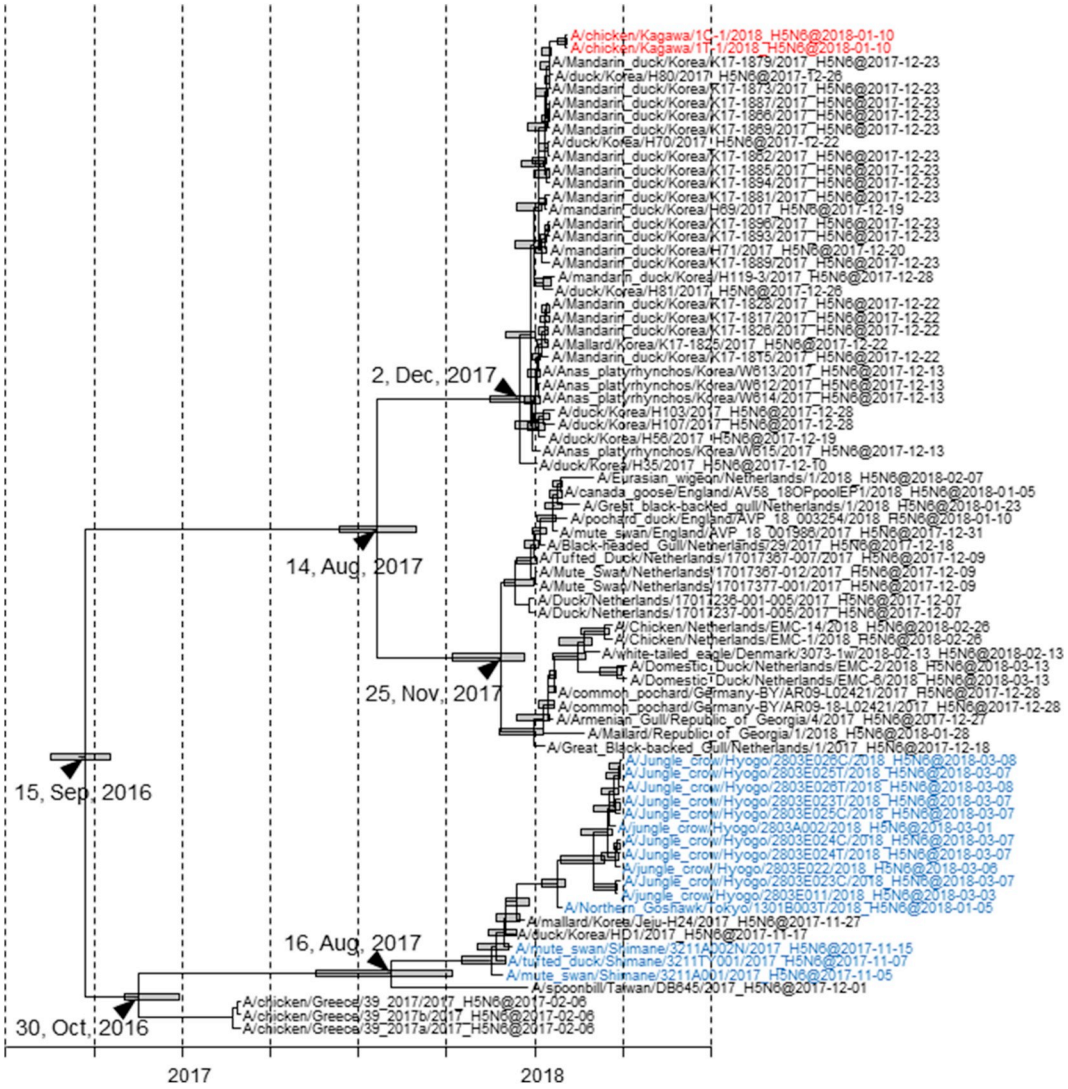

Fig. 4. Detail of the maximum clade credibility phylogenetic tree of the N6 NA genes. The divergence time at the branch is indicated by a black arrow, and the 95% highest posterior density for the divergence time is indicated by a gray box. The Kagawa strain and strains isolated from wild birds are red and blue, respectively.

EID_{50} died during the observation period (Fig. 5a). All of the chickens inoculated with 10^5 or 10^6 EID_{50} of the Tokyo strain died within 5 and 3 days after inoculation, respectively, and 1 of the 5 chickens inoculated with 10^4 EID_{50} of the Tokyo strain died 3 days after inoculation (Fig. 5b). All of the chickens inoculated with 10^6 EID_{50} of the Hyogo strain died within 4 days, as did 1 of 5 and 2 of 5 chickens inoculated with 10^4 or 10^5 EID_{50} of Hyogo strain, respectively (Fig. 5c). For all strains, all chickens inoculated with 10^2 EID_{50} survived the observation period (data not shown). Therefore, the 50% chicken lethal doses of Kagawa, Tokyo, and Hyogo strains were calculated as $10^{4.63}$ EID_{50}, $10^{4.38}$ EID_{50}, and $10^{4.83}$ EID_{50}, respectively. None of the sera collected from surviving chickens on day 14 after inoculation contained detectable antibodies against influenza A virus (data not shown).

The mean viral titer from tracheal swabs collected from dead chickens inoculated with 10^6 EID_{50} of the Kagawa strain (4.44 ± 0.53 $\log_{10}$ EID_{50}/mL) was significantly higher than that from their cloacal swabs (3.52 ± 0.80 $\log_{10}$ EID_{50}/mL) with P value < 0.01 (Fig. 6a and b). The same pattern emerged for chickens inoculated with 10^6 EID_{50} of Hyogo strain (4.62 ± 0.48 $\log_{10}$ EID_{50}/mL vs 3.52 ± 0.80 $\log_{10}$ EID_{50}/mL) with P value < 0.01 (Fig. 6e and f). The mean maximum viral titer of tracheal swabs from live chickens inoculated with the Kagawa strain did not differ from that of wild bird isolates at all inoculation doses (10^4, 10^5, and 10^6 EID_{50}). The same pattern was seen regarding the cloacal swabs from live chickens and the tracheal or cloacal swabs from dead birds, indicating the lack of a significant difference between the Kagawa strain and wild bird isolates regarding viral shedding. No viruses were obtained from the tracheal and cloacal swabs collected from surviving chickens inoculated with the Kagawa, Tokyo, or Hyogo strains on days 7, 10, or 14 after inoculation (data not shown).

Measurement of viral titers in tissues revealed that—unlike the Tokyo and Hyogo strains—the Kagawa strain propagated efficiently in several tissues (Table 2). At 2 days after inoculation of the Kagawa strain at 10^6 EID_{50}, the mean viral titers in kidney and cloaca swabs were significantly higher than those of the Tokyo strain; those in the brain and rectum were significantly higher than those of the Hyogo strain; and those in the muscle and trachea were significantly higher than those of both the Tokyo and Hyogo strains. Viral titers on day 1 after inoculation did not differ among any tissues or strains. No gross lesions were observed in tissues of all chickens at 1 and 2 day(s) after inoculation with each strain.

3.4. Transmission of Kagawa strain in chickens

In the transmission study, the 1 chicken inoculated with 10^6 EID_{50} of Kagawa strain died within 48 h after inoculation, whereas none of

Table 1
Estimated divergence time of each gene of H5N6 HPAIVs during Winter 2017–2018 from the most recent AIVs (except HPAIVs).

Gene	Estimated time (95% HPD interval)		
	H5N6 HPAIVs in Asia (including Kagawa strain)	H5N6 HPAIVs in Asia (including Tokyo and Hyogo strain	H5N6 HPAIVs in Europe
PB2	22 April, 2016 (2 April to 14 June, 2016)	22 April, 2016 (2 April to 14 June, 2016)	19 May, 2017 (29 December, 2016 to 11 September, 2017)
PB1	3 October, 2015 (14 September, 2015 to 29 January, 2016)	3 October, 2015 (14 September, 2015 to 29 January, 2016)	3 October, 2015 (14 September, 2015 to 29 January, 2016)
PA	19 May, 2016 (11 January to 10 June, 2016)	19 May, 2016 (11 January to 10 June, 2016)	30 September, 2016 (5 May to 10 October, 2016)
HA	1 October, 2014 (30 July to 7 November, 2014)	1 October, 2014 (30 July to 7 November, 2014)	1 October, 2014 (30 July to 7 November, 2014)
NP	24 June, 2016 (13 April to 4 August, 2016)	24 June, 2016 (13 April to 4 August, 2016)	24 June, 2016 (13 April to 4 August, 2016)
N6	15 September, 2016 (17 August to 26 October, 2016)	14 August, 2017 (10 June to 21 September, 2017)	14 August, 2017 (10 June to 21 September, 2017)
MP	13 October, 2015 (27 April to 14 December, 2015)	13 October, 2015 (27 April to 14 December, 2015)	13 October, 2015 (27 April to 14 December, 2015)
NS	28 July, 2014 (15 June to 7 November, 2014)	28 July, 2014 (15 June to 7 November, 2014)	28 July, 2014 (15 June to 7 November, 2014)

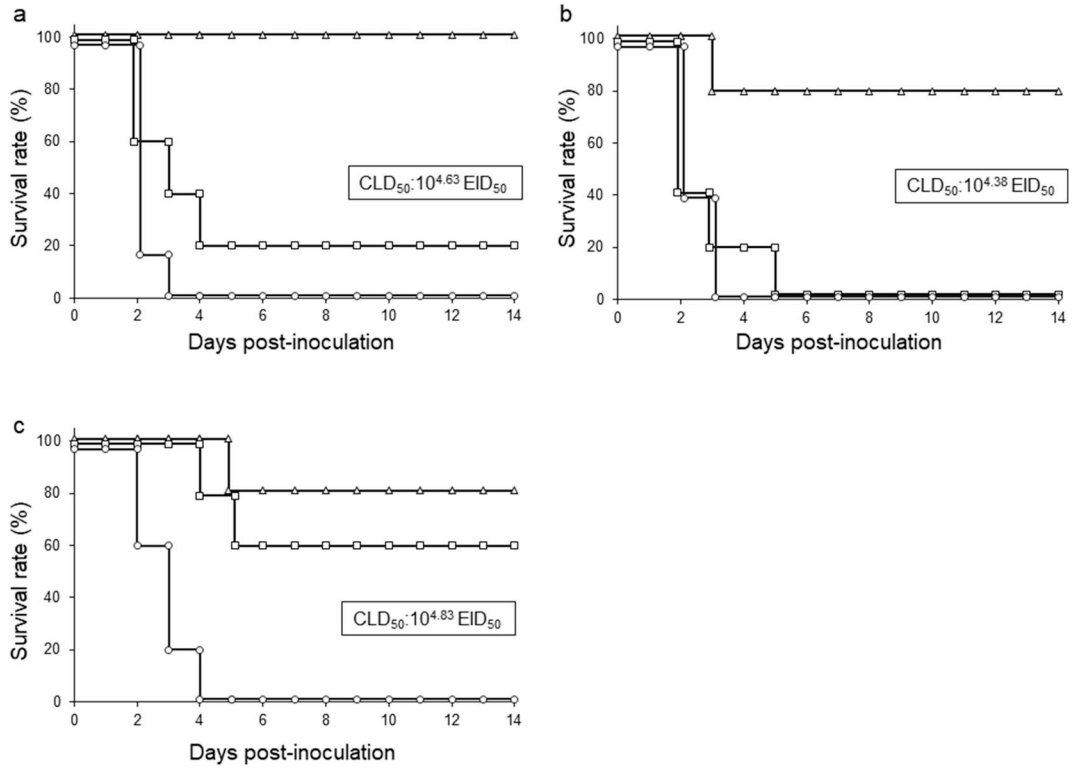

Fig. 5. Survival rates of chickens inoculated intranasally with 10^6, 10^5, or 10^4 EID_{50} of (a) Kagawa strain, (b) Tokyo strain, and (c) Hyogo strain. Circles, squares, and triangles indicate the survival rates of birds inoculated with 10^6, 10^5, and 10^4 EID_{50} of each virus, respectively.

the 6 cohoused chickens died during the 14-day observation period (Fig. 7a). The viral titers of the tracheal and cloacal swabs collected from the dead chicken were 6.07 and 5.20 $\log_{10}$ EID_{50}/mL, respectively. No antibodies against influenza A virus were detected in sera collected from the cohoused chickens that survived for 14 days after placement with the inoculated bird (data not shown), indicating a lack of viral transmission to the cohoused chickens.

We then inoculated each of 3 chickens with 10^6 EID_{50} of Kagawa strain and housed them with 4 naïve chicken. The 3 inoculated chickens died within 66 h after inoculation, and the 4 cohoused chickens died 150 h after being penned with the inoculated birds (Fig. 7b). In addition, 2 of the cohoused chickens demonstrated viral shedding at 102 h after being placed with the inoculated chickens, and the 2 other chickens shed virus at 150 h. The viral titers (mean ± SEM) of the tracheal and cloacal swabs collected from the dead inoculated chickens were 3.99 ± 0.26 and 3.80 ± 1.10 $\log_{10}$ EID_{50}/mL, respectively, and those of the dead cohoused chickens were 4.44 ± 0.55 and 2.81 ± 1.21 $\log_{10}$ EID_{50}/mL, respectively. There were no significant differences between mean viral titers collected from inoculated chicken (s) of two transmission studies. These results show that the 4 cohoused

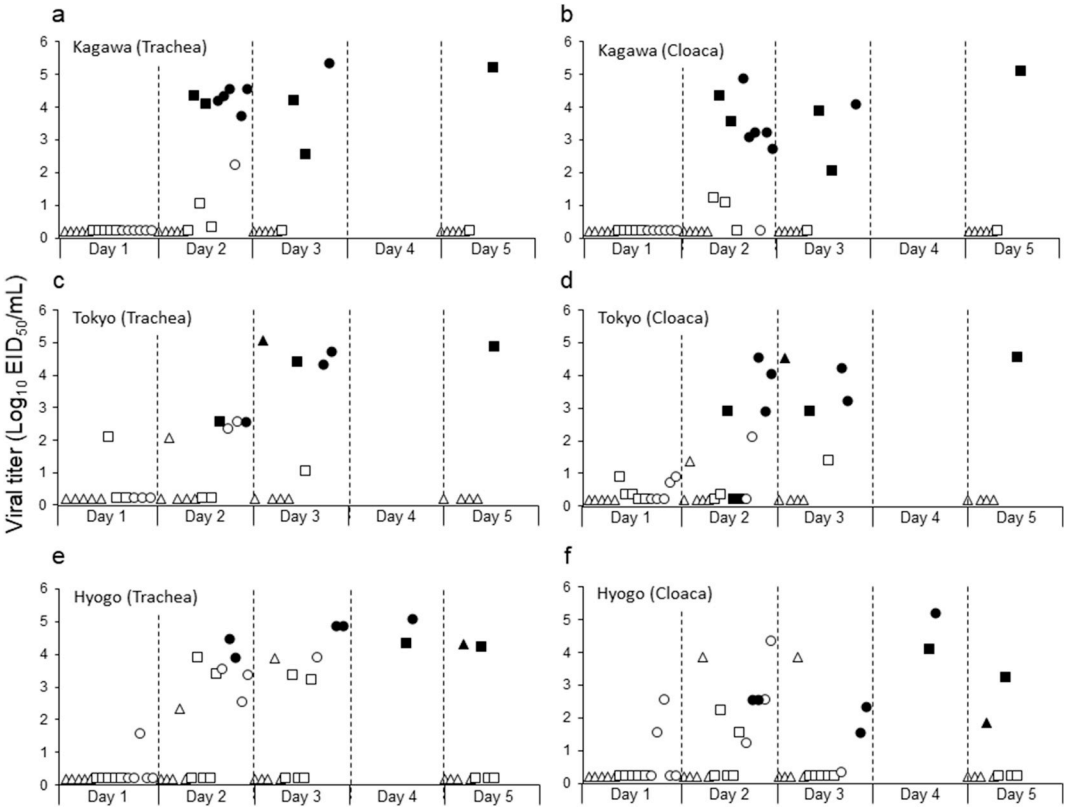

Fig. 6. Viral titers of tracheal and cloacal swabs collected from live and dead chickens that had been inoculated with 10^6, 10^5, or 10^4 EID_{50} of each virus: (a) Tracheal and (b) cloacal swabs collected from chickens inoculated with Kagawa strain. (c) Tracheal and (d) cloacal swabs collected from chickens inoculated with Tokyo strain. (e) Tracheal and (f) cloacal swabs collected from chickens inoculated with Hyogo strain. White and black shapes indicate the results from live and dead chickens, respectively. Circles, squares, and triangles represent the viral titers of chickens inoculated with 10^6, 10^5, or 10^4 EID_{50} of each virus, respectively.

Table 2
Viral titers in organs, tissues, and swabs of chickens inoculated by10^6 EID_{50} of H5N6 HPAIVs.

Sample	Log_{10} EID_{50}/mL at each							
	Kagawa		Tokyo		Hyogo			
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	*P* value vs Kagawa on day 2	
							Tokyo	Hyogo
Pancreas	2.11 ± 1.29 (2/3)†	5.78 ± 1.27 (3/3)	3.42 ± 2.74 (3/3)	4.31 ± 2.41 (3/3)	4.03 ± 1.65 (2/3)	5.76 ± 0.62 (2/3)	0.4029	0.9848
Spleen	4.02 ± 1.50 (3/3)	6.39 ± 0.54 (3/3)	5.24 ± 2.06 (3/3)	6.75 ± 0.38 (3/3)	4.31 ± 3.46 (3/3)	3.89 ± 2.99 (3/3)	0.3954	0.2269
Muscle	1.76 ± 0.80 (2/3)	5.87 ± 0.58 (3/3)	3.42 ± 2.97 (2/3)	4.47 ± 0.54 (3/3)	2.87 ± 1.41 (2/3)	4.09 ± 0.32 (2/3)	0.0382*	0.0314*
Liver	2.75 ± 1.05 (3/3)	6.19 ± 0.28 (3/3)	5.63 ± 2.03 (2/3)	5.48 ± 0.54 (3/3)	5.51 ± 0.62 (2/3)	4.32 ± 1.24 (2/3)	0.1109	0.0724
Trachea	2.28 ± 0.82 (3/3)	6.75 ± 0.38 (3/3)	3.79 ± 2.86 (3/3)	5.68 ± 0.54 (3/3)	4.46 ± 0.11 (2/3)	5.35 ± 0.04 (2/3)	0.0480*	0.0165*
Lung	3.24 ± 1.79 (3/3)	7.43 ± 0.51 (3/3)	4.31 ± 3.01 (3/3)	6.09 ± 1.71 (3/3)	5.39 ± 0.67 (2/3)	4.20 ± 3.18 (3/3)	0.2629	0.1573
Kidney	2.53 ± 1.40 (3/3)	7.24 ± 0.14 (3/3)	3.56 ± 2.54 (3/3)	6.16 ± 0.40 (3/3)	3.37 ± 1.18 (2/3)	3.75 ± 2.80 (3/3)	0.0117*	0.0972
Heart	2.16 ± 1.15 (3/3)	6.05 ± 0.50 (3/3)	4.13 ± 1.50 (2/3)	6.58 ± 1.23 (3/3)	4.01 ± 0.44 (2/3)	7.11 ± 0.13 (2/3)	0.5214	0.0678
Brain	1.67 ± 0.49 (2/3)	5.74 ± 0.22 (3/3)	2.42 ± 2.42 (3/3)	5.25 ± 0.86 (3/3)	2.93 ± 0.86 (2/3)	4.26 ± 0.09 (2/3)	0.3954	0.0034*
Duodenum	1.93 ± 2.27 (2/3)	6.66 ± 0.66 (3/3)	4.37 ± 2.59 (2/3)	5.76 ± 0.65 (3/3)	3.80 ± 0.38 (2/3)	4.36 ± 1.90 (2/3)	0.1686	0.1306
Rectum	2.09 ± 1.53 (3/3)	7.14 ± 0.16 (3/3)	3.022.97 (3/3)	5.57 ± 1.08 (3/3)	3.87 ± 0.94 (2/3)	4.70 ± 0.88 (2/3)	0.0685	0.0148*
Blood	1.66 ± 0.56 (3/3)	-§ (0/3)	4.70 ± 3.06 (2/3)	5.04 ± 0.95 (3/3)	2.62 ± 0.12 (2/3)	3.82 ± 0.88 (2/3)		
Trachea swab	<‡ (0/3)	3.82 ± 1.11 (3/3)	2.07 (1/3)	< (0/3)	< (0/3)	2.53 ± 0.94 (2/3)		0.2767
Cloaca swab	0.32 (1/3)	2.45 ± 0.35 (2/3)	3.07 (1/3)	0.70 ± 0.48 (3/3)	< (0/3)	2.51 ± 1.15 (2/3)	0.0224*	0.9480
Conjunctiva swab	0.32 (1/3)	0.32 (1/3)	2.07 (1/3)	1.32 (1/3)	< (0/3)	1.24 ± 1.31 (2/3)		

†, Titers are shown as means ± standard error (range). Numbers in parentheses: number of chickens whose samples were positive/total number of chickens.
‡, <, no virus was detected from specimens collected during the observation period (detection limit <0.32 log_{10} EID_{50}/mL).
§, -, Blood samples were not collected because chickens died.
*, $P < 0.05$.

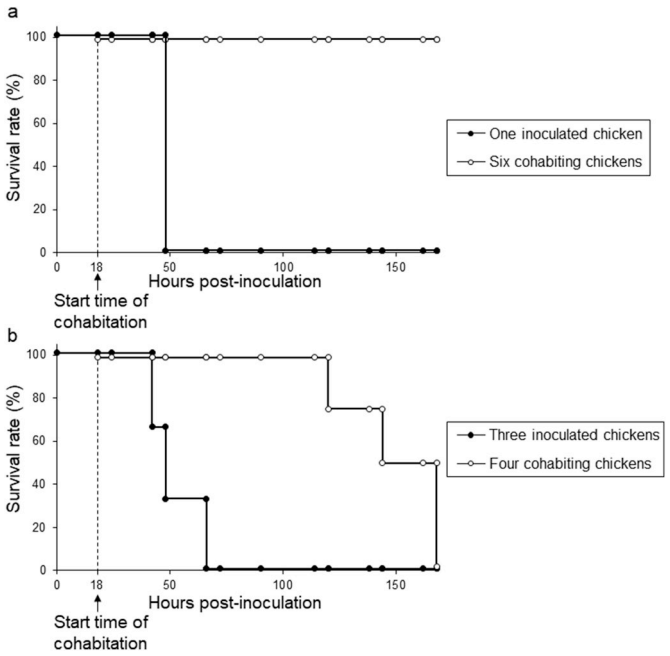

Fig. 7. Survival rates of chickens inoculated intranasally with 10^6 EID_{50} of Kagawa strain and of cohabiting chickens. (a) One chicken was inoculated, and 6 chickens were cohoused. (b) Three chickens were inoculated and 4 chickens were cohoused. Survival rates of inoculated chicken(s) are indicated by black circles; survival rates for cohoused birds are shown as white circles.

chickens were infected through viral transmission from the 3 inoculated chickens.

4. Discussion

Here, we showed that the H5N6 HPAIVs isolated in Japan during Winter 2017–2018 were generated through several reassortments between 2.3.4.4.B H5N8 HPAIVs that circulated in Europe during Winter 2016–2017 and HxN6 AIVs in Europe, similar to the scenario regarding the H5N6 HPAIVs in Korea during the same season (Kim et al., 2018; Lee et al., 2018a). In addition, at least 3 distinct reassortments between H5N8 HPAIVs and HxN6 AIVs reportedly resulted in the appearance of the European H5N6 HPAIVs and 2 types of Korean H5N6 HPAIVs (Lee et al., 2018b); our time-measured phylogenetic analysis suggests that these reassortments might have occurred as depicted in Fig. 8. The PB1, HA, MP, and NS genes of the H5N6 HPAIVs in Asia and Europe during Winter 2017–2018 were derived from the H5N8 HPAIVs in China and Russia during May 2016. The Tokyo and Hyogo strains acquired the PB2, PA, NP, and N6 genes during Summer 2016. In contrast, the Kagawa strain acquired the PB2, PA, and NP genes during Summer 2016 and then N6 during Summer 2017. The identities of the PB2, PA, and NP genes between the Kagawa strain and the Japanese wild bird isolates (98.9%–99.0%, 98.5%–98.7%, and 98.8%–99.0%, respectively) are higher than those of PB1, MP, and NS (98.2%–98.3%, 97.9%–98.1%, and 97.6%–98.0%, respectively), supporting the notion that the origins of PB2, PA, and NP genes were distinct from PB1, MP, and NS genes. European H5N6 HPAIVs obtained their PA and NP genes during Summer 2016 and their PB2 and N6 genes during Summer 2017. However, note that the genetic flow of H5N8 and H5N6 HPAIVs depicted in Fig. 8 is simulated according to the time-measured

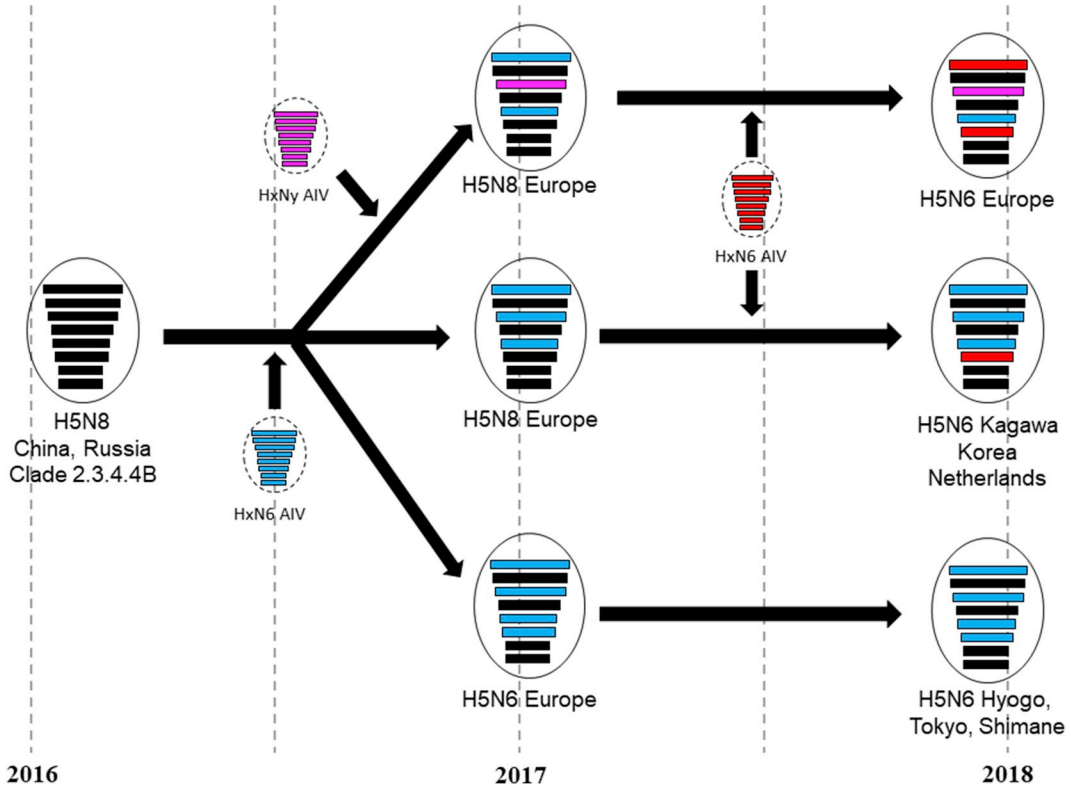

Fig. 8. Evolutionary timeline of generation of the Japanese H5N6 HPAIVs through reassortment between H5N8 HPAIVs and HxN6 AIVs. Black bars represent the lineage of the 2.3.4.4.
B H5N8 HPAIVs in China and Russia in May 2016; the other colored bars represent lineage(s) of AIVs.

phylogenetic tree for each gene. In Fig. 8, AIVs in which all 8 segments are red, blue, or purple have not actually been detected, indicating that even more complicated reassortment events might have occurred.

Phylogenetic analyses revealed that the relationship between Kagawa strain in January 2018 and Korean strains isolated from wild ducks as well as poultry in December 2017, suggesting that the H5N6 HPAIVs were introduced to Japan via migration of wild birds. Considering that the H5N6 HPAIVs were disseminated simultaneously to Europe and Asia by wild migratory birds, they might have originated at a breeding site, possibly in Siberia where migratory birds belonging to several flyways stretching across the Eurasian continent coexist during Summer (Alerstam et al., 2007; Boere and Stroud, 2006); AIVs of various subtypes have been isolated in this area (Sivay et al., 2012; Sharshov et al., 2014). An AIV that carried the N6 NA gene related to the H5N6 HPAIVs during Winter of 2017–2018 was isolated during September 2017 in the Novosibirsk region (A/teal/Ubinskoe_Lake/51/2017; Fig. 2b), which is located in southeastern Siberia, supporting the hypothesis just presented. In addition, the internal genes of H5N6 HPAIVs in Europe and Asia during Winter 2017–2018 originated from AIVs through reassortments that were estimated to have occurred during the Summers of 2014–2017, highlighting the importance of Siberia as the accumulation place of various AIVs. However, few data were available regarding the relationship between the movements of migratory birds and the spread of H5N6 HPAIVs during Winter of 2017–2018 across the Eurasian continent. Therefore, further analysis of genome sequences of AIVs and migration of wild birds in Siberia is needed to elucidate how AIVs/HPAIVs move cross-continentally.

The virulence of clade 2.3.4.4 H5 reassortants reportedly is lower than that of ancestral H5N1 HPAIVs (Lee et al., 2016, 2017; Sun et al., 2016). In addition, our previous experimental infections of several H5 HPAIVs revealed that the lethal infectious dose of the Kagawa, Tokyo, and Hyogo strains in chickens is relatively low because 50% chicken lethal doses of them ($10^{4.38}$ to $10^{4.83}$) were 10-fold more or higher than those of H5N1 HPAIVs during Winter 2010–2011 (Uchida et al., 2012), some H5N8 HPAIVs of Winter 2014–2015 (Kanehira et al., 2015; Tanikawa et al., 2016), and the H5N6 HPAIVs of Winter 2016–2017 (Takemae et al., 2017) under same experimental condition. 10^6 EID_{50} of Kagawa strain killed chickens relatively faster (5 chickens, 2 dpi; 1 chicken, 3 dpi) than Tokyo strain (3 chickens, 2 dpi; 2 chickens, 3 dpi) (Fig. 5), corresponding to higher viral titers in some tissues collected from chickens inoculated with 10^6 EID_{50} of Kagawa strain than Tokyo strain. However, Differences in viral titers in tissues did not explain the lower mortality in 10^4 EID_{50} and 10^2 EID_{50} with Kagawa strain than Tokyo strain, suggesting that capacity to propagate in tissues does not always corelate with the infectivity among the H5N6 HPAIVs. Our current transmission study with a single inoculated chicken revealed that the Kagawa strain was less transmissible than the H5N1 HPAIVs of Winter 2010–2011; in fact, regardless of whether the origins of the H5N1 HPAIVs during Winter 2010–2011 were poultry or wild birds, a single chicken inoculated with 10^6 EID_{50} shed enough virus for transmission to cohoused four chickens in the same experimental setting (Uchida et al., 2012). Furthermore the viral titers of the tracheal and cloacal swabs collected from Kagawa-inoculated chicken (6.1 and 5.2 $\log_{10}$ EID_{50}/mL, respectively) were equal to, or higher than, those of 3 H5N1 HPAIVs (3.3–6.5 and 2.5 to 5.0 $\log_{10}$ EID_{50}/mL, respectively) despite failure of transmission, suggesting that the differences in transmissibility were due to interstrain differences in the 50% chicken lethal doses. Similarly, low pathogenicity and transmissibility were observed for several 2.3.4.4 H5 HPAIVs isolated from wild birds (Bertran et al., 2016; DeJesus et al., 2016; Lee et al., 2016), suggesting that the Kagawa strain was not yet well adapted to chickens. Therefore, these results may explain—at least in part—why the outbreaks in Japan during Winter 2017–2018 were sporadic and constrained.

In conclusion, the results of our current study show that the H5N6 HPAIVs of poultry and wild birds in Japan during Winter 2017–2018 were generated through several distinct reassortments between 2.3.4.4.B H5N8 HPAIVs and HxN6 AIVs that might have occurred during the Summers of 2016 and 2017 in Siberia, where migratory birds wintering in Asia or Europe visit for breeding during summer. The finding that H5N6 HPAIVs with different gene cassettes intruded into Japan highlights the importance of understanding the behavior of AIVs at the breeding site in Siberia, where reassortments might occur, and of elucidating how HPAIVs are disseminated across Eurasian continents. Compared with the H5 HPAIVs that have caused past outbreaks in Japan, the Japanese H5N6 HPAIVs of Winter 2017–2018 were lower in pathogenicity, and the Kagawa strain showed lower transmissibility in chickens, resulting in the limited outbreaks.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.04.011.

Conflicts of interest

The authors declare no conflicts of interests.

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