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# Characterizing the temporal patterns of avian influenza virus introduction into Japan by migratory birds

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**ABSTRACT.** The objectives of the present study were to observe the temporal pattern of avian influenza virus (AIV) introduction into Japan and to determine which migratory birds play an important role in introducing AIV. In total, 19,407 fecal samples from migratory birds were collected at 52 sites between October 2008 and May 2015. Total nucleic acids extracted from the fecal samples were subjected to reverse transcription loop–mediated isothermal amplification to detect viral RNA. Species identification of host migratory birds was conducted by DNA barcoding for positive fecal samples. The total number of positive samples was 352 (prevalence, 1.8%). The highest prevalence was observed in autumn migration, and a decrease in prevalence was observed. During autumn migration, central to southern Japan showed a prevalence higher than the overall prevalence. Thus, the main AIV entry routes may involve crossing the Sea of Japan and entry through the Korean Peninsula. Species identification was successful in 221 of the 352 positive samples. Two major species sequences were identified: the Mallard/Eastern Spot-billed duck group (115 samples; 52.0%) and the Northern pintail (61 samples; 27.6%). To gain a better understanding of the ecology of AIV in Japan and the introduction pattern of highly pathogenic avian influenza viruses, information regarding AIV prevalence by species, the prevalence of hatch-year migratory birds, migration patterns and viral subtypes in fecal samples using egg inoculation and molecular-based methods in combination is required.

**KEY WORDS:** avian influenza, DNA barcoding, Eastern spot-billed duck, Mallard, Northern pintail

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Influenza A viruses are negative-sense RNA viruses classified into the family *Orthomyxoviridae*. These viruses contain eight RNA segments, which encode 11 proteins, namely, PB1, PB1-F2, PB2, PA, hemagglutinin (HA), NP, neuraminidase (NA), M1, M2, NS1 and NS2 [2, 35]. Of these, the surface glycoproteins, HA and NA, have 16 and 9 types, respectively [3, 23, 35]. Influenza A viruses are categorized into subtypes based on their HA and NA combination (for example, H10N7, H3N2 and H4N6). The hosts of these viruses include humans, horses, swine, cats, dogs, marine mammals, poultry and wild birds [35, 38]. In addition, H17N10 has been isolated from Little yellow-shouldered bats (*Sturnira lilium*) in southern Guatemala, and H18N11 has been isolated from the Flat-faced fruit bat (*Artibeus planirostris*) in northern Peru [31, 32]. Influenza A virus infections have been reported in over 100 wild bird species belonging to 13 avian orders [20, 28]. Of these, Anseriformes (dabbling ducks, diving ducks, geese and swans) and Charadriiformes (gulls, terns and shorebirds) constitute the most important reservoirs; however, these species do not exhibit any clinical signs of influenza A virus infection. Viral replication occurs in the intestine, and the viruses are then shed in the

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feces. The viruses are maintained by fecal/oral transmission, especially in breeding grounds, such as Alaska, Canada and Siberia [6, 7, 19, 35, 38]. Influenza A virus of avian origin is usually referred to as avian influenza virus (AIV).

AIVs can also be categorized based on their pathogenicity in chickens. The World Organization for Animal Health (OIE) has adopted the following criteria for establishing high pathogenicity: any influenza A virus that is lethal in six to eight of eight 4-to-8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 m*l* of a 1/10 dilution of bacteria-free, infective allantoic fluid [18] is deemed a highly pathogenic avian influenza virus (HPAIV). The primary subtypes of HPAIV are H5 and H7 [38]. Although virus pathogenicity is determined by its pathogenicity to chickens, HPAIVs show high pathogenicity for wild birds. For example, a mass mortality event caused by HPAIV subtype H5N1 occurred at Qinghai Lake in Qinghai province, China, in 2005. Over a thousand wild birds, including Bar-headed geese (*Anser indicus*), Great cormorants (*Phalacrocorax carbo*), Great black-headed gulls (*Larus ichthyaetus*) and Brown-headed gulls (*Larus brunnicephalus*), were reported dead [9]. In Japan, HPAIV has been isolated from wild birds, such as the Large-billed crow (*Corvus macrorhynchos*; nine individuals, H5N1), in 2004 [29]; Mountain hawk-eagle (*Nisaetus nipalensis*; one individual, H5N1), in 2007 [27]; and Whooper swan (*Cygnus cygnus*; five individuals, H5N1), in 2008 [33]. HPAIV subtype H5N1 was isolated from 63 wild birds during the 2010–2011 winter season [25], and subtype H5N8 was isolated from eight wild birds in 2014–2015 [21]. These HPAIV-infected wild birds included species listed in the Red Data Book of Japan including the Mountain hawk-eagle (*Nisaetus nipalensis*; Endangered [EN]), Peregrine falcon (*Falco peregrine*; Vulnerable [VU]), White-naped crane (*Grus vipio*; VU) and Hooded crane (*Grus monacha*; VU) [13]. HPAIVs may be increasing the extinction risk of endangered Japanese species. Therefore, HPAIV is a threat not only to poultry farming but also to biodiversity in Japan.

Nationwide surveillance of AIV in migratory birds using fecal samples has been conducted in Japan since 2008 as a precautionary measure against HPAIV introduction into Japan. The main target species were dabbling ducks, because experimental HPAIV infection data using dabbling duck species have demonstrated that although infection does not result in clinical signs in these species, HPAIV is shed. For example, three Mallards and three Northern pintails were intranasally infected with A/Whooper Swan/Mongolia/244/05 (H5N1); whereas none of the birds showed clinical signs or mortality, viruses were isolated from oral and cloacal swabs [1].

The results of the nationwide surveillance of AIV in migratory birds using fecal samples were utilized for constructing a potential AIV risk map [17]. The potential risk map indicated high-risk areas for isolation of AIV from wild birds. The risk map showed that the most effective predictor of high-risk areas was the presence of populations of dabbling ducks. In addition, the potential risk map can be used as an HPAIV precautionary measure, because the locations of HPAIV-positive cases in wild birds and poultry coincided with the predicted high-risk areas of the potential risk map [17]. Thus, even AIV positivity data from fecal samples can be used to indicate high-risk areas for HPAIV occurrence in wild birds and poultry. However, the potential risk map could not identify the risk period for virus introduction in each high-risk area or which dabbling duck species serves as the main viral reservoir. If the host dabbling duck species for AIV-positive fecal samples were identified, it would be possible to update the potential risk because nationwide annual census data for dabbling ducks are available [11], and the census data can be used for obtaining detailed migration pattern of the identified host dabbling duck species.

Thus, the objective of the present study was to determine the temporal pattern of AIV introduction by migratory birds and which migratory birds play an important role in introducing the virus into Japan, with the purpose of gaining a better understanding of the ecology of AIV in Japan. This information may contribute to the present understanding of the introduction patterns of HPAIV into Japan.

## MATERIALS AND METHODS

### *Fecal sample collection*

Migratory bird fecal samples, mainly from dabbling duck species, were collected at 52 sites (Fig. 1) determined by the Ministry of Environment, Japan [15]. The 52 sites were divided into two groups: sampling group A (27 sites) and sampling group B (25 sites). Fecal sampling was conducted once every two months during the migration season (October to May) in 2008–2015 following a sampling schedule (Table 1) to obtain monthly nationwide fecal samples. No fecal samples were collected from June to September. Up to five fecal material samples were pooled in a 15 m*l* tube, which was then counted as one fecal sample.

### *Total nucleic acid extraction*

Feces were diluted with an equal amount of phosphate-buffered saline (PBS) to prepare a ~50% fecal suspension. Total nucleic acids (including host genomic DNA and viral RNA) were extracted from the fecal suspension, using the Ambion Mag MAX-96 AI/ND Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA, U.S.A.) or the EZ1 Virus Mini Kit v2.0 (Qiagen, Hilden, Germany). For the Mag MAX-96 AI/ND Viral RNA Isolation Kit, following overnight stationary incubation to obtain a supernatant, 50 $\mu l$ of fecal suspension supernatant was used to extract total nucleic acids according to the manufacturer's instructions. For the EZ1 Virus Mini Kit v2.0, 250 $\mu l$ of the fecal suspension was mixed with 750 $\mu l$ of QIAzol lysis reagent (Qiagen). The solution was then mixed with 200 $\mu l$ of chloroform by vortexing. Subsequent to centrifugation at 12,000 $\times g$, 15 min, 4°C, 400 $\mu l$ of the supernatant was used to extract total nucleic acids according to the manufacturer's instructions. DNA concentration was measured using a Qubit 3.0 Fluorometer (Life Technologies) and the Qubit dsDNA HS Assay Kit (Life Technologies) to confirm that the two types of total nucleic acid solutions were used as the DNA template for identification of host avian species. Concentrations of 0.5 $ng/\mu l$ and 1.1 $ng/\mu l$ were obtained using the Mag MAX-96 AI/ND Viral RNA Isolation Kit and EZ1 Virus Mini Kit v2.0 solutions, respectively.

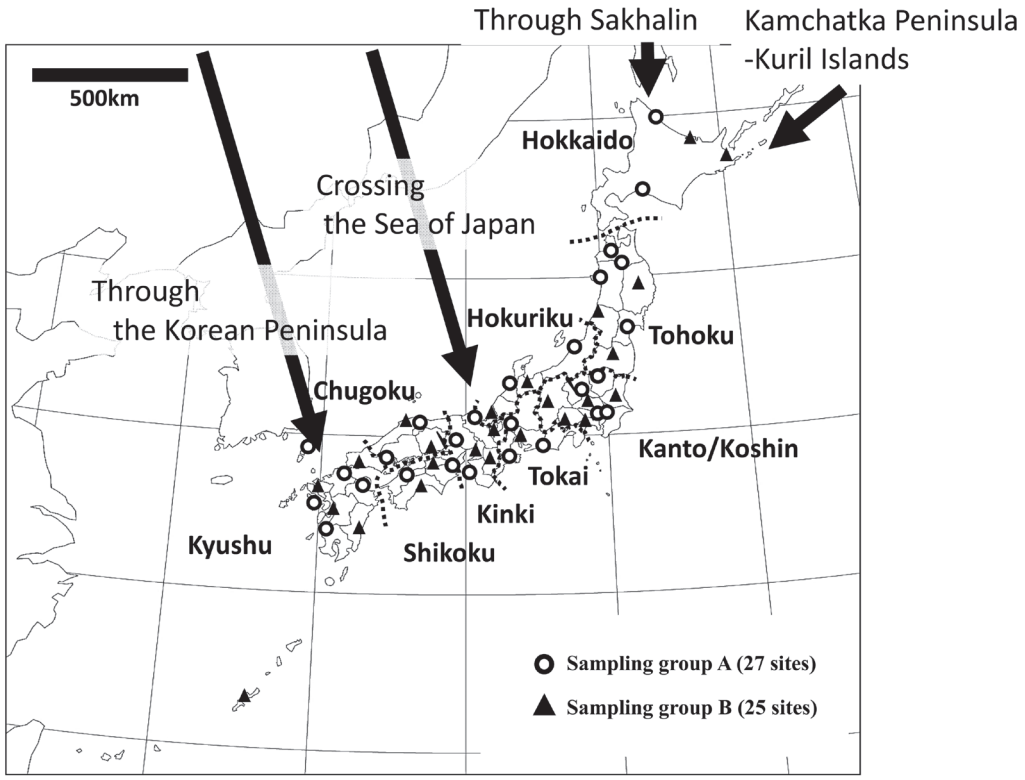


**Fig. 1.** Location of 52 fecal sampling sites. The 52 sites were divided into two groups: sampling group A (27 sites) and sampling group B (25 sites). Dotted lines indicate the border of the nine geographic areas, and the direction of the arrow indicates the four reported main migratory routes into Japan.

**Table 1.** Sampling schedule of nationwide surveillance of avian influenza viruses in migratory birds using fecal samples from 52 sampling sites

| Oct. 2008–May 2011 | Oct. | Nov. | Dec. | Jan. | Feb. | Mar. | Apr. | May | Jun.–Sep. |
|---|---|---|---|---|---|---|---|---|---|
| | autumn migration | | wintering | | | spring migration | | | |
| Sampling group A (27 sites) | ○ | | ○ | | ○ | | ○ | | No sampling |
| Sampling group B (25 sites) | | ○ | | ○ | | ○ | | ○ | |

| Oct. 2011–May 2015 | Oct. | Nov. | Dec. | Jan. | Feb. | Mar. | Apr. | May | Jun.–Sep. |
|---|---|---|---|---|---|---|---|---|---|
| | autumn migration | | wintering | | | spring migration | | | |
| Sampling group A (27 sites) | ○ | | ○ | | ○ | | ○ | ○[a)] | No sampling |
| Sampling group B (25 sites) | ○ | ○ | | ○ | | ○ | | ○[a)] | |

a) Fecal samples were collected only in Hokkaido.

### *AIV gene detection by RT-LAMP*

Total nucleic acid extracts were subjected to reverse transcription loop–mediated isothermal amplification (RT-LAMP) (Eiken Chemical Co., Ltd., Tokyo, Japan) to detect viral RNA. RT-LAMP has been previously applied to detect AIV in the fecal material of migratory birds [26, 39]; the reported detection limit of RT-LAMP for fecal material is $10^{2.5}$ copies [39]. For samples from 2008 and 2009, 5 $\mu l$ of extracted total nucleic acids, the Loopamp RNA Amplification Kit (Eiken Chemical Co., Ltd.) and the primer set provided by Eiken Chemical Co., Ltd. were used for the RT-LAMP reaction following the manufacturer's instructions. For samples from 2010 to 2015, 5 $\mu l$ of extracted total nucleic acid and the Loopamp AIV detection kit (Eiken Chemical Co., Ltd.) were used. A LA-320C Loopamp Real-time turbidimeter (Eiken Chemical Co., Ltd.) was used for the RT-LAMP reaction. The threshold value for viral RNA detection was set at 0.05. Virus isolation from RT-LAMP positive samples was conducted at reference laboratories designated by the Ministry of Environment.

### *Comparison of AIV prevalence by annual migratory season*

AIV prevalence was defined as the ratio of RT-LAMP-positive samples to the total fecal samples, expressed as a percentage.

The prevalence was calculated for each of the seven annual migratory seasons (October 2008–May 2009, October 2009–May 2010, October 2010–May 2011, October 2011–May 2012, October 2012–May 2013, October 2013–May 2014 and October 2014–May 2015). The annual migratory season (October to May) was divided into three terms: October–November, December–February and March–May, in accordance with migration patterns in Japan. October–November is the period of autumn migration, December–February is the period of wintering, and March–May is the period of spring migration. The prevalence was calculated for each of the three terms. Autumn migration prevalence was calculated using the data from 2008–2014. Wintering and spring migration prevalence was calculated using the data from 2008–2015. Chi-squared analyses with pairwise comparisons with Bonferroni corrections were performed to evaluate differences in RT-LAMP positive proportion according to annual migratory seasons and terms (significance was set at $P<0.05$). R version 3.3.2 was used for analysis [22].

### *Comparison of AIV prevalence by geographic area*

Fifty two sampling sites were divided into nine geographic areas using criteria adopted by the Japan Meteorological Agency with minor modifications to detect differences in the temporal change of AIV prevalence by geographic area. The nine geographic areas were as follows: Hokkaido, Tohoku, Kanto/Koshin, Hokuriku, Tokai, Kinki, Chugoku, Shikoku and Kyushu (Fig. 1). The Kyushu area of the present study was the combined area of Kyushu (North), Kyushu (South) and Okinawa used by the Japan Meteorological Agency. AIV prevalence was defined as mentioned above. The prevalence in each geographic area was calculated for each of the three terms (autumn migration, wintering and spring migration).

### *DNA barcoding for host-species identification*

Identification of bird species with virus-positive feces was conducted using DNA barcodes based on the mitochondrial DNA (mtDNA) COI gene sequence [4]. Nested PCR was performed to increase sensitivity using two primer sets: BirdF1 5′-TTCTCCAACCACAAAGACATTGGCAC-3′ and BirdR1 5′-ACGTGGGAGATAATTCCAAATCCTG-3′ were used for the first round of PCR [4], and Bird (HRM)-F 5′-CACGAATAAACAACATAAGCTTCTG-3′ and Bird (HRM)-R2 5′-GAATGTGGTGTTTAGGTTTCGGTC-3′ were used for the second round of PCR. Bird (HRM)-F and Bird (HRM)-R2 were designed based on the sequences of Mallard (*Anas platyrhynchos*), Eastern spot-billed duck (*Anas zonorhyncha*), Teal (*Anas crecca*), Northern pintail (*Anas acuta*), Eurasian wigeon (*Anas Penelope*), Gadwall (*Anas strepera*), Tufted duck (*Aythya fuligula*) and Common pochard (*Aythya ferina*), species commonly observed at the sampling sites. The nested PCR resulted in a product of approximately 400 bp. For the first round of PCR, 50 $\mu l$ of PCR reaction mixture was prepared using the AccuPrime Taq DNA Polymerase System (Invitrogen, Waltham, MA, U.S.A.) containing: 5 $\mu l$ of 10× AccuPrime PCR Buffer 2 (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 15 mM $MgCl_2$, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime protein and 10% glycerol), 1 $\mu l$ of BirdF1 primer (10 $\mu$mol), 1 $\mu l$ of BirdR1 primer (10 $\mu$mol), 0.5 $\mu l$ of AccuPrime Taq DNA Polymerase and 1 $\mu l$ of the extracted total nucleic acid solution. PCR amplification was conducted using the following conditions with the Gene Amp PCR System 9700 (Applied Biosystems, Waltham, MA, U.S.A.): 94°C for 2 min; 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 68°C for 1 min; and a hold at 4°C. The same protocol was used for the second round of PCR with 1 $\mu l$ of the first round PCR reaction mixture as the PCR template. Following the second round of PCR, the PCR product size was verified by electrophoresis on 2% agarose gel stained with Midori Green (Nippon Genetics, Tokyo, Japan). The resulting PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the 3130 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. The sequences were analyzed by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for species identification. However, it should be noted that the Mallard and Eastern Spot-billed duck cannot be differentiated based on mtDNA COI gene sequence because the two species share the same sequence [8, 24]. Hence, if the BLAST search results indicated that the most similar sequence was derived from Mallard or Eastern Spot-billed duck, the result was categorized as "Mallard/Eastern Spot-billed duck group".

## RESULTS

### *Fecal sampling and AIV prevalence*

A total of 1,223 fecal sampling events were conducted throughout the present study period; 19,407 fecal samples were collected, and the total number of RT-LAMP positive samples was 352 (the prevalence of the research period was 1.8% [352/19,407]). The overall results by each annual migratory season are shown in Table 2. The AIV prevalence range was 1.4–2.2%, and there were no significant differences in AIV prevalence by annual migratory season ($P=0.4108$). Virus isolation from RT-LAMP positive samples by egg inoculation was conducted in reference laboratories. Virus isolation was successful in 153 positive samples (43.4%, 153/352), and no HPAIVs were isolated [12, 14, 16]. In addition, cDNA was synthesized from the total nucleic acid extracts of the RT-LAMP positive samples. The isolated AIV and cDNA were cryopreserved for further research.

Figure 2 shows the overall temporal change in AIV prevalence by term from October 2008 to May 2015. The highest prevalence was observed in October–November (autumn migration, 2.8–4.3%) every annual migratory season, followed by a sharp decrease. A similar temporal change pattern was repeated throughout the study period (from October 2008 to May 2015).

The overall results for each term are shown in Table 3. AIV prevalence was 3.5% (204/5,816), 1.3% (121/9,066) and 0.6% (27/4,525) during autumn migration, wintering and spring migration, respectively (Table 3). Significant differences in AIV prevalence were observed between autumn migration and wintering, autumn migration and spring migration, and wintering and spring migration ($P<0.01$).

**Table 2.** Prevalence of avian influenza virus and avian species identification based on DNA barcoding by annual migratory season

| | Oct. 2008 –May 2009 | Oct. 2009 –May 2010 | Oct. 2010 –May 2011 | Oct. 2011 –May 2012 | Oct. 2012 –May 2013 | Oct. 2013 –May 2014 | Oct. 2014 –May 2015 | Total |
|---|---|---|---|---|---|---|---|---|
| No. sampling events[a)] | 182 | 171 | 166 | 175 | 179 | 174 | 176 | **1,223** |
| No. fecal samples | 3,149 | 2,917 | 2,806 | 2,717 | 2,728 | 2,470 | 2,620 | **19,407** |
| No. RT-LAMP positive samples | 69 | 42 | 47 | 44 | 50 | 46 | 54 | **352** |
| Prevalence (%)[b)] | 2.2 | 1.4 | 1.7 | 1.6 | 1.8 | 1.9 | 2.1 | **1.8** |
| No. successful virus isolation events[c)] | 19 | 14 | 12 | 27 | 27 | 27 | 27 | **153** |
| No. successful DNA barcoding results | 26 | 17 | 27 | 35 | 28 | 38 | 50 | **221** |
| Species | | | | | | | | |
| Mallard/Eurasian Spot-billed duck group | 5 | 7 | 16 | 18 | 19 | 25 | 25 | 115 (52.0%[d)]) |
| Northern pintail | 12 | 7 | 4 | 11 | 4 | 8 | 15 | 61 (27.6%[d)]) |
| Teal | 6 | 1 | 3 | 4 | 2 | 3 | 7 | 26 (11.8%[d)]) |
| Eurasian wigeon | 3 | 1 | 4 | 2 | 3 | 1 | 1 | 15 (6.8%[d)]) |
| Others | 0 | 1 (Carrion crow) | 0 | 0 | 0 | 1 (Jungle crow) | 2 (Commons shoveler, Common pochard) | 4 (1.8%[d)]) |

a) Total number of fecal sampling events conducted in the 52 sampling sites. b) (No. RT-LAMP positive/ No. fecal samples) ×100. c) Press release from the Ministry of Environment. d) (No. identified species/ No. successful DNA barcoding results) ×100.

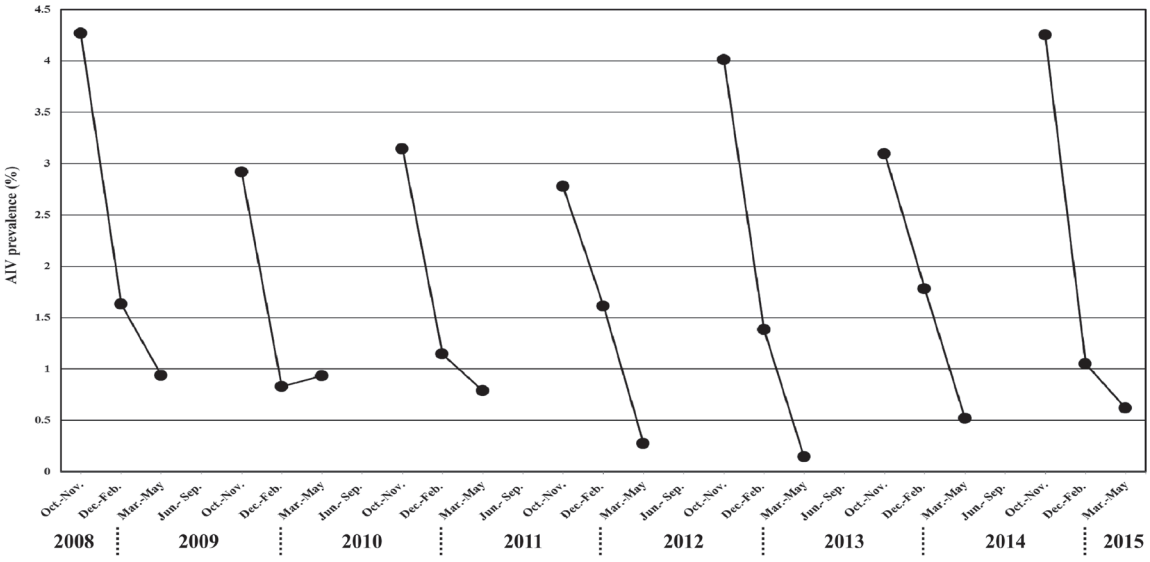


**Fig. 2.** Temporal change in the avian influenza virus (AIV) prevalence of migratory bird fecal samples from October 2008 to May 2015.

The overall results for each geographic area are shown in Table 3. During autumn migration, the Tokai, Hokuriku, Chugoku and Kyushu areas had a higher prevalence than the overall prevalence in autumn migration (3.5%). During wintering, the Hokuriku, Kanto/Koshin, Hokkaido and Kyushu areas had a prevalence equal to or higher than the overall prevalence in wintering (1.3%). During spring migration, the Tokai, Hokuriku, Tohoku and Kyushu areas had a prevalence higher than the overall prevalence in spring migration (0.6%).

### *Species identification by DNA barcoding*

DNA barcoding was applied to the RT-LAMP positive samples. Species identification was successful in 221 samples, but failed in 131 samples because of the lack of available sample for species identification, no PCR amplification or unclear sequence data. The breakdown of the identified avian species is as follows: Mallard/Eastern Spot-billed duck group, 115 samples (52.0%); Northern pintail, 61 samples (27.6%); Teal, 26 samples (11.8%); Eurasian wigeon, 15 samples (6.8%), and other species, 4 samples (1.8%, Commons shoveler (*Anas clypeata*) one sample; Common pochard (*Aythya ferina*), one sample; Large-billed crow (*Corvus macrorhynchos*), one sample; and Carrion crow (*C. corone*), one sample) (Table 2).

**Table 3.** Prevalence of avian influenza virus by terms

| Term | Autumn migration[a)] (Oct.–Nov.) | Wintering[b)] (Dec.–Feb.) | Spring migration[b)] (Mar.–May.) | Overall |
|---|---|---|---|---|
| Prevalence of each term (%) (No. RT-LAMP positive/No. fecal samples) | 3.5 (204/5,816) | 1.3 (121/9,066) | 0.6 (27/4,525) | 1.8 (352/19,407) |
| Prevalence of each area (%) (No. RT-LAMP positive/No. fecal samples) | | | | |
| Hokkaido | 2.5 (15/592) | **1.3 (8/594)** | 0.3 (2/636) | 1.4 (25/1,822) |
| Tohoku | 2.8 (16/564) | 1.1 (9/821) | **1.0 (3/301)** | 1.7 (28/1,686) |
| Kanto/Koshin | 1.7 (14/845) | **1.9 (27/1,432)** | 0.3 (2/578) | 1.5 (43/2,855) |
| Hokuriku | **6.3 (29/460)** | **2.2 (17/758)** | **1.2 (4/324)** | **3.2 (50/1,542)** |
| Tokai | **6.8 (33/482)** | 1.0 (7/699) | **1.5 (5/333)** | **3.0 (45/1,514)** |
| Kinki | 1.5 (11/710) | 1.0 (13/1,248) | 0.2 (1/494) | 1.0 (25/2,452) |
| Chugoku | **4.2 (26/619)** | 0.8 (6/784) | 0.4 (2/474) | **1.8 (34/1,877)** |
| Shikoku | 3.4 (16/477) | 1.0 (8/791) | 0.2 (1/489) | 1.4 (25/1,757) |
| Kyushu | **4.1 (44/1,067)** | **1.3 (26/1,939)** | **0.8 (7/896)** | **2.0 (77/3,902)** |

a) The data of 2008, 2009, 2010, 2011, 2012, 2013 and 2014 were convined. b) The data of 2008, 2009, 2010, 2011, 2012, 2013, 2014 and 2015 were convined. Bold and underline: The prevalence equal to or higher than overall prevalence of each term.

## DISCUSSION

The present study illustrates the nationwide prevalence of AIV in migratory birds during wintering in Japan. The results of the present study show 1.4–2.2% AIV prevalence depending on annual migratory season and that the overall prevalence during the research period (from 2008 to 2015) was 1.8%. AIV prevalence was highest in October–December (the period of autumn migration) and then decreased significantly. The same pattern of temporal change in AIV prevalence was observed every year, although HPAIVs were isolated from wild birds in the annual migratory season of October 2010–May 2011 and October 2014–May 2015 [21, 25]. A similar phenomenon was observed in the Pacific Flyway, the migration route from breeding grounds in Alaska to wintering grounds in California and Mexico [5]. This phenomenon in the Pacific Flyway can be explained by immune system development and limited transmission in the wintering ground [5, 20, 35]; the underdeveloped immune system of hatch-year birds is not able to limit AIV infection. Thus, hatch-year birds are infected with the virus at the breeding grounds and then carry the virus to the wintering grounds. The bird immune system then develops and becomes resistant to infection during wintering [5]. The sharp decrease in AIV prevalence in Japan may be caused by a similar reason. To confirm this, it will be necessary to conduct live-bird trapping or sampling of hunted birds to evaluate AIV prevalence in hatch-year birds. Age estimation can be conducted during live bird trapping or sampling of hunted birds.

### *There are four main migratory routes into Japan*

Through the Kamchatka Peninsula-Kuril Islands, through Sakhalin, crossing the Sea of Japan and through the Korean Peninsula (Fig. 1) [37]. However, the AIV introduction route and whether a single route or multiple routes are used are unknown. The results of the present study suggest that AIV is introduced into Japan through all four routes because the highest prevalence was observed in autumn migration in most of the geographic areas and then the prevalence decreased. During autumn migration, the geographic areas showing a prevalence equal to or higher than 3.5% (the overall prevalence of autumn migration) were located in the central to southern parts of Japan, i.e., Hokuriku (6.3%), Tokai (6.8%), Chugoku (4.2%) and Kyushu (4.1%). The reasons for this finding are unclear. One possible reason is that dabbling ducks, which have been reported as the most effective predictor for areas at high risk for AIV, could mainly migrate into Japan by crossing the Sea of Japan and through the Korean Peninsula. The geographic areas showing a prevalence equal to or higher than the overall prevalence appear to change from southern Japan to northern Japan. This could be related to the movement of dabbling ducks prior to spring migration. According to satellite-tracking data on Mallards, the ducks from southern Japan travel northward and cross the Sea of Japan [36]. Thus, it might be possible that AIV also moves from south to north with the ducks. However, further data accumulation from satellite tracking and bird banding and data exchange with neighboring countries are necessary to understand AIV entry into Japan and AIV movement in Japan.

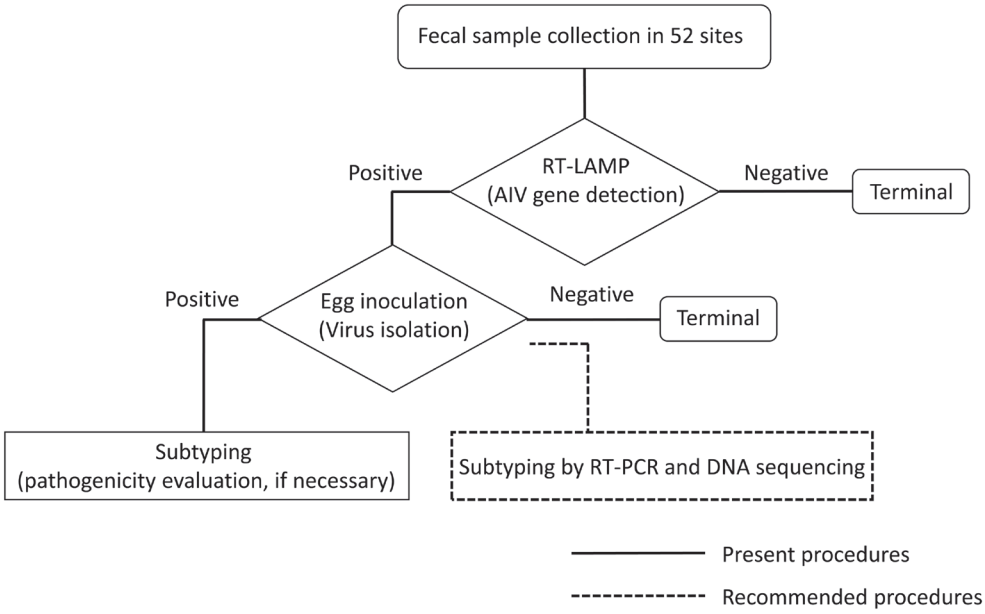


**Fig. 3.** Present and recommended procedures for AIV surveillance using fecal samples in Japan.

*Surveillance using fecal samples has a number of advantages*

Handling and capture of birds is not required, a large number of samples can be collected rapidly and easily, and virus isolation techniques from fecal samples are well-established [34]. However, fresh samples (1–4 days following evacuation) are necessary to isolate viruses, if egg inoculation is used [34]. In the present study, AIV isolation by egg inoculation and subtyping was successful in 153 RT-LAMP-positive fecal samples out of 352 RT-LAMP-positive samples. In contrast, AIV isolation by egg inoculation and subtyping failed in 199 RT-LAMP-positive fecal samples. It might be possible to subtype the remaining 199 RT-LAMP-positive fecal samples by molecular-based methods (RT-PCR and DNA sequencing). Therefore, we recommend adding molecular-based methods in the future for subtyping in fecal samples in which AIV isolation fails, to increase the efficacy of the present surveillance system [Fig. 3], as it might be possible to detect HPAIV sequences from fecal samples in which AIV isolation failed. In fact, another research group reported the isolation of HPAIV (H5N1) from duck fecal samples in 2010 in Japan [25].

A previously reported potential AIV risk map showed that the most effective predictor of AIV high-risk areas was the presence of populations of dabbling ducks [17]. There are five common dabbling duck species wintering in Japan, namely, Mallard, Eastern spot-billed duck, Northern pintail, Teal and Eurasian wigeon [10]. The DNA barcoding results of the present study showed that *CO1* gene sequences of six types of dabbling duck species were present in the RT-LAMP-positive fecal samples, including two major *CO1* gene sequences: the Mallard/Eastern Spot-billed duck group (52.0%, 115/221) and the Northern pintail (27.6%, 61/221). Considering the reported common dabbling duck species wintering in Japan, the Mallard, Eastern spot-billed duck and Northern pintail might play an important role in introducing AIV into Japan and could be priority species for fecal sampling. However, further studies are necessary to decide the priority species for fecal sampling. The DNA barcoding method applied in the present study cannot distinguish between Mallard and Eastern Spot-billed duck, because the two species have the same *CO1* gene sequence; therefore, these 115 sequences were categorized as the Mallard/Eastern Spot-billed duck group. We were unable to estimate the prevalence of each dabbling duck species in the present study, because host species information was not available for all collected fecal samples. Several sampling options exist for evaluating the prevalence according to species, such as live bird trapping or sampling of hunted birds [5, 30]. Although it might be difficult to conduct live-bird trapping on a regular basis (ideally a monthly basis) throughout Japan during the winter, sampling of hunted birds for surveillance might be relatively applicable for greater coverage of Japan.

In conclusion, we demonstrate that AIV prevalence decreases significantly from the autumn migration period to the spring migration period and that the same temporal change pattern of AIV prevalence is repeated every year in Japan. Multiple AIV introduction routes were confirmed, and crossing of the Sea of Japan and entry through the Korean Peninsula might be the main routes. In addition, Mallards, Eastern Spot-billed ducks and Northern pintails might play an important role in introducing AIV into Japan; these three species could be the main target species for AIV surveillance in Japan.

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## REFERENCES

1. Brown, J. D., Stallknecht, D. E., Beck, J. R., Suarez, D. L. and Swayne, D. E. 2006. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. *Emerg. Infect. Dis.* **12**: 1663–1670. [Medline] [CrossRef]
2. Chen, W., Calvo, P. A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J. R. and Yewdell, J. W. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat. Med.* **7**: 1306–1312. [Medline] [CrossRef]
3. Fouchier, R. A., Munster, V., Wallensten, A., Bestebroer, T. M., Herfst, S., Smith, D., Rimmelzwaan, G. F., Olsen, B. and Osterhaus, A. D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**: 2814–2822. [Medline] [CrossRef]
4. Hebert, P. D., Cywinska, A., Ball, S. L. and deWaard, J. R. 2003. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* **270**: 313–321. [Medline] [CrossRef]
5. Hill, N. J., Takekawa, J. Y., Cardona, C. J., Meixell, B. W., Ackerman, J. T., Runstadler, J. A. and Boyce, W. M. 2012. Cross-seasonal patterns of avian influenza virus in breeding and wintering migratory birds: a flyway perspective. *Vector Borne Zoonotic Dis.* **12**: 243–253. [Medline] [CrossRef]
6. Hinshaw, V. S., Webster, R. G. and Turner, B. 1980. The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. *Can. J. Microbiol.* **26**: 622–629. [Medline] [CrossRef]
7. Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R. G. and Kida, H. 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. *Arch. Virol.* **140**: 1163–1172. [Medline] [CrossRef]
8. Jin, S. D., Hoque, M. R., Seo, D. W., Kim, I. K., Jo, C., Paek, W. K. and Lee, J. H. 2012. Phylogenetic relationships among dabbling duck species in Korea using COI Gene variations in mtDNA. *Jpn. Poult. Sci.* **49**: 163–170. [CrossRef]
9. Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X. W., Zhang, X. L., Zhao, D., Wang, G., Feng, Y., Ma, J., Liu, W., Wang, J. and Gao, G. F. 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* **309**: 1206. [Medline] [CrossRef]
10. Ministry of Environment. The Annual Census on Waterfowl (Anatidae) Population (in Japanese). https://www.biodic.go.jp/gankamo/seikabutu/index.html [accessed November 22, 2016].
11. Ministry of Environment. The nationwide annual census data of migratory birds (in Japanese). http://www.env.go.jp/nature/dobutsu/bird_flu/migratory/index.html [accessed November 22, 2016].
12. Ministry of Environment. 2013. The surveillance report of avian influenza in wild birds in 2012–2013 (in Japanese). http://www.env.go.jp/nature/dobutsu/bird_flu/attach/result20130906.pdf [accessed November 22, 2016].
13. Ministry of Environment. 2014. Red data book 2014. Threatened Wildlife of Japan (Ministry of Environment ed.), Gyosei Corporation, Tokyo.
14. Ministry of Environment. 2014. The surveillance report of avian influenza in wild birds in 2013–2014 (in Japanese). http://www.env.go.jp/nature/dobutsu/bird_flu/attach/result20140924.pdf [accessed November 22, 2016].
15. Ministry of Environment. 2015. Technical manual for highly pathogenic avian influenza in wild birds (in Japanese). http://www.env.go.jp/nature/dobutsu/bird_flu/manual/pref_0809/full.pdf [accessed November 22, 2016].
16. Ministry of Environment. 2015. The surveillance report of avian influenza in wild birds in 2014–2015 (in Japanese). http://www.env.go.jp/nature/dobutsu/bird_flu/attach/result20150901.pdf [accessed November 22, 2016].
17. Moriguchi, S., Onuma, M. and Goka, K. 2013. Potential risk map for avian influenza A virus invading Japan. *Divers. Distrib.* **19**: 78–85. [CrossRef]
18. OIE. 2015. Avian influenza (infection with avian influenza viruses). Chapter 2.3.4. OIE Terrestrial Manual 2015.
19. Okazaki, K., Takada, A., Ito, T., Imai, M., Takakuwa, H., Hatta, M., Ozaki, H., Tanizaki, T., Nagano, T., Ninomiya, A., Demenev, V. A., Tyaptirganov, M. M., Karatayeva, T. D., Yamnikova, S. S., Lvov, D. K. and Kida, H. 2000. Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. *Arch. Virol.* **145**: 885–893. [Medline] [CrossRef]
20. Olsen, B., Munster, V. J., Wallensten, A., Waldenström, J., Osterhaus, A. D. and Fouchier, R. A. 2006. Global patterns of influenza a virus in wild birds. *Science* **312**: 384–388. [Medline] [CrossRef]
21. Ozawa, M., Matsuu, A., Tokorozaki, K., Horie, M., Masatani, T., Nakagawa, H., Okuya, K., Kawabata, T. and Toda, S. 2015. Genetic diversity of highly pathogenic H5N8 avian influenza viruses at a single overwintering site of migratory birds in Japan, 2014/15. *Euro. Surveill.* **21**: 20.
22. R Development Core Team 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org [accessed November 22, 2016].
23. Röhm, C., Zhou, N., Süss, J., Mackenzie, J. and Webster, R. G. 1996. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. *Virology* **217**: 508–516. [Medline] [CrossRef]
24. Saitoh, T., Sugita, N., Someya, S., Iwami, Y., Kobayashi, S., Kamigaichi, H., Higuchi, A., Asai, S., Yamamoto, Y. and Nishiumi, I. 2015. DNA barcoding reveals 24 distinct lineages as cryptic bird species candidates in and around the Japanese Archipelago. *Mol. Ecol. Resour.* **15**: 177–186. [Medline] [CrossRef]
25. Sakoda, Y., Ito, H., Uchida, Y., Okamatsu, M., Yamamoto, N., Soda, K., Nomura, N., Kuribayashi, S., Shichinohe, S., Sunden, Y., Umemura, T., Usui, T., Ozaki, H., Yamaguchi, T., Murase, T., Ito, T., Saito, T., Takada, A. and Kida, H. 2012. Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in the 2010–2011 winter season in Japan. *J. Gen. Virol.* **93**: 541–550. [Medline] [CrossRef]
26. Shivakoti, S., Ito, H., Murase, T., Ono, E., Takakuwa, H., Yamashiro, T., Otsuki, K. and Ito, T. 2010. Development of reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for detection of avian influenza viruses in field specimens. *J. Vet. Med. Sci.* **72**: 519–523. [Medline] [CrossRef]
27. Shivakoti, S., Ito, H., Otsuki, K. and Ito, T. 2010. Characterization of H5N1 highly pathogenic avian influenza virus isolated from a mountain hawk eagle in Japan. *J. Vet. Med. Sci.* **72**: 459–463. [Medline] [CrossRef]
28. Stallknecht, D. E., Nagy, E., Hunter, D. B. and Slemons, R. D. 2007. Avian influenza. pp. 108–130. *In*: Infectious Disease of Wild Birds. (Thomas, N. J., Hunter, D. B. and Atkinson, C. T. eds.), Blackwell Publishing, Oxford.
29. Tanimura, N., Tsukamoto, K., Okamatsu, M., Mase, M., Imada, T., Nakamura, K., Kubo, M., Yamaguchi, S., Irishio, W., Hayashi, M., Nakai, T., Yamauchi, A., Nishimura, M. and Imai, K. 2006. Pathology of fatal highly pathogenic H5N1 avian influenza virus infection in large-billed crows (*Corvus macrorhynchos*) during the 2004 outbreak in Japan. *Vet. Pathol.* **43**: 500–509. [Medline] [CrossRef]
30. Terregino, C., De Nardi, R., Guberti, V., Scremin, M., Raffini, E., Martin, A. M., Cattoli, G., Bonfanti, L. and Capua, I. 2007. Active surveillance for avian influenza viruses in wild birds and backyard flocks in Northern Italy during 2004 to 2006. *Avian Pathol.* **36**: 337–344. [Medline] [CrossRef]
31. Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D. A., Chen, L. M., Recuenco, S., Ellison, J. A., Davis, C. T., York, I. A., Turmelle, A.

S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M. R., Tang, K., Rowe, L. A., Sammons, S., Xu, X., Frace, M., Lindblade, K. A., Cox, N. J., Anderson, L. J., Rupprecht, C. E. and Donis, R. O. 2012. A distinct lineage of influenza A virus from bats. *Proc. Natl. Acad. Sci. U.S.A.* **109**: 4269–4274. [Medline] [CrossRef]
32. Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., Chen, L. M., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., McBride, R., Carney, P. J., Gilbert, A. T., Chang, J., Guo, Z., Davis, C. T., Paulson, J. C., Stevens, J., Rupprecht, C. E., Holmes, E. C., Wilson, I. A. and Donis, R. O. 2013. New world bats harbor diverse influenza A viruses. *PLoS Pathog.* **9**: e1003657. [Medline] [CrossRef]
33. Uchida, Y., Mase, M., Yoneda, K., Kimura, A., Obara, T., Kumagai, S., Saito, T., Yamamoto, Y., Nakamura, K., Tsukamoto, K. and Yamaguchi, S. 2008. Highly pathogenic avian influenza virus (H5N1) isolated from whooper swans, Japan. *Emerg. Infect. Dis.* **14**: 1427–1429. [Medline] [CrossRef]
34. U.S. Avian Influenza Interagency Working Group. 2006. Environmental sampling (feces/water). pp. 54–60. *In*: An Early Detection System for Highly Pathogenic H5N1 Avian Influenza in Wild Migratory Birds. U.S. Department of Agriculture, Washington, D.C.
35. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. and Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**: 152–179. [Medline]
36. Yamaguchi, N. and Higuchi, H. 2008. Migration of birds in east Asia with reference to the spread of avian influenza. *Glob. Environ. Res.* **12**: 41–54.
37. Yoneda, K. 2011. The role of wild birds in the epidemiology of H5N1 highly pathogenic avian influenza virus. *J. Vet. Epidemiol.* **15**: 45–56 (in Japanese). [CrossRef]
38. Yoon, S. W., Webby, R. J. and Webster, R. G. 2014. Evolution and ecology of influenza A viruses. *Curr. Top. Microbiol. Immunol.* **385**: 359–375. [Medline]
39. Yoshida, H., Sakoda, Y., Endo, M., Motoshima, M., Yoshino, F., Yamamoto, N., Okamatsu, M., Soejima, T., Senba, S., Kanda, H. and Kida, H. 2011. Evaluation of the reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a screening method for the detection of influenza viruses in the fecal materials of water birds. *J. Vet. Med. Sci.* **73**: 753–758. [Medline] [CrossRef]