Epidemiological surveys of the avian influenza virus infection in wild birds and countermeasures for the infection to Japanese zoological collections

(野鳥における鳥インフルエンザウイルス感染症の疫学調査と
動物園飼育鳥類における感染対策)

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ABBREVIATIONS

AIV	Avian Influenza Virus
CITES	Washington Convention (Convention on International Trades in Endangered Species of Wild Fauna and Flora)
CO1	mitochondrial cytochrome oxidase subunit 1
HA	hemagglutinin
HPAI	Highly Pathogenic Avian Influenza
IUCN	International Union for Conservation of Nature
JAZA	Japanese Association of Zoos and Aquariums
LPAI	Low Pathogenic Avian Influenza
MAFF	Ministry of Agriculture, Forestry and Fisheries
MOE	Ministry of the Environment
NA	neuraminidase
OIE	World Organization for Animal Health
PBS	phosphate-buffered saline
RT-LAMP	reverse transcription loop-mediated isothermal amplification
USDA	United States Department of Agriculture
VU	Vulnerable

PREFACE

Avian influenza (AI) is caused by influenza A virus which are negative-sense RNA viruses classified into the family *Orthomyxoviridae*. These viruses contain eight RNA segments, which encode 11 protein, namely, PB1, PB1-F2, PB2, PA, hemagglutinin (HA), NP, neuraminidase (NA), M1, M2, NS1 and NS2 [13, 100]. Of these, the surface glycoproteins, HA (H1-H16) and NA (N1-N9), have 16 and 9 subtypes, respectively [22, 75, 100]. Influenza A viruses are categorized into subtypes based on their HA and NA combination. Even among them, H5 or H7 viruses which have highly pathogenic strains call “Highly Pathogenic Avian Influenza (HPAI)”. On the other hand, H5 or H7 viruses which have low pathogenic properties call “Low Pathogenic Avian Influenza (LPAI)”.

Today infection of the AIVs have been reported in not only domestic and wild birds but also many species of mammals including humans, pigs, horses, mink, stone marten, felids and marine mammals and captive birds kept in zoos and aquariums [100]. Thus, the AI is thought that it is one of the most important infectious disease for the public health. However, according to the Japanese government or science community responsible for animal health, the AI has been regarded as one of typical and nearly recent re-emerging infectious diseases of avian species since 2004 [2, 3, 30, 66]. There is only correspondence manual when AI occurred. Adding to the changing of the avian fauna or ecosystems in Japan, there are a large number (524 bird species belonging to 23 avian orders) of captive birds kept in over 150 zoological gardens and/or aquariums throughout the country (htttp://www.jaza.jp/z_map/z_seek00.html). If the outbreak of the diseases occurs, it will impact the captive individuals as well.

The AI has been regarded as a highly contagious disease of birds, especially poultry, in world since the late 19th century, but the AIVs are isolated from wild birds, particularly migratory waterfowl belonging to the orders Anseriformes and Charadriiformes as natural reservoirs for the viruses in the World [23]. Therefore, the objectives of the present study were to observe the temporal pattern of AIV introduction into Japan and to determine which migratory birds play an important role in introducing AIV (see CHAPTER 1).

In addition, there is limited information about AIV epidemiology of shorebirds (Charadriidae and Scolopacidae families) in the East Asia/Australian Flyway. Thus, we investigated the prevalence of AIVs in shorebirds flown to Hokkaido, Japan where is the stopover site of the flyway to understand

the ecology of AIV translocation in the Flyway (see CHAPTER 2).

Furthermore, I provide a recent overview of the AI and/or their responsible agents recorded from the free-ranging and captive species in world including Japan, and potential strategies of countermeasures for an epidemic risk in facilities kept with captive birds of zoological collections are shown (see CHAPTER 3).

CHAPTER 1

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

1.1. Introduction

Influenza A viruses are negative-sense RNA viruses classified into the family *Orthomyxoviridae*. The 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 [13, 100]. Of these, the surface glycoproteins, HA and NA, have 16 and 9 types, respectively [22, 75, 100]. 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 [100, 104]. In addition, H17N10 has been isolated from Little yellow-shouldered bats (*Sturniara lilium*) in southern Guatemala, and H18N11 has been isolated from the Flat-faced fruit bat (*Artibeus planirostris*) in northern Peru [90, 91]. Influenza A virus infections have been reported in over 100 wild bird species belonging to 13 avian orders [65, 83]. 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 feces. The viruses are maintained by fecal/oral transmission, especially in breeding grounds, such as Alaska, Canada and Siberia [29, 33, 64, 100, 104]. 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 *ml* of a 1/10 dilution of bacteria-free, infective allantoic fluid [63] is deemed a highly pathogenic avian influenza virus (HPAIV). The primary subtypes of HPAIV are H5 and H7 [104]. 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 [43]. In Japan, HPAIV has been isolated from wild birds, such as the Large-billed crow (*Corvus macrorhynchos*; nine individuals, H5N1), in 2004 [86]; Mountain hawk-eagle (*Nisaetus nipalensis*; one individual, H5N1), in 2007 [80]; and Whooper swan (*Cygnus cygnus*; five individuals, H5N1), in 2008 [94]. HPAIV subtype H5N1 was isolated from 63 wild birds during the 2010–2011 winter season [78], and subtype H5N8 was isolated from eight wild birds in 2014–2015 [68]. 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) [48]. 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 [10].

The results of the nationwide surveillance of AIV in migratory birds using fecal samples were utilized for constructing a potential AIV risk map [56]. 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 [56]. 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 [50], 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.

1.2. 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 [50]. 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 15 *ml* 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 μl 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 μl of the fecal suspension was mixed with 750 μl of QIAzol lysis reagent (Qiagen). The solution was then mixed with 200 μl of chloroform by vortexing. Subsequent to centrifugation at 12,000 $\times g$, 15 min, 4°C, 400 μl 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/μl* and 1.1 *ng/μl* were obtained using the Mag MAX-96 AI/ND Viral RNA Isolation Kit and EZ1 Virus Mini Kit v2.0 solutions, respectively.

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 [79, 105]; the reported detection limit of RT-LAMP for fecal material is 102.5 copies [105]. For samples from 2008 and 2009, 5 *μl* 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 *μl* of extracted total nucleic acid and the Loopamp AIV detection kit (Eiken Chemical Co., Ltd.) were used. ALA-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 [73].

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) CO1 gene sequence [27]. 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 [27], 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 μl of PCR reaction mixture was prepared using the AccuPrime Taq DNA Polymerase System (Invitrogen, Waltham, MA, U.S.A.) containing: 5 μl 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 μl of BirdF1 primer (10 μmol), 1 μl of BirdR1 primer (10 μmol), 0.5 μl of AccuPrime Taq DNA Polymerase and 1 μl 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 μl 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 [36, 76]. 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".

1.3. 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 [47, 49, 51]. 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.

Fig. 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. Significant differences in AIV prevalence were observed between autumn migration and wintering, autumn migration and spring migration, and wintering and spring migration ($P<0.01$).

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 a higher than the overall prevalence in wintering (1.3%). During spring migration, the Tokai, Hokuriku, Tohoku and Kyusyu 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).

1.4. 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 [68, 78]. A similar phenomenon was observed in the Pacific Flyway, the migration route from breeding grounds in Alaska to wintering grounds in California and Mexico [28]. This phenomenon in the Pacific Flyway can be explained by immune

system development and limited transmission in the wintering ground [28, 65, 100]; 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 [28]. 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) [103]. 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 [102]. 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.

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 [95]. However, fresh samples (1–4 days following evacuation) are necessary to isolate viruses, if egg inoculation is used [95]. In the present study, AIV isolation by egg inoculation and subtyping was successful in 153 out of 352 RT-LAMP-positive fecal 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 [78].

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 [56]. There are five common dabbling duck species wintering in Japan, namely, Mallard, Eastern spotbilled duck, Northern pintail, Teal and Eurasian wigeon [49]. 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 [28, 87]. 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.

1.5. 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 reported 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.

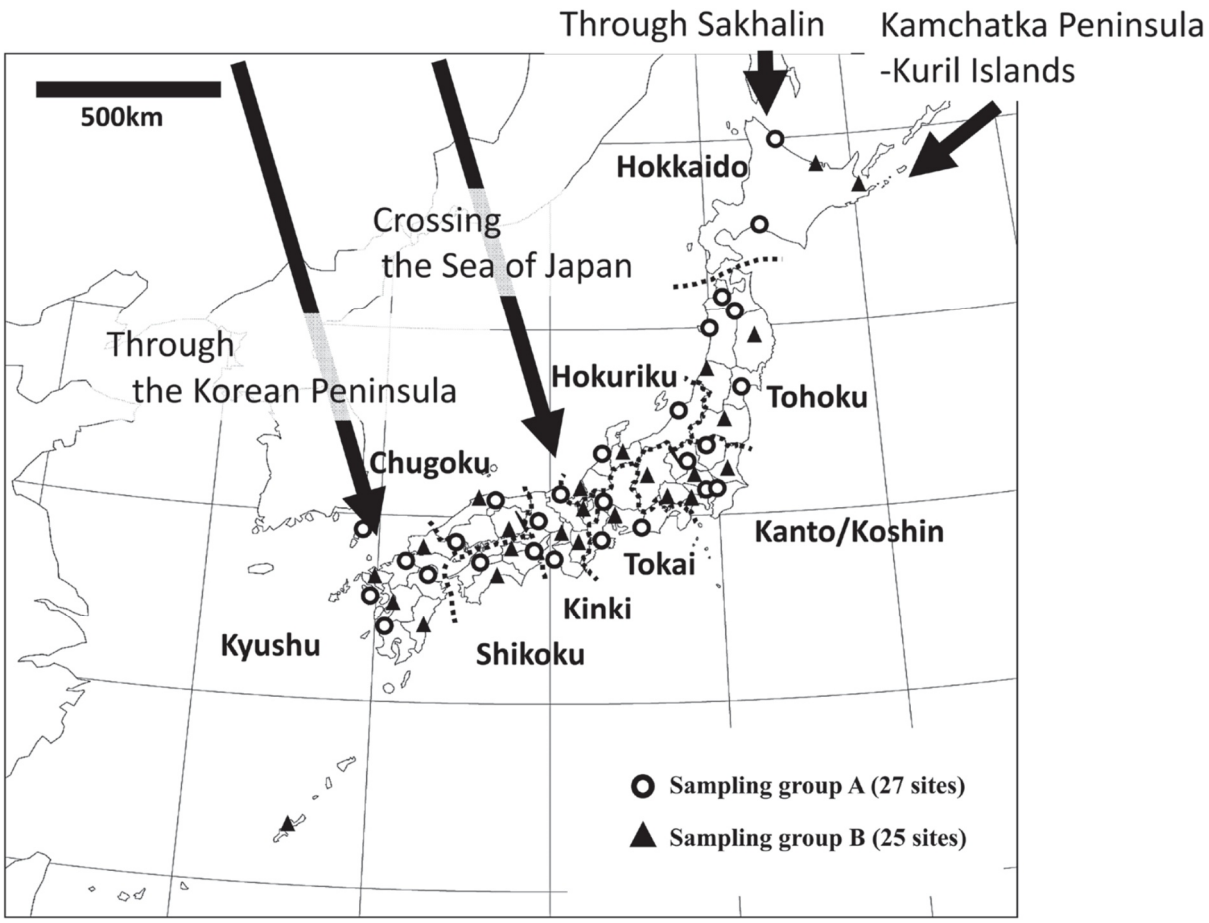

Fig. 1. Location of 52 fecal sampling sites. The 52 sites were divided into two groups: sampling group A (27 sites) and sampling group B (25 sites). Dotted lines indicate the border of the nine geographic areas, and the direction of the arrow indicates the four reported main migratory routes in Japan.

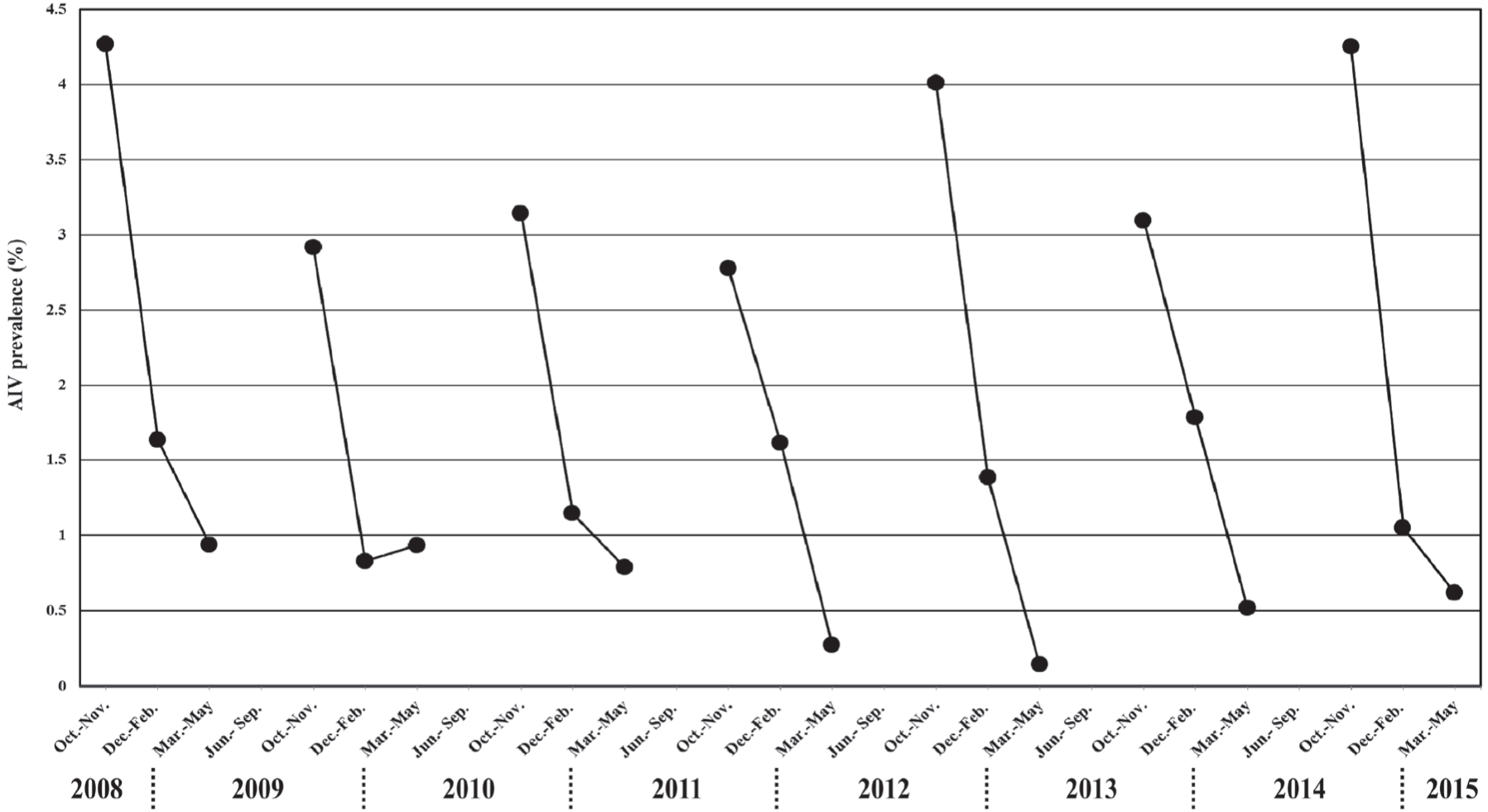

Fig. 2. Temporal change in the avian influenza virus (AIV) prevalence of migratory bird fecal samples from October 2008 to May 2015.

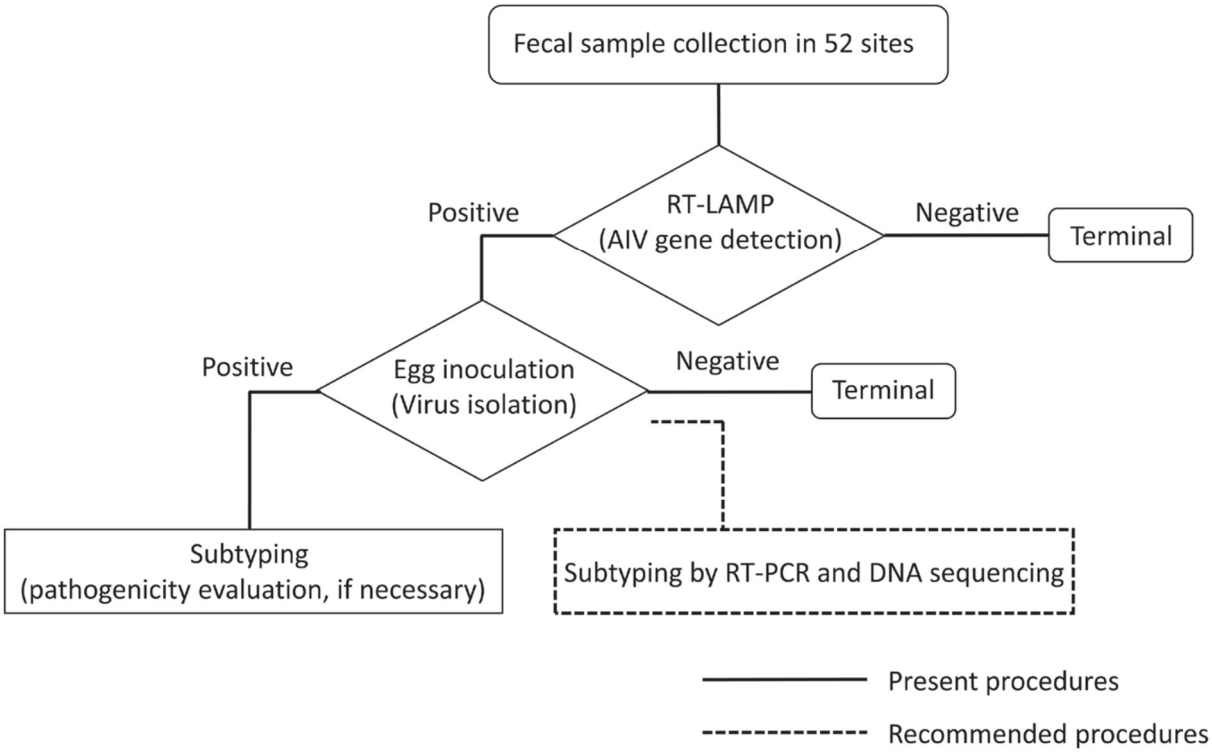

Fig. 3. Present and recommended procedures for AIV surveillance using fecal samples in Japan.

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.

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	**2**
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)])
Northan Pingtail	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

Table 3. Prevalence of avian virus by terms

Term	Autim 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.

CHAPTER 2

Epidemiological survey of avian influenza virus infection in shorebirds captured in Hokkaido, Japan

2.1. Introduction

Avian influenza virus (AIV) of the *Orthomyxoviridae* family has been detected in over 100 bird species belonging to 13 avian orders, and the Anseriformes and Charadriiformes orders constitute the most important reservoirs of AIV [65, 82, 83]. In particular, shorebirds (the family Scolopacidae and Charadriidae) could play an important role in global AIV translocation because they breed in the Northern Hemisphere during summer and migrate to the Southern Hemisphere during winter. There are eight recognized flyways of shorebird species [9], and while various epidemiological surveys have been performed on the East Atlantic, Mediterranean/Black Sea, West Asia/Africa, and Atlantic America flyways [24, 26, 37, 45, 46, 69, 82, 101], there is limited information about virus epidemiology in the East Asia/Australasia flyway, and although AIV prevalence data have been collected in Alaska, which is a part of the flyway and breeding ground of shorebirds, no data are available on AIV prevalence at the stopover sites in the flyway. Hokkaido is one of the stopover sites in this flyway. In summer to autumn, shorebirds migrate to Hokkaido from Siberia and Alaska on their way to wintering grounds, mainly Oceania (autumn migration) (Fig. 4). Moreover, they migrate to Hokkaido from the wintering ground in Spring on their way to breeding grounds (spring migration). Sampling during autumn migration could show the highest prevalence of AIV because they migrate from Siberia and Alaska where there are various kinds of AIV strains in the environment. Hence, we explored the prevalence of these viruses in shorebirds flown to Hokkaido, Japan to gain a better understanding of AIV translocation in this flyaway.

2.2. Materials and methods

Between July and September (during autumn migration) of 2006 to 2010, 1,698 shorebirds belonging to 26 species were captured and released in two sites of Hokkaido (1,332 individuals in Lake Komuke and 366 individuals in Lake Furen, Table 4. and Fig. 4) using mist nets. The two locations are major monitoring sites for nationwide shorebird populations.

All procedures were conducted by licensed bird banders and were authorized by the Ministry of Environment, Japan and the Yamashina Institute for Ornithology, Japan. and all operations were

permitted by the Ministry of Education, Culture, Sports, Science and Technology, Japan for academic research purposes.

Cloacal and tracheal swabs were collected from the captured birds. The swabs were preserved in the BD™ Universal Viral Transport medium (Becton Dickinson, Franklin Lakes, NJ), transported to the National Institute for Environmental Studies, and stored at -80 °C until total nucleic acid extraction. Total nucleic acid was extracted from the viral transport medium using the EZ1 Virus Mini Kit v2.0 (Qiagen, Hilden, Germany) following previously reported procedures [66] and subjected to reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Eiken Chemical Co., Ltd., Tokyo, Japan), which is used as the standard method for nationwide AIV survey in Japan. RT-LAMP primer set was designed for the M gene [105]. The reported sensitivity of RT-LAMP for fecal materials in $10^{2.5}$ copies of viral RNA and that for infectious allantoic fluid of embryonated chicken eggs is $10^{2.9}$ copies of viral RNA [105].

Full-lengths of hemagglutinin gene (HA) and neuraminidase gene (NA) were amplified in RT-LAMP positive samples for virus subtyping. One-step RT-PCR was performed to prepare the templates for HA and NA gene amplification using the primers Uni12 and Uni13 [15, 31, 74, 81]. A One-step RT-PCR reaction mixture was prepared using a Takara PrimeScript High Fidelity RT-PCR Kit (TAKARA BIO INC., Shiga, Japan). One-step RT-PCR was conducted with a Gene Amp PCR System 9700 (Applied Biosystems, Waltham, MA, USA) using the following conditions: one cycle of 42°C for 30 min and 94 °C for 2 min.; 40 cycles of 98°C 10 sec, 30 °C 30 sec, 72 °C 7 min; one cycle of 72 °C for 7 min, and a hold step at 4 °C. The reaction mixtures were diluted 1:50 in TE buffer, and the diluted PCR reaction mixtures (prepared using KOD-Plus-Ver.2 [TOYOBO LIFE SCIENCE, Tokyo, Japan]) were used for PCR amplifications of HA and NA using the reported primer sets [93]. PCR amplifications were conducted with a Gene Amp PCR System 9700 using the following conditions: 94 °C for 2 min; 35 cycles of 98 °C for 10 sec, 50 °C for 30 sec, and 68 °C for 2 min, followed by hold step at 4 °C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and direct sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the 3130 Genetic Analyzer (Applied Biosystems). The sequences were analyzed by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for virus subtyping.

2.3. Results and Discussion

Among the 1,698 swab samples collected between July and September of 2006–2010, one AIV-positive sample was detected from a lesser sand plover (C. mongolus) captured in Lake Komuke in September 2010. Full-lengths of HA and NA genes, as well as PA, NP, MP, and NS genes, were successfully amplified from the AIV-positive sample (Table 6). However, PB1 and PB2 genes could not be amplified. Information on the highest homology of each gene is presented in Table 5. All the sequences showing the highest homology were isolated from Anseriformes species. Shorebirds migrate to Japan one month earlier than Anseriformes. Therefore, the lesser sand plover could have been infected by virus from Anseriformes species at the breeding ground and not in Japan.
HA gene sequence showed the highest identity with H10 sequence, and NA gene sequence exhibited the highest identity with N7 sequence. According to the Influenza Research Database (https://www.fludb.org/brc/home.spg?decorator=influenza), there were 17 complete genome information of subtype H10N7 isolated in Asia during the study period. The phylogenic trees for the HA and NA genes were constructed using the 17 sequences obtained in Asia and 15 sequences obtained in North America using the neighbor-joining method [77] with a bootstrap test of 1000 replicates [21] (Figs. 5 and 6). MEGA X [40] was used to construct the trees. Moreover, phylogenetic trees for PA, NP, MP, and NS genes were constructed using 50 sequences with high homology (Figs. 7-10). The phylogenic analysis results showed that HA and NA detected in the present study were related to H10N7 isolated in Bangladesh and China in 2009. Moreover, sequences of the other four genes, PA, NP, MP, and NS, detected from the lesser sand plover, had high homology with sequences found in Asia. Thus, the subtype H10N7 detected in the present study belongs to the Eurasian lineage and the related virus strain existed in Asia in 2009.

The H4N8 subtype of avian influenza virus was reportedly isolated from red-necked stint (Calidris ruficollis) captured in Lake Komuke [11], Hokkaido. Our result showed that lesser sand plover is the second shorebird to have tested positive for AIV in Japan. Other possible hosts are found in Table 4 such as ruddy turnstone (Arenaria interpres) and red knot (C. canutus). However, the capture number of these species was relatively low in the present study. Thus, further evaluation of AIV prevalence in these species is essential to understand AIV translocation in the East Asia/Australasia flyway.

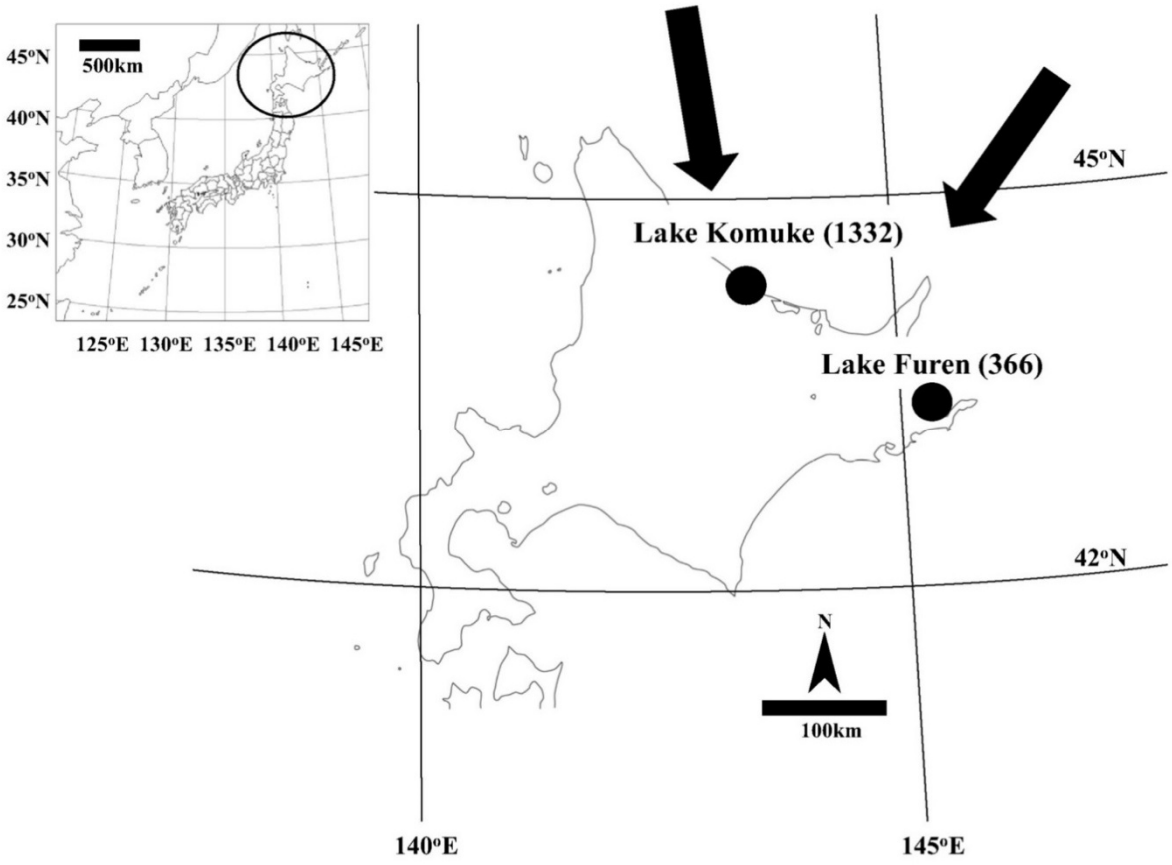

Fig. 4. Location map of capture sites in Hokkaido, Japan, from 2006 to 2010. The number in parentheses indicate the captured bird number in each site. Arrows indicate the direction of autumn migration of shorebirds.

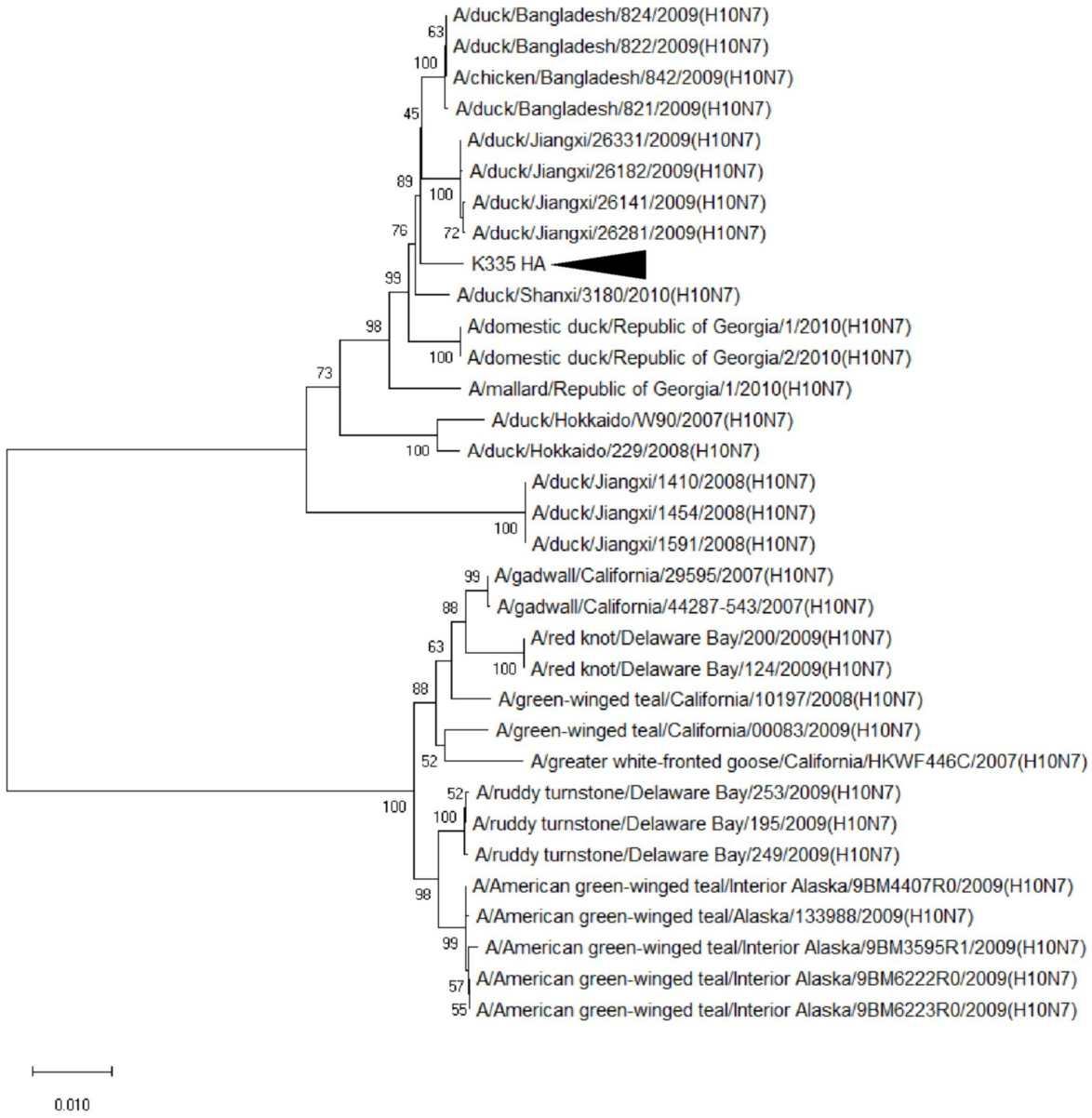

Fig. 5. Phylogenic tree of subtype H10N7 sequences using the HA gene sequence obtained from the lesser sand plover captured in Lake Komuke, Hokkaido, Japan in 2010 and the sequences of the H10N7 isolated during the study period in Asia and North America. The sequence of the present study is indicted by a black arrow.

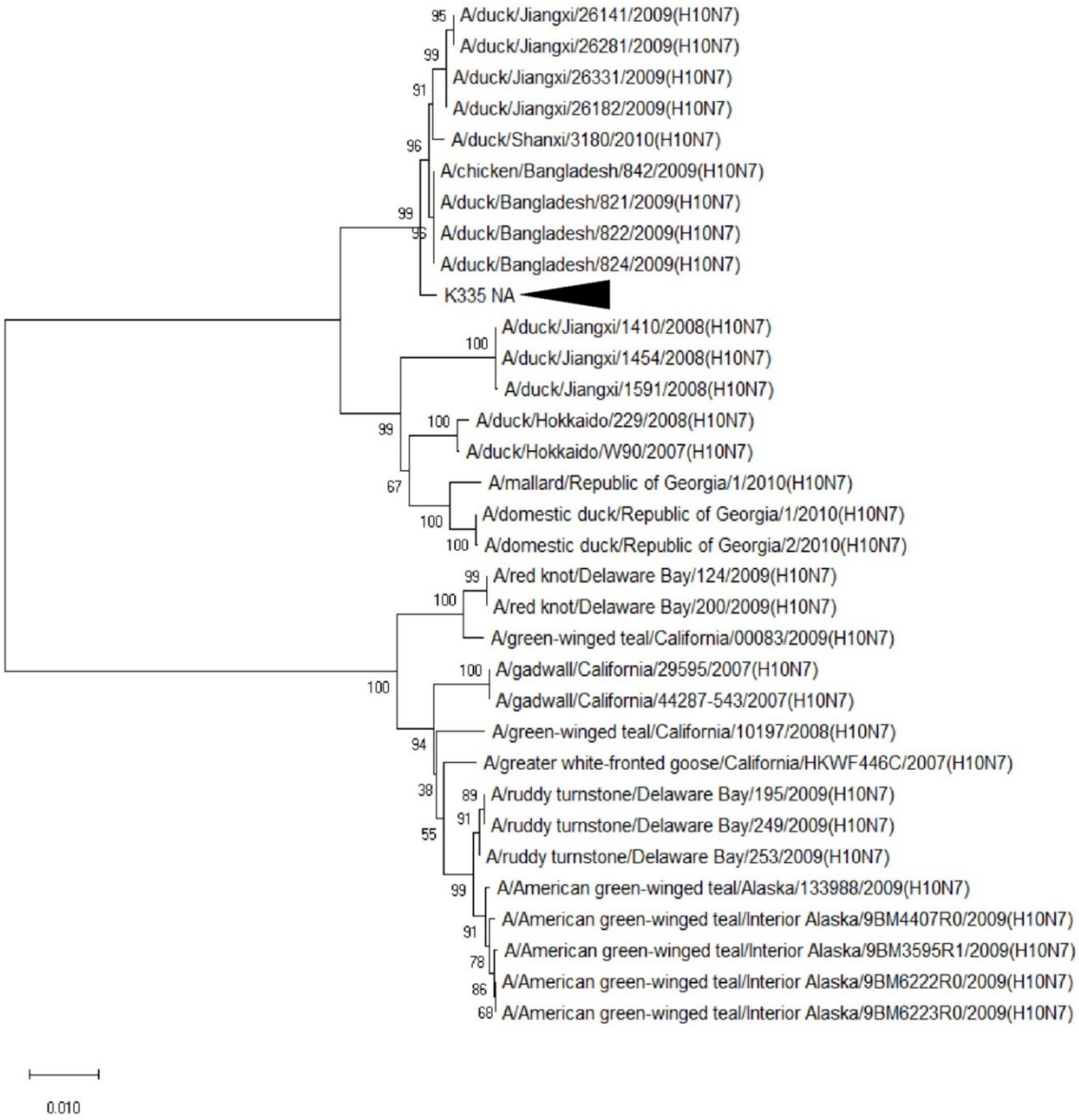

Fig. 6. Phylogenic tree of subtype H10N7 sequences using the NA gene sequence obtained from the lesser sand plover captured in Lake Komuke, Hokkaido, Japan in 2010 and the sequences of the H10N7 isolated during the study period in Asia and North America. The sequence of the present study is indicted by a black arrow.

Table 4. List of shorebird species captured in Hokkaido, Japan, from 2006 to 2010.

	AIV detection		2006		2007		2008		2009		2010		TOTAL		
			Aug., Sep.	Aug.	Jul., Aug.	Aug., Sep.	Jul.,	Aug., Sep.	Aug.	Aug., Sep.		Aug., Sep.			
	*Reported	Present study	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	All
Scolopacidae															
Ruddy Turnstone (*Arenaria interpres*)	+[a, b, c, d, f]		2		4		1	1		1		2	7	4	11
Dunlin (*Calidris alpina*)	+[a, f]			4		21		7		12		10		54	54
Red Knot (*C. canutus*)	+[a, b, d, h]			1	1			2					1	3	4
Curlew Sandpiper (*C. ferruginea*)					1								1		1
Red-necked Stint (*C. ruficollis*)	+[e]		18	91	7	88	14	104	9	212		422	48	917	965
Long-toed Stint (*C. subminuta*)			2	5	1	4		3		6		37	3	55	58
Temminick's Stint (*C. temminckii*)								1						1	1
Great Knot (*C. tenuirostris*)												2		2	2
Common Snipe (*Gallinago gallinago*)				4		1		1		1		4		11	11
Latham's Snipe (*G. hardwickii*)				14	3					2		6	3	22	25
Broad-billed Sandpiper (*Limicola falcinellus*)				1		7		8	1	9		12	1	37	38
Bar-tailed Godwit (*Limosa lapponica*)	+[g]					3	1					3	1	6	7
Black-tailed Godwit (*L. limosa*)				1								4		5	5
Whimbrel (*Numenius phaeopus*)										1		1		2	2
Red-necked Phalarope (*Phalaropus lobatus*)						1		13		2				16	16
Ruff (*Philomachus pugnax*)				6				1						7	7
Grey-tailed Tattler (*Tringa brevipes*)			94	6	127		31	6	42	19		15	294	46	340
Wood Sandpiper (*T. glareola*)						2		1				2		5	5
Common Sandpiper (*T. hypoleucos*)				8				3				7		18	18
Greenshank (*T. nebularia*)				15		3		2		1		5		26	26
Marsh Sandpiper (*Tringa stagnatilis*)				1		2						4		7	7
Terek Sandpiper (*Xenus cinereus*)			1	1	3	2		1	1			5	5	9	14
Charadriidae															
Little ringed Plover (*Charadrius dubius*)				1				1		2				4	4
Great Sand Plover (*C. leschenaultii*)							1						1		1
Lesser Sand Plover (*C. mongolus*)		+	1	11		6		7		11		38	1	73	74
Grey Plover (*Pluvialis squatarola*)						1				1				2	2
TOTAL			118	170	147	141	210		53	280	579		366	1332	1698

*a: Gaidet et al. 2012, b: Stallknecht et al. 2012, c: Maxted et al. 2016, d: Maxted et al. 2012, e: Bui et al. 2012, f: Pearce et al. 2012, g: Ip et al. 2008, h: Johnson et al. 2014

Table 5. The result of homology search with BLAST in HA, NA, PA, NP, MP, and NS genes obtained from the lesser sand plover captured in Lake Komuke in September 3rd, 2010

		Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
HA	A/mallard/Korea/1242/2010(H10N6)	3040	3040	1	0	0.9917	JN817576.1
NA	A/common teal/Hong Kong/MPM1740/2011(H7N7)	2604	2604	1	0	0.9986	KF259638.1
	A/common teal/Hong Kong/MPM1670/2011(H7N7)	2604	2604	1	0	0.9986	KF259636.1
PA	A/duck/Guizhou/888/2006(H6N5)	3890	3890	1	0	0.9935	CY109281.1
NP	A/wild waterfowl/Hong Kong/MPM2121/2011(H7N7)	2748	2748	1	0	0.998	KF259824.1
	A/common teal/Hong Kong/MPM1645/2011(H7N1)	2748	2748	1	0	0.998	KF259819.1
MP	A/wild bird/Korea/A344-2/2009(H5N1)	1797	1797	1	0	0.9969	JX236015.1
	A/wild duck/Korea/SNU50-5/2009(H5N1)	1797	1797	1	0	0.9969	JX497771.1
NS	A/muscovy duck/Vietnam/LBM348/2013(H3N8)	1537	1537	1	0	0.9976	LC028124.1
	A/duck/Zhejiang/D486/2013(H9N2)	1537	1537	1	0	0.9976	KF357835.1

Table 6. The primer sequences for amplifying PA, NP, MP, and NS genes and PCR condition.

	Foreward	Reverse
PA	ATGGAAGACTTTGTGCGACAATGCTTCA	TTTCAGTGCATGTGTGAGGAAGGAGTTG
NP	ATGGCGTCTCAAGGCACCAAACGATCT	TTAATTGTCATACTCCTCTGCATTGTC
MP	ATGAGTCTTCTAACCGAGGTCGAAACG	TACTCCAGCTCTATGTTGACAAAATGACC
NS	ATGGAYTCCAACACTGTGTCAAGCTTTC	TTTATCATTAAATAAGCTGAAACGAGA

The PCR reaction mixtures were prepared using KOD-Plus-Ver.2 (TOYOBO LIFE SCIENCE, Tokyo, Japan).
The PCR amplifications were conducted with a Gene Amp PCR System 9700 (Applied Biosystems).
The PCR conditions were as follows: 94 ^{o}C for 2 min; 35 cycles of 98 ^{o}C for 10 sec, 50 ^{o}C for 30 sec, and 68 ^{o}C for 2 min, followed by hold step at 4 ^{o}C.

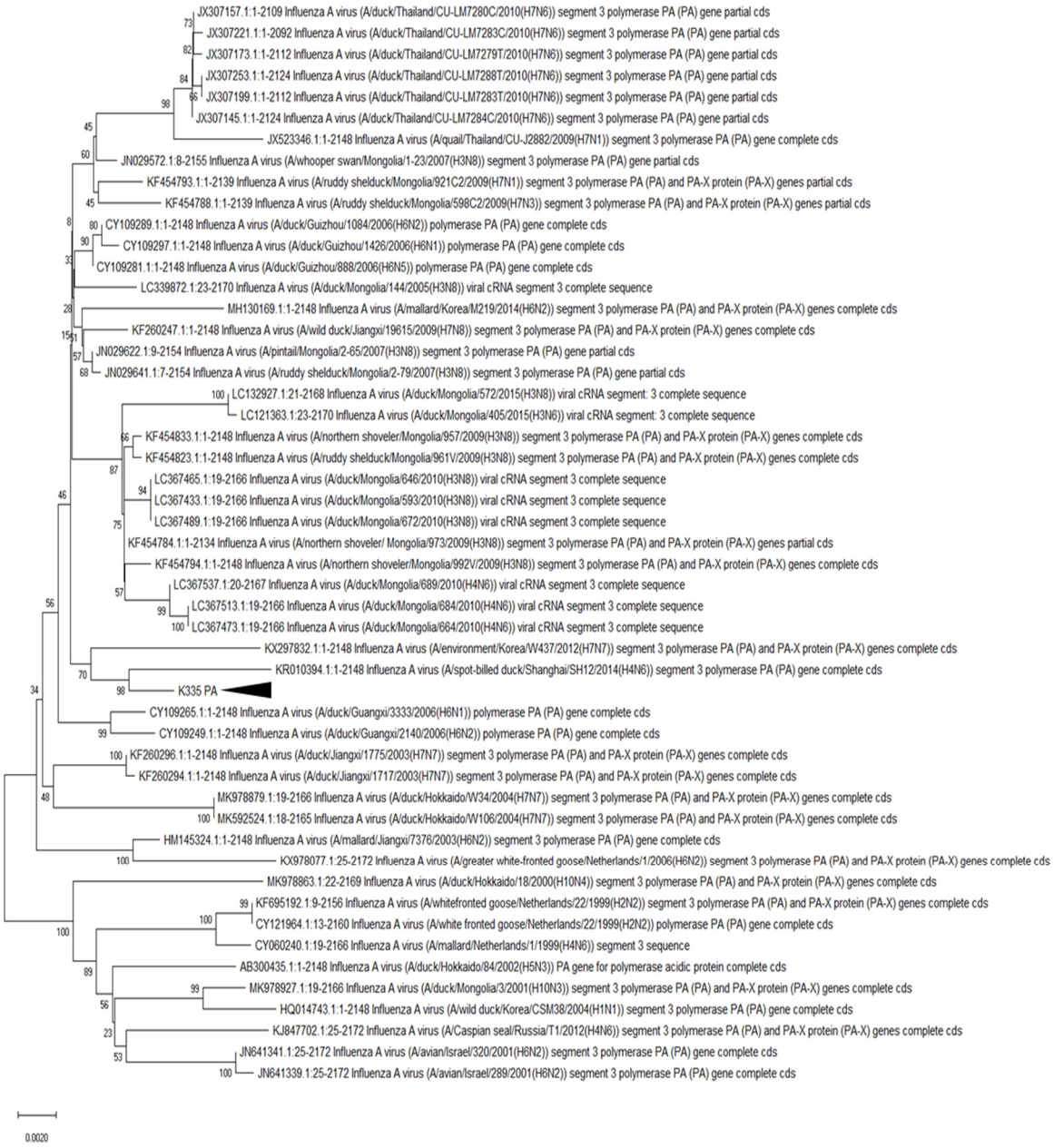

Fig. 7. Phylogenic tree of the PA gene sequence obtained from the lesser sand plover captured in Lake Komuke, Hokkaido, Japan in 2010 using 50 sequences with high homology. The sequence of the present study is indicted by a black arrow.

Fig. 8. Phylogenic tree of the NP gene sequence obtained from the lesser sand plover captured in Lake Komuke, Hokkaido, Japan in 2010 using 50 sequences with high homology. The sequence of the present study is indicted by a black arrow.

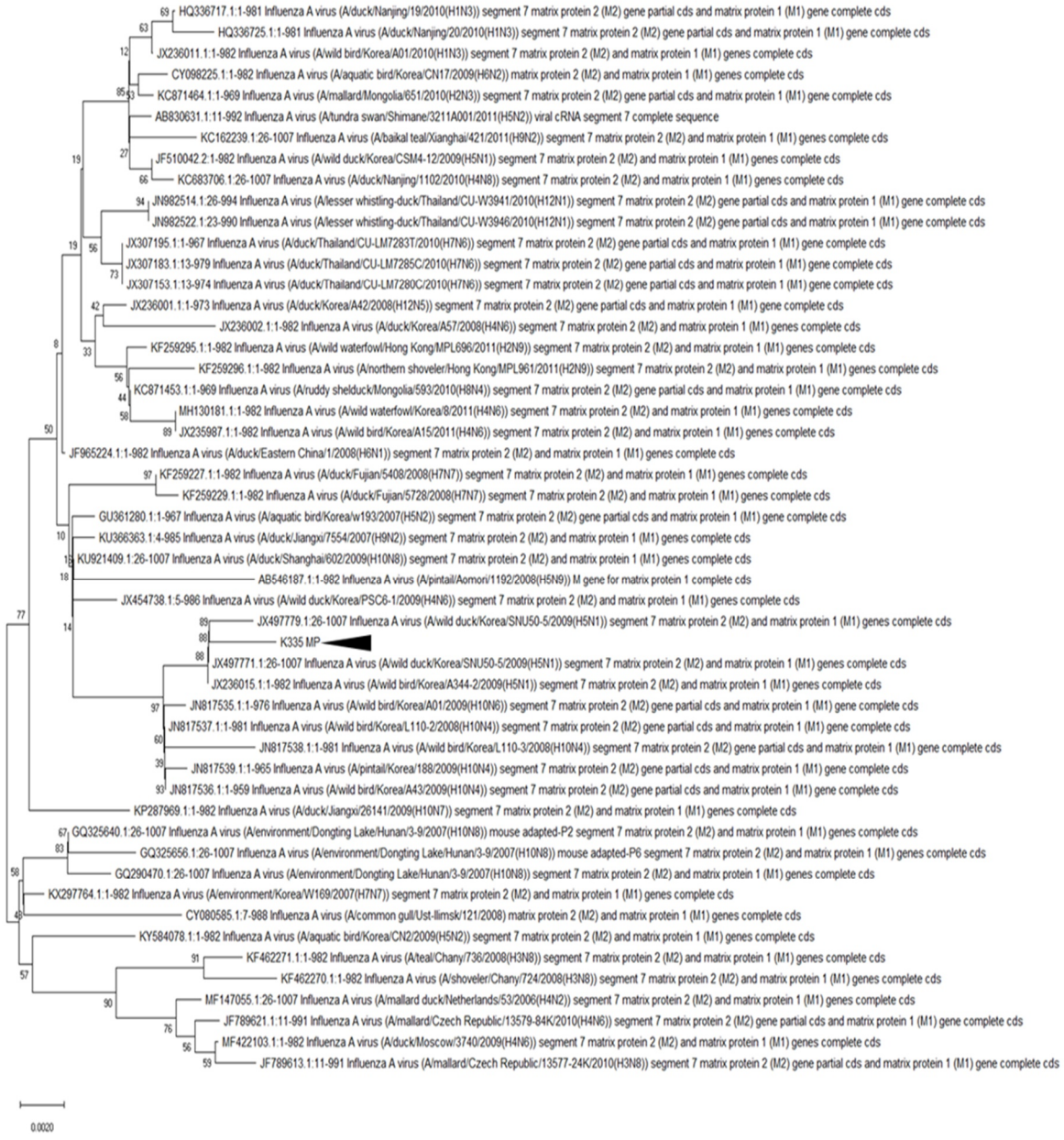

Fig. 9. Phylogenic tree of the MP gene sequence obtained from the lesser sand plover captured in Lake Komuke, Hokkaido, Japan in 2010 using 50 sequences with high homology. The sequence of the present study is indicted by a black arrow.

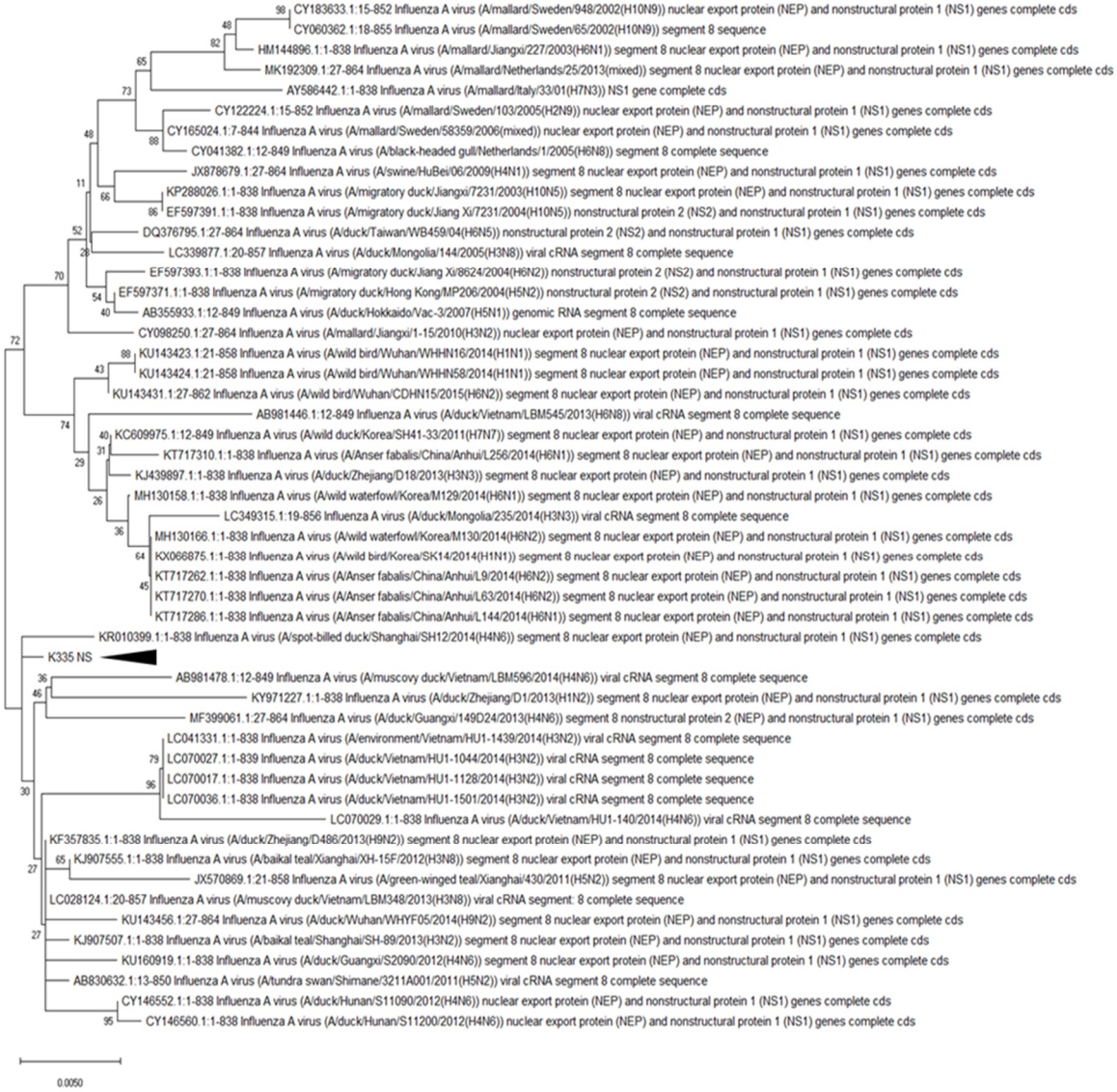

Fig. 10. Phylogenic tree of the NS gene sequence obtained from the lesser sand plover captured in Lake Komuke, Hokkaido, Japan in 2010 using 50 sequences with high homology. The sequence of the present study is indicted by a black arrow.

CHAPTER 3

Countermeasures for avian influenza outbreaks among captive avian collections at zoological gardens and aquariums in Japan

3.1. Background

Despite considerable environmental changes over the last 160 years [2, 3], Japan is an important transit country for a wide range of migratory avian species. It is situated on the East Asian Flyway, a principal migratory route connecting Northeast Asia with Southeast Asia. Major branches pass through the Nansei-Shoto Islands, Kyushu, Honshu and Hokkaido into Northeast Russia, and via Kyushu and the Korean Peninsula into Eastern China [67]. So far, Japan has escaped the outbreaks of infectious disease that have significantly impacted bird populations in other parts of the world [2, 3, 30]. During these outbreaks, tens of thousands of birds become sick and, in extreme cases, even die [23]. In addition to the changing avian fauna and ecosystems in Japan, a large number of captive birds are kept in over 150 zoological gardens and/or aquariums throughout the country (http://www.jaza.jp/z_map/z_seek00.html). It is therefore likely that these captive birds would also be affected if a disease outbreak should occur.

Although there are numerous infectious diseases that affect both free-ranging and captive avian species, this review contains a brief overview of the situation regarding avian influenza (AI) in Japan, as well as suggestions for the implementation of countermeasures for the prevention and management of potential AI outbreaks.

First described in the late 19th century, AI is a highly contagious viral disease affecting birds, especially poultry, worldwide. AI viruses are generally isolated from wild birds, particularly migratory waterfowl belonging to the orders Anseriformes and Charadriiformes, which are considered natural reservoirs of the viruses [23]. However, since 2004, highly pathogenic (HP) AI has been classified as a typical and re-emerging infectious disease of avian species by the Japanese government and the science community responsible for animal health [2, 3, 30, 66].

Therefore, the current review provides an overview of recent changes in AI and its causative agents in both free-ranging and captive avian species worldwide, including Japan, and provides potential strategies to manage epidemic risk in facilities with captive birds or zoological collections.

3.2. General characteristic of Avian Influenza and its causative agents

AI is caused by influenza type A viruses, a group of negative-sense, single-stranded RNA viruses belonging to the family *Orthomyxoviridae*. Influenza A virus genomes contain eight segments encoding 11 proteins. Segments one to six encode PB2, PB1, PB1-F2, PA, hemagglutinin (HA), nucleoprotein NP, and neuraminidase (NA) in decreasing order in size. The seventh and eighth segments encode M1, M2, NS1, and NS2. The viruses are further categorized into various subtypes based on the combination of HA (H1–H16) and NA (N1–N9) antigens. Various influenza A virus subtypes occur in wild birds, especially aquatic species, and may also infect mammals such as humans and pigs [2, 66, 83].

AI viruses are globally distributed and are likely found everywhere that potential host species are present [66]. AI or its causative viruses have been reported in over 100 free-ranging bird species belonging to 13 avian orders [66, 83]. Among these, birds belonging to the orders Anseriformes, including dabbling ducks, diving ducks, geese, and swans, and the order Charadriiformes, including gulls, terns, and shorebirds, constitute the most important viral reservoirs [83]. However, most reports of AI have been in birds from the family Anatidae (Anseriformes), with AI viruses having been isolated from over 30 duck and goose species worldwide [83].

The pathogenicity of AI viruses varies significantly according to the subtype. The viruses are deemed highly pathogenic (HP) or low pathogenic based on several factors: i) the outcome of intravenous pathogenic index assays in chickens, ii) the amino acid sequence at the hemagglutinin cleavage site, or iii) the ability of the virus to cause cytopathic effects in cell culture in the absence of trypsin. To date, highly pathogenic strains have been restricted to the H5 and H7 subtypes, although most H5 and H7 viruses display low pathogenic properties. Cleavage of the hemagglutinin protein is of paramount importance in determining virulence, but the combination of genes, including the nucleoprotein and polymerase genes, is also a consideration. The HPAI viruses contain alterations in their cleavage sites that allow the precursor hemagglutinin to be processed by a variety of ubiquitous intracellular proteases found in many body tissues, resulting in the potential for systemic, multi-organ infections [16, 55, 63].

3.3. Recent outbreak of Highly Pathogenic Avian Influenza

Since 2017, outbreaks of HPAI in poultry have occurred in 19 countries in Asia, two countries in

North America, 28 countries in Europe, seven countries in Africa, and in Russia and Serbia (Table 7) [44]. As a result of several HPAI outbreaks in Japan since 2004, the Ministry of Agriculture, Forestry, and Fisheries (MAFF) carries out an annual review of all outbreaks (Table 8).

The most common pathogenic HPAI virus subtypes are H5N1, H5N6, and H7N7. However, there are slight differences in the predominant subtypes for each continent. For example, subtype H5N1 is frequently found in Asian countries, while subtype H5N8 is more common in Europe. In comparison, H5N1 and H5N8 are the predominant subtypes in most African countries. Differences in the frequency of the various subtypes among countries and/or continents appear to be associated with proximity to the different migratory bird flyways. The major migratory routes include the East Asian/Australasian, the Central Asian, and the Black Sea/Mediterranean flyways [89, 98]. Because all three flyways share common northern destinations, there is potential for AI viruses to spread from locations in Siberia and Alaska to other parts of the world, including Japan [85].

HPAI infections, most of which were caused subtype H5N8 viruses, were recorded in 33 zoological gardens across 15 countries in Europe and Asia between October 2016 and March 2017 [20]. In Asia, most of these infections occurred in local water fowl species in India, including rosy pelican (*Pelecanus onocrotalus*), bar-headed goose (*Anser indicus*), greylag goose (*Anser anser*), and painted stork (*Mycteria leucocephala*), housed at Gwailor Zoo, the National Zoological Park, Tata Zoo, Mysore Zoo, and Nandankanann Zoological Park [44]. In Europe, however, the HPAI infections occurred in various bird species from zoological collections in several different countries, including emu (*Dromaius novaehollandiae*) at Ueckermünde Zoo in Germany, swans at Liberec Zoo in the Czech Republic, and a Dalmatian pelican (*Pelecanus crispus*) at Schönbrunn Zoo in Austria. Within the zoological collections, the HPAI outbreaks were not restricted to avian species, with tigers (*Panthera tigiris*), leopards (*Panthera pardus*), and lions (*Panthera leo*) also affected in Thailand and China. The feeding of infected chickens to these large carnivores was likely responsible for the secondary infections [13, 32, 39, 88]. On both continents, the possibility of infection being contracted from wild birds was considered likely.

Several fatal or severe cases of HPAI infection (subtypes H5N1 or H5N6) have been recorded in Japanese zoological collections between 2010 and 2016. These included a mute swan (*Cygnus olor*) at Takaoka Kojo Park in Toyama, three black swans (*C. atratus*) and three snowy owls (*Bubo scandiacus*) at Omoriyama Zoo in Akita Prefecture, and three black swans, four cackling geese

(*Branta hutchinsii*), two mallard ducks (*Anas platyrhynchos*), and a Eurasian wigeon (*Anas penelope*) at Higashiyama Zoo in Aichi Prefecture [1, 58, 59, 60, 61, 92].

In comparison, there have been very few reported cases of HPAI infection in free-ranging birds in Japan, with only three whooper swans (*Cygnus cygnus*) affected between 2007 and 2009 [2]. However, after the introduction of a nationwide surveillance program for detection of AI virus in birds, a total of 60 individuals belonging to 15 wild bird species, including both migratory and resident species, were found to have contracted HPAI virus infections between 2010 and 2011 (Table 7) [30].

3.4. Nationwide surveillance of Avian Influenza viruses in Japan

Because of the role of free-ranging birds in AI virus epidemics in Japan, a nationwide surveillance program to detect the viruses in fecal or blood samples from wild birds was introduced by the Ministry of Environment in 2008 [66]. According to the survey results, including unpublished data, the general AI virus prevalence ranged from 1.4%–2.2% in the 10-year period between 2008 and 2018. The highest prevalence rates were recorded between October and December each year, just after the autumn migration. Onuma et al. [66] showed that three routes were likely responsible for the introduction of the AI viruses to Japan, with the direct crossing of the Sea of Japan and entry through the Korean Peninsula identified as the two main routes. In addition, the study showed that the so-called mallards-eastern spot-billed duck group (*Anas platyrhynchos* and/or *Anas zonorhyncha*) and Northern pintails (*Anas acuta*) were the species most likely to have carried the AI viruses into Japan.

However, as mentioned above, the viruses have also been isolated from species belonging to the order Charadriiformes [23]. Thus, an investigation into the prevalence of AI viruses in Charadriiformes shorebirds from Hokkaido, the stopover site along the flyway, was performed [38]. Blood samples were collected from 1749 individual birds; however, AI virus was only detected in one individual identified as a lesser sand plover (*Charadrius mongolus*), corresponding to an overall prevalence of 0.06%. Hence, shorebirds are unlikely to be vehicles of AI virus transmission in Japan [38].

3.5. Countermeasures for management of Avian Influenza virus infection in zoological collections in Japan

As per the Domestic Animal Infectious Disease Control Law of Japan, if an AI outbreak occurs in

poultry such as chickens or ducks, the affected facilities come under the control of regional livestock hygiene service centers. In comparison, in the case of infections in other birds, including wild birds and those housed in zoological collections, the Technical Manual for Highly Pathogenic Avian Influenza in Wild Birds (translated title) [55] is applied as a general countermeasure against infection. In addition, for zoos and aquariums, the Guidelines of Countermeasures for Highly Pathogenic Avian Influenza Infection for Captive Breeding Birds (translated title) [54] should be referenced. The Guidelines suggest that each step of the countermeasures should be followed for the three outbreak situation levels. These levels are: level 1, outbreak has not yet occurred; level 2, poultry outbreak; level 3, outbreak in a zoological collection. Unfortunately, there is no clear guideline on whether to preserve by treatment or to euthanize birds from zoos and aquariums in the event of AI virus infection in birds included on the International Union for Conservation of Nature Red List of Threatened Species or mentioned in the Convention on International Trade in Endangered Species. Hence, clear guidelines should be published in the near future.

Furthermore, we recommend that the Japanese government should consider implementing an AI vaccination program for endangered avian species, such as that recommended by the World Organisation for Animal Health (OIE) [7, 18, 42, 62, 84, 96, 97]. According to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals published by the OIE [63], most North American and European zoos adopt the program using the water-in-oil adjuvanted H5N2 vaccine (Nobilis®) or H5N9 vaccine (Poulvac®). Captive birds were vaccinated twice via subcutaneous or intramuscular injection with a 3–6 weeks interval. In general, a “high” or “good” titer was obtained in >60% of vaccinated birds [5, 8, 17, 41, 70]. However, a similar vaccination program has not been permitted in Japan.

3.6. Conclusion

In conclusion, there are several suitable countermeasures for the prevention and management of AI outbreaks in zoological gardens and aquariums in Japan. Based on the systematic and nationwide surveys conducted in Japan since 2008, the prevalence of AI virus appears to be highest during the autumn migration period. In addition, mallard ducks, eastern spot-billed ducks, and northern pintails play an important role in introducing AI virus into Japan. Hence, zoos and aquariums should attempt to prevent duck species, especially the three species mentioned above, from gaining access to their

facilities during the autumn migration period. Further, depending on population numbers, a control of these duck species may need to be carried out at affected facilities. Because there are also resident populations of mallard and eastern spot-billed ducks in Japan, it would be preferable to prevent these species from entering zoos and aquariums at any time, especially during the autumn migration and wintering periods. Finally, in addition to general sanitary and quarantine procedures mentioned above, an AI virus vaccination program is needed in Japan.

Table 7. Recent outbreaks of the HPAI in the world since 2017.

Country	Subtype of HPAI			
Asia				
Japan	H5N6			
Korea	H5N6	H5N8		
China	H5N1	H5N6	H5N8	H7N9
Taiwan	H5N2	H5N6	H5N8	
Hong Kong	H5N6			
Malaysia	H5N1			
Vietnam	H5N1	H5N6		
Laos	H5N1			
Philippines	H5N6			
Cambodia	H5N1	H5N6		
Myanmar	H5*	H5N1		
Nepal	H5N1	H5N8		
Indonesia	H5N1			
India	H5N1	H5N8		
Bangladesh	H5*	H5N1		
Iran	H5N1	H5N8		
Bhutan	H5N1			
Iraq	H5N8			
Israel	H5N8			
North America				
America	H5*			
Mexico	H7N3			
Europe				
Italy	H5N8			
Netherland	H5N5	H5N6		
United Kingdom	H5N8			
Portugal	H5N8			
Spain	H5N8			
Bulgaria	H5*	H5N8		
Sweden	H5N6			
Poland	H5N5			
Croatia	H5N5			
Czech Republic	H5N5	H5N8		
Ireland	H5N6			
Macedonia	H5*	H5N8		
Belgium	H5N8			
Germany	H5N5	H5N6	H5N8	
Denmark	H5N6			
Montenegro	H5N5			
Switzerland	H5N8			
Finland	H5*	H5N6	H5N8	
Ukraine	H5N8			
Serbia	H5N5			
Greece	H5N5	H5N6		
Luxembourg	H5N8			
Cyprus	H5N8			
Bosnia and Her Chegovina	H5N8			
Slovakia	H5N8			
Lithuania	H5N8			
Slovenia	H5N5	H5N8		
Africa				
South Africa	H5N8			
Niger	H5N8			
Cameroon	H5N8			
Togo	H5N1			
Uganda	H5N8			
Congo	H5*			
Zimbabwe	H5N8			
Russia and NIS coutries				
Russia	H5*	H5N2	H5N8	
Kazakhstan	H5N8			

*: Subtype of the AI viruses are unknown.

Table 8. Outbreaks of the HPAI in Japan between 2005 and 2018.

	2005-2006	2006-2007	2007-2008	2008-2009	2009-2010	2010-2011	2011-2012	2012-2013	2013-2014	2014-2015	2015-2016	2016-2017	2017-2018
Poulty	41(9*)	4	0	7	0	24	23	0	0	5	0	12	1
Wild birds	0	0	3	0	0	60	0	0	0	13**	0	200	46
TOTAL	41(9*)	4	3	7	0	84	23	0	0	18	0	212	47

*: Number of antibody-positive cases.

**: Number of positive cases partly derived from the materials of feces and environmental samples (eg., water, soil etc).

CONCLUSION

According to the systematic and nationwide surveys since 2008, the AI virus prevalence shows highest at the autumn migration period. In addition, Mallards, Eastern Spot-billed ducks, and Northern Pintails play an important role in introducing AIV into Japan. Hence, zoo and aquarium managers should avoid approaching to the facilities by duck species, especially, the 3 avian species mentioned above, at the autumn period. Depending on aggregation state, they do not hesitate to control such ducks as soon as possible. Among the 3 species, because there are also resident populations of Mallards and Eastern Spot-billed in Japan, it is recommended that the managers had better block check invasion of the both ducks to their facilities all seasons, especially, during the autumn migration period and wintering periods. Adding to general sanitary and quarantine procedures mentioned above, an emergent vaccination program is needed.

ACKNOWLEDGEMENT

First of all, I would like to express my sincere thanks to Specially Appointed Prof. Manabu Onuma, Graduate School of Veterinary Medicine, Rakuno Gakuen University (RGU), Japan, who is the Chief Researcher of the National Institute for Environmental Studies (NIES), Japan, as well. Actually, the present results of the chapters 1 and 2 of this thesis are derived from the research project performed by the NIES, and I have joined the project partly. Therefore, my sincere thanks are extended to the NIES, especially to the Director of the Ecological Risk Assessment and Control Section Dr. Kouichi Goka, for permission of my participation into the research project.

And, I would also like to thank Prof. Mitsuhiko Asakawa, as main supervisor, and Profs. Rikio Kirisawa and Hidetoshi Higuchi, as co-supervisors, the Graduate School of Veterinary Medicine, RGU, Japan, respectively.

Finally, my thank also owed to the staff of the Kobe Animal Kingdom, Japan, which is specialized for keeping zoological avian species and I have been worked in, for giving me not only a kind support to complete this thesis, but also a primary motive to start the present studies including countermeasures for infectious diseases to zoological collections.

This study was supported, in part, by the Strategic Research Foundation at Private Universities (2013-2017) and by a Grant-in-Aid for Scientific Research (C-23570120 and C-26460513) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

This work was supported partly by the Environment Research and Technology Development Fund (SII-1) of the Environmental Restoration and Conservation Agency of Japan.

REFERENCES

1. Akita Prefecture. 2016. HPAI in Omoriyama Zoo. http://www.pref.akita.lg.jp/uploads/public/archive_0000038843_00_14.pdf (in Japanese)

2. Asakawa, M., Nakade, T., Murata, S., Ohashi, K., Osa, Y. and Taniyama, H. 2013. Recent viral diseases of Japanese anatid with a fatal case of Marek's disease in an endangered species, white-fronted goose (*Anser albifrons*). pp. 37-48. *In*: Ducks: Habitat, Behavior and Diseases (Hambrick, J. and Gammon, L. T. eds.) Nova Science Publishers, Inc., USA.

3. Asakawa, M., Nakamura, S. and Brazil, M. A. 2002. An overview of infectious and parasitic diseases in relation to the conservation biology of the Japanese avifauna. *J. Yamashina Inst. Ornithol.* 34: 200-221.

4. Amonsin, A., Payungporn, S., Theamboonlers, A., Thanawongnuwech, R., Suradhat, S., Pariyothorn, N., Tantilertcharoen, R., Damrongwantanapokin, S., Buranathai, C., Chaisingh, A., Songserm, T. and Poovorawan, Y. 2006. Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. *Virology* 344(2):480-491.

5. Alert, V. J., Bellon, F. H., Busquets, N., Alcántara, G., Delclaux, M., Pizarro, B., Sánchez, C., Sánchez, A., Majó, N. and Darji, A. 2011. Comprehensive Serological Analysis of Two Successive Heterologous Vaccines against H5N1 Avian Influenza Virus in Exotic Birds in Zoos. *Clin. Vaccine Immunol*. 18(5): 697-706.

6. Alexander, D. J. 2000. A review of avian influenza in different bird species. *Vet. Microbiol.* 74:3-13.

7. American Zoo and Aquarium Association, Animal Health Committee. 2005. Avian Influenza:Guidelines for prevention in North American zoos. https://www.jumpjet.info/Emergency-Preparedness/Disaster-Mitigation/NBC/Bio/Avian_Influenza_Guidelines_for_Zoos.pdf

8. Bertelsen, M. F., Klausen, J., Holm, E., Grøndahl, C. and Jørgensen, P. H. 2007. Serological response to vaccination against avian influenza in zoo-birds using an inactivated H5N9 vaccine. *Vaccine* 25(20):4345-4349.

9. Boere, G. C. and Stroud, D. A. 2006. The Flyway Concept: What It Is and What It Isn't. pp. 40–47. *In*: Waterbirds around the World (Boere, G. C., Galbraith, C. A. and Stroud, D. A. eds.) The Stationery Office, Edinburgh, UK.

10. 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.

11. Bui, V. N., Ogawa, H, Xininigen., Karibe, K., Matsuo, K., Awad, S. S., Minoungou, G. L., Yoden, S., Haneda, H., Ngo, L. H., Tamaki, S., Yamamoto, Y., Nakamura, K., Saito, K., Watanabe, Y., Runstadler, J., Huettmann, F., Happ, G. M. and Imai, K. 2012. H4N8 subtype avian influenza virus isolated from shorebirds contains a unique PB1 gene and causes severe respiratory disease in mice. *Viroligy* 423:77-88.

12. Chen, Q., Wang, H., Zhao, L., Ma, L., Wang, R., Lei, Y., Li, Y., Yang, G., Chen, G., Li, L., Jin, T., Liu, X., Xu, X., Wong, G., Liu, L., Liu, Y., Shi, W., Bi, Y. and Gao, G. F. 2016. First documented case of avian influenza (H5N1) virus infection in a lion. *Emerg. Microbes. Infect.* 5(12):e125-127.

13. 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.

14. Clerc, M. G., Lebarbenchon, C. and Thomas, F. 2007. Recent expansion of highly pathogenic avian influenza H5N1: a critical review. *Ibis.* 149: 202-214.

15. Desselberger, U., Racaniello, V. R., Zazra, J. J. and Palese, P. 1980. The 3’ and 5’-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene* 8: 315-328.

16. Desvaux, S., Marx, N., Ong, S., Gaidet, N., Hunt, M., Manuguerra, J. C., Sorn, S., Peiris, M., Werf, S. V. and Reynes, J. M. 2009. Highly Pathogenic Avian Influenza Virus (H5N1) Outbreak in Captive Wild Birds and Cats, Cambodia. *Emerg. Infect. Dis.* 15(3):475-478.

17. Dolka, B., Żbikowski, A., Dolka I., Szeleszczuk, P. 2016. The response of mute swans (Cygnus olor, Gm. 1789) to vaccination against avian influenza with an inactivated H5N2 vaccine. *Acta. Vet. Scand.* 58:74.

18. EAZWV. 2007. Transmissible Diseases Handbook Ⅺ.AVIAN INFLUENZA. http://c.ymcdn.com/sites/www.eazwv.org/resource/resmgr/Files/Transmissible_Diseases_Handbook/Main_Chapters/11_Avian_Influenza.pdf

19. Ellis, T. M., Bousfield, R., Bisset, L., Dyrting, K., Luk, G., Tsim, S., Sturm-Ramirez, K., Webster, R. G., Guan, Y., and Malik Peiris, J.S. 2004. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathol.* 2004(33): 492–505.

20. FAO (Food and Agriculture Organization of the United Nations). 2017. Highly pathogenic H5 avian influenza in 2016 and 2017. http://www.fao.org/3/a-ie8068e.pdf

21.Felsentein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

22. 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.

23. Friend, M. and Franson, J. C. 1999. Field manual of wildlife diseases. Genaral field procedures and diseases of birds. *Geological Survey Madison Wi Biological Resources Div.* 181-184..

24. Gaidet, N., Mamy, A. B. O. E., Cappelle, J., Caron, A., Cumming, G. S., Grosbois, V., Gil, P., Hammoumi, S., de Almeida, R. S., Fereidouni, S. R., Cattoli, G., Abolnik, C., Mundava, J., Fofana, B., Ndlovu, M., Daiwara, Y., Hurtado, R., Newman, S. H., Dodman, T. and Balanca, G. 2012. Investigating avian influenza infection hotspots in old-world shorebirds. *PLoS One*. 7:e46049.

25. Globig, A., Starick, E., Homeier, T., Pohlman, A., Grund, C., Wolf, P., Zimmermann, A., Wolf, C., Heim, D., Schlößer, H., Zander, S., Beer, M., Conraths, F. J. and Harder, T. C. 2016. Epidemiological and Molecular Analysis of an Outbreak of Highly Pathogenic Avian Influenza H5N8 clade 2.3.4.4 in German Zoo: Effective Disease Control with Minimal Culling. *Transboundary Emerg. Dis.* 64(6): 1813-1824.

26. Hall, J. S., Hallgrimsson, G. T., Suwannanarn, K., Sreevatsen, S., Ip, H. S., Magnusdottir, E., TeSlaa, J. L., Nashold, S. W. andDusek, R. J. 2014. Avian influenza virus ecology in Iceland shorebirds: intercontinental reassortment and movement. *Infect. Genet. Evol*. 28: 130-136.

27. Hebert, P. D., Cywinska, A., Ball, S. L. and de Waard, J. R. 2003. Biological identifications through DNA barcodes. *Proc. Biol. Sci*. 270: 313-321.

28. 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

29. 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.

30. Hirayama, T., Ushiyama, K., Osa, Y. and Asakawa, M. 2013. Recent infectious diseases or

theirresponsible agents recorded from Japanese wild birds. pp. 83-95. *In*: Birds: Evolutionand Behavior, Breeding Strategies, Migration and Spread of Disease (Ruiz, L and Iglesias, F. eds.) Nova Science, USA.

31. Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. and Perez, D. R. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol*. 146(12): 2275-2289.

32. Hu, T., Zhao, H., Zhang, Y., Zhang, W., Kong, Q., Zhang, Z., Cui, Q., Qiu, W., Deng, B., Fan, Q. and Zhang, F. 2016. Fatal influenza A (H5N1) virus Infection in zoo-housed Tigers in Yunnan Province, China. *Sci. Rep.* 6: 25845.

33. 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.

34. Ip, H. S., Flint, P. L., Franson, J. C., Dusek, R. J., Derksen, D. V. Gill, R. E. Jr., Ely, C. R., Pearce, J. M., Lanctot, R. B., Matsuoka, S.M., Irons, D. B., Fischer, J. B., Oates, R. M., Petersen, M. R., Fondell, T. F., Rocque, D. A., Pedersen, J. C. and Rothe, T. C. 2008. Prevalence of Influenza A viruses in wild migratory birds in Alaska: patterns of variation in detection ata a crossroads of intercontinental flyways. *Virol. J*. 5: 71.

35. JAZA. 2006. Infectious disease Handbook of Zoo and Aquarium animals: Highly pathogenic avian influenza (in Japanese)
http://member.jaza.jp/pdf/04_operation/kansen_hb_h25_03/B14.pdf

36. 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 variation in mtDNA. *Jpn. Poult. Sci*. 49: 163-170.

37. Johnson, J. A., DeCicco, L. H., Ruthrauff, D. R., Krauss, S. and Hall, J. S. 2014. Avian influenza virus antibodies in Pacific Coast Red Knots (*Calidris canutus reselaari*). *J. Wildl. Dis.* 50: 671-675.

38. Kakogawa, M., Onuma, M., Saito, K., Watanabe, Y., Goka, K. and Asakawa, M. in submission. Epidemiological survey of avian influenza virus infection in shorebirds captured in Hokkaido, Japan. *J. Wildl. Dis.*

39. Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R. A. M., Amonsin, A., Payungporn, S., Noppornpanth, S., Wattanodorn, S., Theamboonlers, A., Tantilertcharoen, R., Pattanarangsan, R., Arya, N., Ratanakorn, P., Osterhaus, A. D. M. E. and Poovorawan, Y. 2004. Avian Influenza H5N1 in Tigers and Leopards. *Emerg. Infect. Dis.* 10(12): 2189- 2191.

40. Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*. 35:1547-1549.

41. Lécu, A., Langhe, D. C., Petit, T., Bernard, F. and Swam, H. 2009. Serologic response and safety to vaccination against avian influenza using inactivated H5N2 vaccine in zoo birds. *J. Zoo. Wildl. Med.* 40(4): 731-743.

42. Leong, H. K., Goh, C. S., Chew, S. T., Lim, C. W., Lin, Y. N., Chang, S. F., Yap, H. H. and Chua, S. B. 2008. Prevention and Control of Avian Influenza in Singapore. *Ann. Acad. Med. Singap.* 37(6):504-509.

43. 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.

44. MAFF (Ministry of Agriculture, Forestry and Fisheries, Japan). 2019. The world outbreaks of avian influenza in poultry since 2017.http://www.maff.go.jp/j/syouan/douei/tori/pdf/AI_wd.pdf (in Japanese)

45. Maxted, A. M., Luttrell, M. P., Goekjian, V. H., Brown, J. D., Niles, L. J., Dey, A. D., Kalasz, K.

S.,Swayne, D. E. and Stallknecht, D. E. 2012. Avian influenza virus infection dynamics in shorebird hosts. *J.Wildl. Dis*. 48: 322-334.

46. Maxted, A. M., Sitters, H. P., Luttrell, M. P., Dey, A. D., Kalasz, K. S., Niles, L. J. and Stallknecht, D. E. 2016. Spring Migration Stopover Ecology of Avian Influenza Virus Shorebird Hosts at Delaware Bay. *Avian Dis*. 60(1 Suppl):394-405.

47. MOE (Ministry of Environment, Japan). 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

48. MOE. 2014. Red data book 2014. Threatened Wildlife of Japan (Ministry of Environment Ed.) Gyousei Corporation, Tokyo.

49. MOE.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

50. MOE. 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

51. MOE.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

52. MOE.2016. The nationwide annual census data of migratory birds (in Japanese).
http://www.env.go.jp/nature/dobutsu/bird_flu/migratory/index.html

53. MOE. 2016. The Annual Census on Waterfowl (Anatidae) Population (in Japanese).
http://www.biodic.go.jp/gankamo/seikabutu/index.html

54. MOE. 2017. Correspondence guidelines for highly pathogenic avian influenza of breeding birds in

zoos (in Japanese).
https://www.env.go.jp/press/files/jp/107492.pdf

55. MOE. 2017. Correspondence technical manual pertaining to highly pathogenic avian influenza in the wild bird (in Japanese).
http://www.env.go.jp/nature/dobutsu/bird_flu/2910_torifurumanual.pdf

56. Moriguchi, S., Onuma, M. and Goka, K. 2013. Potential risk map for avian influenza A virus invading Japan. *Divers. Distrib*. 19: 78-85.

57. Nagarajan, S., Kumar, M., Murugkar, H.V., Tripathi, S., Shukla, S., Agarwal, S., Dubey, G., Nagi, R. S., Singh, V. P. and Tosh, C. 2017. Novel Reassortant Highly Pathogenic Avian Influenza (H5N8) Virus in Zoos, India. *Emerg. Infect. Dis.* 23(4): 717-719.

58. Nagoya-shi. 2016. HPAI in Higashiyama zoo (First report).
http://www.higashiyama.city.nagoya.jp/blog/archives/001/201711/08%20281212%20.pdf (in Japanese)

59. Nagoya-shi. 2016. HPAI in Higashiyama zoo (Second report).
http://www.higashiyama.city.nagoya.jp/blog/archives/001/201711/10%20281213%20.pdf (in Japanese)

60. Nagoya-shi. 2016. HPAI in Higashiyama zoo (Third report).
http://www.higashiyama.city.nagoya.jp/blog/archives/001/201711/16%20281220%20.pdf (in Japanese)

61. Nagoya-shi. 2016. HPAI in Higashiyama zoo (Fourth report).
http://www.higashiyama.city.nagoya.jp/blog/archives/001/201711/17%20281228%20.pdf (in Japanese)

62. OIE (World Organization for Animal Health) 2007. AVIAN INFLUENZA VACCINATION. https://www.oie.int/doc/ged/D4410.PDF

63. OIE. 2015. Avian influenza (infection with avian influenza viruses). Chapter 3.3.4. pp. 821-843 *In*: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018.

64. 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., Yamnukova, 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.

65. 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.

66. 2017. Characterizing the temporal patterns of avian influenza virus introduction into Japan by migratory birds. *J.Vet.Med.Sci.* 79(5): 943-951.

67. Ornithological Society of Japan. 2012. Check-List of Japanese Birds 7th Ed.

68. 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.

69. Pearce, J. M., Ruthrauff, D. R. and Hall, J. S. 2012. Paired serologic and polymerase chain reaction analyses of avian influenza prevalence in Alaskan shorebirds. *J. Wildl. Dis.* 48:812-814.

70. Philippa, J., Baas, C., Beyer, W., Bestebroer, T., Fouchier, R., Smith, D., Schaftenaar, W. and Osterhaus, A. 2007. Vaccination against highly pathogenic avian influenza H5N1 virus in zoos using an adjuvanted inactivated H5N2 vaccine. *Vaccine.* 25(19): 3800-3808.

71. Philippa, J. D. W., Munster, V. J., Bolhuis, H., Bestebroer, T. M., Schaftenaar, W., Beyer, W. E. P., Fouchier, R. A. M., Kuiken, T. and Osterhaus, A. D. M. E. 2005. Highly pathogenic avian influenza (H7N7): Vaccination of zoo birds and transmission to non-poultry species. *Vaccine.* 23(50): 5743-5750.

72. Quirk, M. 2004. Zoo tigers succumb to avian influenza. *The Lancet Infect. Dis.* 4(12): 716.

73. R Development Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
http://www.R-project.org

74. Robertson, J. S. 1979. 5’ and 3’ terminal nucleotide sequences of the RNA genome segments of influenza virus. *Nucleic Acids Res*. 6: 3745-3757.

75. 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.

76. 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.

77. Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.

78. 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.

79. 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.

80. 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.

81. Skehel, J. J. and Hay, A. J. 1978. Nucleotide sequences at the 5’ terminal of influenza virus RNAs and their transcripts. *Nucleic Acids Res*. 5: 1207-1219.

82. Stallknecht, D. E., Luttrell, M. P., Poulson, R., Goekjian, V., Niles, L., Dey, A., Krauss, S. and Webster, R. G. 2012. Detection of avian influenza viruses from shorebirds: evaluation of surveillance and testing approaches. *J. Wildl. Dis*. 48: 382-393.

83. 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.

84. Sweden. 2006. Vaccination programme for birds kept in zoos against avian influenza submitted by Sweden for approval by the commission Rev.1. https://ec.europa.eu/food/sites/food/files/animals/docs/ad_control-measures_ai_birds_vaccine_swe.pdf

85. Takemae, N., Tsunekuni, R., Sharshov, K., Tanikawa, T., Uchida, Y., Ito, H., Soda, K., Usui, T., Sobolev, I., Shestopalov, A., Yamaguchi, T., Mine, J., Ito, T. and Saito, T. 2017. Five distinct reassortants of H5N6 highly pathogenic avian influenza A viruses affected Japan during the winter of 2016–2017. *Virology* 512: 8-20.

86. 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.

87. 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.

88. Thanawongnuwech, R., Amonsin, A., Tantilertcharoen, R., Damrongwatanapokin, S., Theamboonlers, A., Payungporn, S., Nanthapornphiphat, K., Ratanamungklanon, S., Tunak, E., Songserm, T., Vivatthanavanich, V., Lekdumrongsak, T., Kesdangsakonwut, S., Tunhikorn, S. and Poovorawan, Y. 2005. Probable Tiger-to-Tiger Transmission of Avian Influenza H5N1. *Emerg. Infect. Dis.* 11(5): 699-701.

89. Tian, H., Zhou, S., Dong, L., van Boeckel, T. P., Cui, Y., Newman, S. H., Takekawa, J. Y., Prosser, D. J., Xiao, X., Wu, Y., Cazelles, B., Huang, S., Yang, R., Grenfell, B. T. and Xu, B. 2015. Avian influenza H5N1 viral and migration networks in Asia. *Proc. Natl. Acad. Sci.* 112(1): 172-177.

90. 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. USA*. 109: 4269-4274.

91. 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.

92. Toyama Prefecture. 2011. HPAI in Takaoka Kojo Park.

http://www.pref.toyama.jp/sections/1612/tori_influenza/files/message4.pdf (in Japanese)

93. Tseng, C. H., Tsai, H. J. and Chang, C. M. 2014. A complete molecular diagnostic procedure for applications in surveillance and subtyping of avian influenza virus. *Biomed. Res. Int.* 2014:653056.

94. 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.

95. 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 Bird. USDA, Washington, D. C.

96. USDA Animal and Plant Health Inspection Service. 2015. Concept of Operations Plan: Management of an Avian Influenza Outbreak at a Zoological Institution. https://www.aphis.usda.gov/animal_health/downloads/animal_diseases/ai/hpai-zoo-conops.pdf

97. USDA, APHIS, AZA. 2009. Management Guidelines for Avian Influenza: Zoological Parks & Exhibitors Outbreak Management Plan. https://zahp.aza.org/wp-content/uploads/2015/06/AI-Outbreak-Management-Plan-for-Zoological-Parks-and-Exhibitors.v322.1.31.17.pdf

98. Verhagen, J. H., van der Jeugd, H. P., Nolet, B. A., Slanterus, R., Kharionov, S. P., de Vries, P. P., Vuong, O., Majoor, F., Kuiken, T. and Fouchier, R. A. 2015. Wild bird surveillance around outbreaks of highly pathogenic avian influenza A (H5N8) virus in the Netherlands, 2014, within the context of global flyways. *Euro. Surveill*. 20(12): 21-32.

99. Wang, G., Zhan, D., Li, L., Lei, F., Liu, B., Liu, D., Xiao, H., Feng, Y., Li, J., Yang, B., Yin, Z., Song, X., Zhu, X., Cong, Y., Pu, J., Wang, J., Liu, J., Gao, G. F. and Zhu, Q. 2008. H5N1 avian influenza re-emergence of Lake Qinghai: phylogenetic and antigenic analyses of the newly isolated viruses and roles of migratory birds in virus circulation. *J. Gene Virol*. 89(Pt3): 697.

100. 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.

101. Winker, K., Spackman, E. and Swayne, D. E. 2008. Rarity of influenza A Virus in spring shorebirds, southern Alaska. *Emerg. Infect. Dis.* 14:1314-1316.

102. 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.

103. Yoneda, K. 2011. The Role of Wild Birds in the Epidemiology of H5N1 Highly Pathogenic Avian Influenza Virus (in Japanese). *J. Vet. Epidemiol*. 15: 45-46

104. 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.

105. 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.

ABSTRACT

I provide an epidemiological data of the avian influenza (AI) and/or their responsible agents recorded from the free-ranging and captive species in world including Japan, and potential strategies of countermeasures for an epidemic risk in facilities kept with captive birds of zoological collections are shown.

At first, the objectives of the first study were to observe the temporal pattern of the 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, 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 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 (CHAPTER 1).

There is limited information about virus epidemiology of shorebirds (family *Charadriidae* and *Scolopacidae*) in the East Asia/Australasia flyway. Thus, we investigated the prevalence of AIVs in shorebirds flown to Hokkaido, Japan, the stopover site of the flyway, to understand the ecology of AIV translocation in the flyway from 2006 to 2010. In total, 1,749 shorebirds belonging to 27 species were captured and released into two different sites using mist nets. Tracheal and cloacal swabs were collected using cotton swabs. The RNA of influenza A viruses was detected using reverse transcription loop-mediated isothermal amplification. One AIV-positive sample was obtained from a lesser sand plover (*Charadrius mongolus*) captured in September 2010 in Lake Komuke. Full-lengths of HA, NA,

PA, NP, MP, and NS genes were successfully amplified from the AIV-positive sample. All sequences showed the highest identity with sequences obtained from virus strains of Anseriformes species. Shorebirds migrated to Japan one month earlier than Anseriformes species. Therefore, the lesser sand plover could have been infected by the virus from Anseriformes species in the breeding ground. HA sequence showed the highest identity with H10 sequence, whereas NA sequence exhibited the highest identity with N7 sequence. Phylogenic analysis showed that the detected subtype H10N7 belongs to the Eurasia lineage and the related strain might have widely spread in Asia in 2009 (CHAPTER 2).

Japan is situated along the East Asian Flyway, which is an important migratory route. Outbreaks of infectious disease could impact bird populations along this route, and is expected to have a negative influence on captive bird populations. Here, we provide a brief overview of situation regarding AI in both free-ranging and captive avian species in Japan. We also suggest suitable countermeasures for the prevention and management of AI outbreaks in zoological gardens and aquariums, with special reference to the control of free-ranging duck population and/or individuals and the nationwide surveillance of AI viruses. Furthermore, we have disclosed the prominence of vaccination program for zoological collections in Japan (CHAPTER 3).

ABSTRAT IN JAPANESE (和文要旨)

本研究では野鳥および日本を含む世界でのフリーレンジングや飼育種における鳥インフルエンザの疫学的調査およびデータ収集を実施し、動物園における飼育鳥の疫学的リスクに対する有効的な対策を示した。

まず、第一の研究での目的は日本への季節的な鳥インフルエンザウイルス(AIV)パターンを観察し、どの渡り鳥がAIV伝播に重要な役割を担っているか決めることである。2008年10月から2015年3月までの間に全国52箇所で計19,407の糞便サンプルを収集した。そのサンプルから採取された核酸よりRT-LAMP法を用いてウイルスRNAを検出した。陽性サンプルは計352件(陽性率1.8%)であった。最も高い陽性率は秋の渡り時期に観察され、日本の中部から南部において全体の陽性率より高い陽性率を示していた。したがって、主なAIV伝播経路は日本海の縦断あるいは朝鮮半島を通過してくる経路が考えられる。また計352の陽性サンプルの内221サンプルで鳥種の同定に成功した。2つの主な種のシーケンスは同定され、マガモ/カルガモグループ(61サンプル、27.6%)とオナガガモ(115サンプル、52.0%)のものであった。より詳しく日本におけるAIVの生態および高病原性鳥インフルエンザウイルスの伝播パターンを把握するためには、種ごとのAIV陽性率、孵化年の渡り鳥の陽性率、渡りのパターンおよび卵接種と分子法を用いた糞便サンプルのウイルス亜型同定の情報が求められる(CHAPTER1)。

東アジア／オーストラリアの渡りルートでのシギ・チドリ類におけるAIVの疫学的情報は少ない。したがって、2006年から2010年の間、AIVの伝播生態を把握するために飛来地である北海道に飛来するシギ・チドリ類のAIVの陽性率について調査した。27種・計1,749羽のシギ・チドリ類を異なる2箇所でかすみ網を用いて捕まえ放鳥した。喉頭およびクロアカスワブは綿スワブを用いて採取した。インフルエンザAウイルスのRNAはRT-LAMP法を用いて検出した。2010年9月にコムケ湖で捕獲されたメダイチドリ(*Charadrius mongolus*) から唯一のAIV陽性サンプルが検出された。そのAIV陽性サンプルからHA、NA、PA、NP、MPおよびNS遺伝子全ての増幅に成功した。全てのシーケンスはカモ目のウイルス株から得られたシーケンスと最も高く一致していた。シギ・チドリ類はカモ類よりも1ヶ月早く日本へ渡ってくる。したがって、このメダイチドリは繁殖地にいるカモ類のウイルスに感染した可能性がある。HAシーケンスはH10シーケンスと最も高く一致しており、NAシーケンスはN7シーケンスと最も高く一致していた。系統学的解析ではユーラシア系統に属し、2009年にアジアで

広く拡散された株に関連したH10N7亜型と示された(CHAPTER2)。

重要な渡りルートである東アジア渡りルートに日本は位置している。このルートでの感染症の発生は鳥類の生息数に影響を与え、飼育鳥にも悪影響を与えかねない。そこで日本におけるフリーレンジングおよび飼育鳥種における鳥インフルエンザの状況の概要を示した。また放し飼いのカモの数や個体管理や全国的なAIVの監視を元に動物園や水族館での鳥インフルエンザの発生の予防および管理についての有効的な対策を提案した。さらに、日本の動物園動物におけるワクチン接種プログラムも提案した(CHAPTER3)。