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Avian influenza and Newcastle disease viruses from northern pintail in Japan: Isolation, characterization and inter-annual comparisons during 2006–2008

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ABSTRACT

Since wild ducks constitute a vital element in the epizootiology of avian influenza viruses (AIVs) as well as avian paramyxoviruses (APMVs) and play a key role in the ecology and inter-species transmission of these viruses, it is crucial to elucidate the diversity and prevalence of these viruses within these bird populations. This report shows the presence, antigenic diversity, and inter-annual prevalence variations of AIVs in apparently healthy northern pintail (Anas acta) wintering in Japan. We also provide evidence that this host carries APMV-1: Newcastle disease virus (NDV) and other haemagglutinating viruses. Composite samples ($n = 2381$) of fresh fecal materials were collected from northern pintail during November 2007–March 2008 at different locations of Tohoku district, main Island, Japan. We isolated 47 haemagglutinating viruses, out of which 25 were identified as AIVs, representing 9 combinations of 5 different haemagglutinin (HA) and 6 neuraminidase (NA) subtypes. Both H5 and H7 subtypes were identified and found to be low pathogenic. A further 11 viruses were grouped into APMV-1 (NDV). The rest of the viruses ($n = 11$) remained to be identified. Some of the HA subtypes and NA subtypes detected during the first season reoccurred in the second season, as well as some of their combinations; yet, several new subtypes and combinations appeared during the second season. These findings indicate that different subtypes of AIVs, NDV and other haemagglutinating viruses circulate subclinically in the pintail populations sampled. Pintails should be regarded, potentially, as important spreaders of AIVs and NDVs, particularly due to their extensively ramified flyways, which include various inter-continental routes.

1. Introduction

It is evident that aquatic wild birds are of central importance to the ecology of influenza A viruses at large. They are asymptomatic carriers of not only essentially all haemagglutinin (HA) and neuraminidase (NA) combinations of influenza A viruses (Horimoto and Kawaoka, 2001; Webster, 1998), but also of different members of avian paramyxoviruses (APMVs) (Hanson et al., 2005; Hua et al., 2005; Shortridge et al., 1980; Stallknecht et al., 1991). Viruses from both groups are economically highly significant with regard to poultry industry globally (Alexander, 2000).

Outbreaks caused by highly pathogenic avian influenza (HPAI) H5N1 virus occurred in Japan in chicken farms (Mase et al., 2005), and this pathogen was isolated in Japan from wild birds, as well, including migratory swans (World Organization for Animal Health www.oie.int/downld/AVIAN INFLUENZA/A_AI-Asia.htm, accessed 28 August 2008). Low pathogenic avian influenza (LPAI) virus subtype H5N2 has also been detected from commercial layer chicken farms in 2005, and caused culling of 5.68 million chickens, with the hope of viral spread prevention (Okamatsu et al., 2007).

Various migratory waterfowl, especially ducks, are known to tolerate and perpetuate avian influenza viruses (AIVs) – both highly and low pathogenic – thus having the capacity to convey them over Japan, other parts of Asia, and across the Northern Hemisphere. They often have direct interface with domestic free-grazing waterfowl, as well, bringing about reciprocal virus transmissions. As a part of an ongoing AIV surveillance during successive winter seasons (November–March), which started in 2003 in Japan (Jahangir et al., 2008), we here report the findings we obtained during a second period of the study (November 2007–March 2008) regarding the diversity and prevalence of AIVs as well as New-

Abbreviations: APMVs, avian paramyxoviruses; AIV, avian influenza virus; AIVs, avian influenza viruses; BLAST, basic local alignment search tool; CEF, chicken embryo fibroblast; CEI, chicken embryo inoculation; CK, chicken kidney; CPE, cytopathic effect; DEPC, diethyl pyrocarbonate; HA, haemagglutinin; HI, haemagglutination inhibition; HPAI, highly pathogenic avian influenza; NA, neuraminidase; NDV, Newcastle disease virus; NI, neuraminidase inhibition; NP, nucleoprotein; PBS, phosphate buffered saline; RBC, red blood cell.

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castle disease virus (NDV) isolated from northern pintails (*Anas acuta*). We regard this duck – a typically wintering waterfowl in Japan – to be an important carrier of various myxoviruses, particularly AIV and NDV, considering its vast geographical distribution, worldwide. Also, we present a comparative analysis, both temporally and spatially, regarding inter-annual variations in prevalence and diversity of AIVs and NDV within this host species. We thus cover two successive winter seasons – 2006–2007 and 2007–2008 – during which we repeatedly probed certain aquatic habitats commonly occupied by pintails. Along that period of time, allover, we obtained 63 AIV isolates. We found all of them – albeit including some H5 and H7 subtypes – to be low pathogenic, and discuss their occurrences, reoccurrences and inter-annual variability throughout 2006–2008. Different factors shaping this dynamics are presented.

Naturally occurring infections of AIVs were reported in more than 90 species of 13 avian orders (Olsen et al., 2006; Stallknecht and Shane, 1988). Most of the species are associated with aquatic habitats and two avian orders, the Anseriformes (ducks, geese, and swans) and the Charadriiformes (gulls, terns, and shorebirds). The prevalence of AIVs in these natural hosts varies largely (Hanson et al., 2003; Krauss et al., 2004; Webster et al., 1992). Although all antigenic subtypes of AIVs have been isolated from wild waterfowls, it is reported that all of these subtypes are not equally represented among wild bird populations (Stallknecht and Brown, 2007). For example, among 16 HA subtypes of AIVs, only H3, H4, and H6 subtypes were predominantly isolated from wild ducks in North America (Sharp et al., 1993). In contrast, subtypes belonging to H5, H7, and H9 were isolated less frequently (Stallknecht et al., 1990; Krauss et al., 2004). Moreover, H8 subtype was found to exhibit least prevalent rate (Stallknecht and Shane, 1988; Krauss et al., 2004). Temporally and spatially as well, antigenic diversity of AIVs in wild birds is dynamic. The prevalence and antigenic diversity of AIVs may vary between years, months, and weeks, even day to day (Runstadler et al., 2007). Many factors related to the host species, geographical locations, seasonality and the environment (Munster et al., 2005; Runstadler et al., 2007) may contribute to these variations.

In wild birds and poultry throughout the world, AIVs representing 16 HA and 9 NA subtypes have been identified (Fouchier et al., 2005), and in almost all possible combinations have been isolated from waterfowl and shorebirds (Hinshaw et al., 1980; Kawaoka et al., 1988, 1990). HPAI strains are believed to possibly derive from low pathogenic H5 and H7 viruses harbored by wild birds (Horimoto et al., 1995; Rohm et al., 1995; Suarez et al., 2004). Invasion of a low pathogenic progenitor virus into domestic poultry is regarded to bring about mutations and genetic recombinations with resultant highly pathogenic variant containing multiple basic amino acids at the cleavage site of the HA molecule (Horimoto et al., 1995; Kawaoka et al., 1984; Zanella et al., 2001). As AIVs, H5N1 in particular, have crucial effect on poultry and the potential to evolve into a pandemic strain, scrutiny of the viruses and their host range is essential (Olsen et al., 2006). Although wild birds are recognized as source and reservoir for all subtypes of AIVs, and most strains harbored by wild birds are low pathogenic, the uncovering of HPAI subtype H5N3 that caused death of approximately 1300 common terns in South Africa (Becker, 1966), as well as HPAI viruses subtype H5N1 in wild birds, for example in Hong Kong (Ellis et al., 2004), and quite recently in dead swans in Japan (World Organization for Animal Health www.oie.int/downld/AVIANINFLUENZA/A_AI-Asia.htm, accessed 28 August 2008) reinforces the need for a much more detailed understanding of AIV's natural history. Based on these facts and perspectives, our study was conducted in aquatic wild bird. It includes, in addition to the isolation of AIVs, another important avian myxovirus, namely NDV.

2. Materials and methods

2.1. Sampling and processing of samples

A total of 2381 composite fecal samples were collected from a wintering duck species, the northern pintail, during November 2007 to March 2008 in Japan. Fecal materials were collected at five different wintering sites in Tohoku district, main Island, Japan viz. Minase river of Akita prefecture, Lake Izunuma and Lake Uchinuma of Miyagi prefecture and Lake Ogawara and Tsubo river of Aomori prefecture, where aquatic wild birds roam during winter time (Jahangir et al., 2008). Although other aquatic birds (waterfowl and gulls) concomitantly occupy the sites we probed, pintails were by far dominant, and the sampling practice we applied was adequately useful for distinguishing between feces of pintails and other species. Four freshly voided fecal materials were pooled into one tube and regarded as one composite sample (further details concerning the methodology of sampling are presented in Appendix A).

Samples were processed as described (Jahangir et al., 2008) with little modification. Briefly, 25% suspension of fecal sample was made with phosphate buffered saline (PBS pH 7.2) supplementing with penicillin (10,000 units/ml), streptomycin (10,000 mg/ml), gentamycin (5000 mg/ml), and amphotericin B (50 mg/ml) and kept at 4 °C overnight to facilitate antimicrobial activity. Supernatant was yielded after refrigerated centrifugation at 12,000 × *g* for 3 min and used for inoculation into chicken embryo.

2.2. Virus isolation

Virus isolation was done by chicken embryo inoculation (CEI). Eggs were obtained from parent stocks for layer (Koiwai Farm, Iwate, Japan) and incubated for 10 days prior to inoculation. Two hundred microliter of supernatant of each sample was inoculated into the allantoic cavity of two 10-day-old chicken embryos. Embryos were incubated at 37 °C for 72 h and candled twice daily to verify their livability. Death within 24 h after inoculation was regarded as nonspecific. Soon after found dead or upon 72 h incubation, embryos were chilled and allantoic fluid was evaluated by haemagglutination test for the presence of haemagglutinating activity (Hirst, 1941). For negative samples, five allantoic fluids were pooled (300 μl from each) and repassaged into two additional eggs at the dose rate of 750 μl/egg, in order to ascertain negativity. Portion of allantoic fluid for each individual sample was stored at −80 °C for further passage whenever a pooled sample showed haemagglutination. All positive supernatants were eventually kept at −80 °C.

2.3. Analysis of Newcastle disease virus

Allantoic fluids showing haemagglutinating activity were subjected to haemagglutination inhibition (HI) test with NDV specific antiserum. The alpha method (i.e., variable virus concentration and constant antibody titer) of HI test was done to identify and differentiate NDV from possible presence of AIVs, other APMVs and egg drop syndrome (EDS) virus. The test was conducted by the conventional microtiter method. Briefly, two-fold serial dilution of 25 μl HA positive allantoic fluid was made with PBS in U bottom microtiter plate (Nunc). Pretitrated 25 μl antiserum/well was added and allowed to stand at room temperature for 60 min to facilitate antigen antibody reaction. About 50 μl of 0.5% chicken red blood cell (RBC) suspension was added into each well and again allowed to stand at room temperature for 40 min. The result was observed without any aids. Positive and negative controls were run simultaneously with the test samples to validate the test.

2.4. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Briefly, viral RNA was extracted from haemagglutinating virus-positive allantoic fluid, except those confirmed as NDV by HI test, using TRIzol LS (Invitrogen, Carlsbad, CA) or Isogen LS (Nippon Gene, Toyama, Japan), a commercially available mixture of guanidium isothiocyanate, according to the manufacturer's instructions. RNA extract was suspended in 10 μl of diethyl pyrocarbonate (DEPC)-treated water. First-strand cDNA was generated in a total volume of 30 μl. The reaction mixture consisted of 4.5 μl of RNA template, 3 μl of 10× PCR buffer II, 12 μl of diethylnitrophyenyl thiophosphate mixture (dNTP 2.5 mM each), 6 μl of 25 mM $MgCl_2$, 30 units of RNase inhibitor, 75 units of Moloney Murine Lukemia virus (M-MLV) reverse transcriptase (GeneAmp RNA PCR Kit, Applied Biosystems, Tokyo, Japan) and 1.5 μl of 20 pmol/μl Uni12 universal primer (Hoffmann et al., 2001). The mixture was incubated at room temperature for 15 min to anneal the primer and then heated at 42 °C for 90 min followed by an enzyme inactivation step at 99 °C for 5 min. Samples were then cooled to 4–5 °C by immediately being placed on ice. Polymerase chain reaction (PCR) was performed in a total volume of 25 μl with influenza A virus nucleoprotein (NP) specific primers described previously (Lee et al., 2001). Once the virus was found positive for NP, further analysis for subtyping was done with the subtype specific primers (Lee et al., 2001). One microliter of the cDNA was mixed with 24 μl PCR master mixture that included 10× PCR buffer 2.5 μl, 25 mM $MgCl_2$ 1 μl, dNTP mix (2.5 mM each) 2 μl, TaqDNA polymerase (5 U/μl) 0.125 μl (GeneAmp RNA PCR Kit, Applied Biosystems, Tokyo, Japan), DEPC treated water 16.375 ml, and 100 pmol forward and reverse primer 1 μl each. Primer sets used for NA subtyping were kindly provided by Dr. K. Tsukamoto National Institute of Animal Health, Tsukuba, Japan. Thermal cycling began with initial 4 min denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 s (denaturation), 55 °C for 40 s (annealing), and 72 °C for 40 s (extension) for H2–4, H11, H14, and H15. Conditions for the primers H1, H5–10, H12, H13, and N1–N9 were the same, except that the annealing temperature was 50 °C. The final primer extension was done at 72 °C for 10 min. All samples were kept at 4 °C until the PCR product was analyzed through electrophoresis on 1% agarose gel. PCR products on the gel were visualized through ethidium bromide staining. Apparent size of each PCR product was compared with DNA markers of known sizes (Gene Ruler 100 bp DNA Ladder Plus, Fermentas, Life Sciences).

2.5. Cloning of HA genes of H1 isolates

Full length amplifications of the HA segment were done as described earlier (Hoffmann et al., 2001). The purified product was ligated into a plasmid vector pCR2.1 using TA cloning kit (Invitrogen, San Diego, CA). After transformation in competent *Escherichia coli* INVαF (Invitrogen, San Diego, CA), the positive clone was selected by blue white colony selection in Luria Bertani (LB) agar followed by visualization of specific insert resulting from EcoRI digestion of purified plasmid. Sequencing was performed using vector specific primers initially. Basic local alignment search tool (BLAST) was used to confirm HA subtype of the obtained sequence data through existing sequence data into GenBank.

2.6. Antigenic characterization of AIV isolates

Antigenic characterizations of AIV isolates were done by the HI and neuraminidase inhibition (NI) tests, using a panel of reference antisera against 12 HA and 9 NA subtypes, respectively (Jahangir et al., 2008). HI and NI tests were conducted as described (Alexander, 2004; Aymard-Henry et al., 1973; van Deusen et al., 1983; Webster and Campbell, 1972). HI test was performed using the alpha method as described in Section 2.3 except for using of HA subtype specific polyclonal antiserum instead of NDV antiserum.

NI test was conducted in 1.5 ml microtube by means of the microtiter method (van Deusen et al., 1983). Briefly, 5 μl of 10-fold-diluted virus was mixed with equal volume of pretitrated N subtype specific polyclonal chicken antisera in a corresponding microtube labeled earlier and incubated at room temperature for 60 min. The tube was shaken for 1 min after adding 10 μl of fetuin solution and incubated overnight at 37 °C. Before adding 10 μl of periodate solution, the tube was kept on ice for 2 min. After shaking, the tube was kept at room temperature for 20 min. About 100 μl of arsenite reagent was mixed and vortexed until the brown color disappeared. The mixture was boiled for 15 min after adding 250 μl of thiobarbituric acid. Results were observed by naked eye examination.

2.7. Analysis of HA cleavage site of H5 and H7 isolates

Amplification of 300 base pairs (bp) covering the HA cleavage site of H5 and H7 isolates was performed by PCR, using the primers described previously (Jahangir et al., 2008; Munch et al., 2001). Amplified products were purified from excised gel and resuspended in 10 μl TE buffer pH 8.0. The nucleotide sequencing reaction mixture (20 μl per reaction) contained 9.5 μl of water, 4 μl of 5× buffer (BigDye Terminator v1.1, v3.1 4× sample buffer, PerkinElmer Applied Biosystems, Tokyo, Japan), 4 μl of reaction mixture (BigDye Deoxy Terminator Cycle Sequencing FS Ready Reaction Kit v3.1, PerkinElmer Applied Biosystems), 2 μl of primer 4 pmol/μl (internal primer for H5 as described by Jahangir et al. (2008) and for H7 same used in PCR by Munch et al. (2001)) and 0.5 μl of purified product. The thermal conditions applied were as follows: denaturation for 1 min at 96 °C and 25 cycles of PCR amplification with each cycle consisting of 45 s denaturation, at 96 °C, 40 s of annealing at 55 °C and 4 min of elongation at 60 °C. The PCR products were purified with a BigDye XTerminator Purification Kit (Applied Biosystems, Japan) according to the related instructions. The sequence was read by automated ABI310 Gene Analyzer (Applied Biosystems, Japan) as described (Sanger et al., 1977). The nucleotide sequences were analyzed by the GENETYX-Mac version 10.0; (Software Development Corp., Tokyo, Japan) and aligned by the Clustal W method, using the Mega 4.0 software (Tamura et al., 2007). Oligonucleotides used in this study were synthesized by Oligonucleotides synthesis service (FASMAC, Tokyo, Japan)

3. Results

3.1. Haemagglutinating viruses isolated during November 2007–March 2008

A total of 2381 composite fecal samples from northern pintails were investigated for virus isolation through chicken embryo inoculation and then virus identification by serological and molecular techniques. Temporal and spatial distributions of composite fecal samples and isolated AIVs plus non-AIV haemagglutinating viruses are presented in Table 1. The highest portion (40.28%, namely 959/2381) of samples was obtained from Minase river, whereas 21.29% (507/2381), 17.18% (409/2381), 14.28% (340/2381), and 6.97% (166/2381) of the samples were collected from Lake Ogawara, Lake Izunuma, Tsubo river and Lake Uchinuma, respectively.

Maximal number (580/2381) of samples were collected in March, whereas least number (376/2381) in February 2008. The number of samples collected from each site and at each time varies due to changing numbers of roaming pintail and availability of fecal materials. Among 2381 composite samples, 1.97% (47/2381) samples were found positive for the presence of haemagglutinating

Table 1
Composite fecal sampling and isolation of AIVs and other haemagglutinating viruses from northern pintails in Tohoku district, Japan during winter November 2007–March 2008 ($n = 2381$).

Sampling site	Sampling period November 2007–March 2008					Total by site
	November	December	January	February	March	
Minase river	2/5/204[a]	0/5/248	0/1/239	0/0/106	10/2/162	12/13/959
Lake Izunuma	0/0/121	0/0/92	0/1/69	1/0/88	1/1/39	2/2/409
Lake Uchinuma	0/0/13	0/0/14	0/0/46	0/0/32	0/0/61	0/0/166
Lake Ogawara	0/0/101	2/1/53	2/0/110	1/0/101	2/3/142	7/4/507
Tsubo river	0/0/47	0/0/25	0/0/43	0/0/49	4/3/176	4/3/340
Total by month	2/5/486	2/6/432	2/2/507	2/0/376	17/9/580	25/22/2381
Total haemagglutinating virus isolation						47/2381 (1.97%)
Total AIV isolation						25/2381 (1.05%)
Total non-AIV haemagglutinating virus isolation						22/2381 (0.92%)

[a] AIV/non-AIV haemagglutinating virus/total number of composite fecal samples.

viruses. By HI test with NDV specific antiserum 11 out of 47 viruses were identified as NDV. Based on RT-PCR with NP gene specific primer, 25 samples were categorized as AIV, with overall isolation rate of 1.05% (25/2381). Rest of the haemagglutinating positive samples ($n = 11$) remained to be identified.

Out of 25 isolated AIVs, 17 isolates (68%) were obtained from samples collected in March 2008, whereas 8% AIVs (2/25) were obtained in each of the other months (Table 1). None of the antigenic subtypes found were detected twice from the same sampling site during the study period, except for H7N7, which was isolated from Lake Ogawara in January and March 2008 (Table 2). On the other hand, 48% (12/25) of the total AIVs were isolated from Minase river, followed by Lake Ogawara – 28% (7/25), and Tsubo river – 16% (4/25) (Table 1). None of the samples from Lake Uchinua were found to have positive haemagglutinating activity. Temporally, the relative prevalence of AIVs over the sampling period ranges from 0.39% to 2.93%. About 2.93% of 580 composite samples collected in March 2008 were found positive whereas only 0.39% out of 507 samples collected in January was found positive. The relative prevalence rates for November, December, and February were 0.41%, 0.46% and 0.53%, respectively (Table 2). None of the subtypes of AIVs were isolated from two or more sampling sites, except H7N7 which was isolated from three out of five sampling sites viz. Tsubo river, Lake Ogawara, and Izunuma (Table 2). Occurrence of non-AIV haemagglutinating viruses has similarity with that of AIVs in terms of sampling site and month. For example, 40.91% (9/22) of the total non-AIV haemagglutinating viruses were isolated from the samples collected in March 2008 and 59.09% (13/22) viruses derived from pintail wintering at Minase river (Table 1).

3.2. Analysis of HA and NA subtypes of AIV isolates obtained during November 2007–March 2008

Samples tested positive for AIV with NP specific RT-PCR ($n = 25$) were further analyzed by RT-PCR with HA and NA subtype specific primers. Antigenic analysis was done by HI and NI tests using polyclonal antisera. Both techniques (molecular and serological tests) resulted in identical findings, in terms of HA and NA subtypes. The distribution of HA and NA subtypes and their combinations are depicted in Fig. 1 and Table 3, respectively. A total of nine combinations of five HA and six NA subtypes were observed among the 25 isolates. The combinations and their proportional rates were H7N7 (36%), H1N2 (20%), H1N1 (8%), H4N6 (8%), H5N2 (8%), H11N9 (8%), H1N3 (4%), H5N3 (4%), and H5N9 (4%) (Table 3). No H5N1 subtype combination was observed. H1 subtype specific primers described earlier (Lee et al., 2001) failed to amplify specific gene. Then full length HA gene was amplified using the primer described by Hoffmann et al. (2001) and cloned into pCR2.1 vector (TA Cloning Kit.). BLAST search with nucleotides obtained after sequencing of purified plasmid signifies that the gene represents the H1 subtype of AIV.

3.3. Antigenic analysis of NDV isolated during November 2007–March 2008

Among 22 non-AIV haemagglutinating viruses, the majority of viruses ($n = 13$) were derived from composite samples collected at Minase river, followed by Lake Ogawara ($n = 4$), Tsubo river ($n = 3$), and Lake Izunuma ($n = 2$) (Table 4). Lake Uchinuma was negative in terms of virus isolation. Based on HI test with antibody against

Table 2
Temporal and spatial distribution of subtypes of AIVs isolated from northern pintails in Tohoku district, Japan during November 2007–March 2008.

Sampling time	Virus isolation[a]	Prevalence (%)	Subtypes
November	2/486	0.41	H5N2[b] (2)
December	2/432	0.46	H4N6[c] (2)
January	2/507	0.39	H5N3[c] (1), H7N7[c] (1)
February	2/376	0.53	H1N1[c] (1), H7N7[d] (1)
March	17/580	2.93	H1N2[b] (5), H7N7[b,c] (5 + 2)[‡] H1N1[d] (1), H1N3[e] (1), H5N9[e] (1), H11N9[e] (2)
Total	25/2381		

( ) Values in the parentheses indicate number of isolate.
[a] Positive/sample tested.
[b] Minase river.
[c] Lake Ogawara.
[d] Lake Izunuma.
[e] Tsubo river.
[‡] H7N7 isolates derived from two locations, namely b and c with number of isolate 5 and 2 respectively.

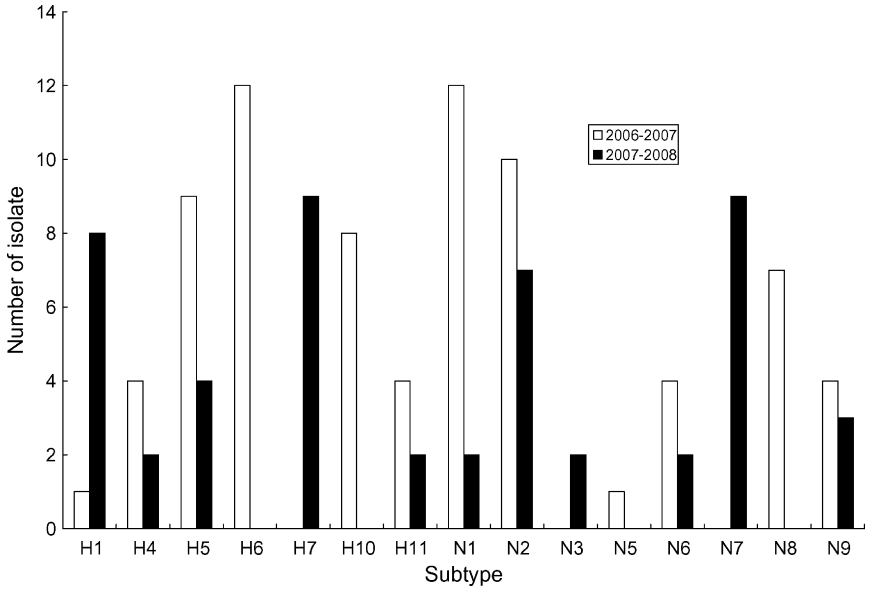

Fig. 1. Inter-annual comparison of distribution of haemagglutinin and neuraminidase subtypes of AIVs isolated from northern pintail in Tohoku district, Japan during two successive winter seasons: 2006–2007 and 2007–2008.

NDV, 50% (11/22) of the viruses were grouped into NDV (Table 4). The rest 11 viruses remained to be identified. All of the non-AIV haemagglutinating viruses isolated from Lake Ogawara and Tsubo river of Aomori prefecture were identified as NDV, whereas only 4 out 13 viruses from Minase river were likewise identified.

3.4. Characterization of H5 and H7 subtypes isolated during November 2007–March 2008

Pathogenicity test using molecular technique is regarded valuable (aside intravenous pathogenicity index), and was here applied. The sequence analysis of HA cleavage sites of the H5 and H7 isolates – namely the subtypes which might potentially be highly pathogenic – revealed that all those isolates were low pathogenic. Amino acid motifs deduced from nucleotide sequences obtained were PQRETR*G (H5 subtype) and ETPKGR*G (H7 subtype) (*cleavage point). All H7 isolates have residue proline (P) at position −4 from cleavage site, which is reported to be highly conserved among H7 subtypes (Lee et al., 2006).

3.5. Inter-annual variations of AIVs in pintails

Antigenic diversity along with proportional prevalence rate of AIVs isolated from northern pintails in two winter seasons from November 2006 to March 2008 is depicted in Table 3 and Fig. 1. During the first winter season (November 2006–March 2007) a total of 38 AIVs with 11 combinations of each of the 6 different HA and NA subtype were observed. In contrast, only 25 AIVs with 9 combinations of 5 different HA and 6 NA were isolated in the same season of the later year (2007–2008). In both winter seasons (2006–2008) H1N1, H4N6, H5N2, and H11N9 subtypes were commonly isolated, although their proportional prevalence rate varied. In winter 2006–2007, the proportional prevalence rate of H5N2

Table 3
Inter-annual comparison of different antigenic subtypes of AIV isolated from northern pintails in Tohoku district, Japan during two successive winter seasons: 2006–2007 and 2007–2008.

Subtype	Proportional prevalence (%)[a]			
	Season 1[b]		Season 2[c]	
H1N1	2.63	LO[d]	8.0	LI, LO
H1N2	–[e]		20.0	MR
H1N3	–		4.0	TR
H4N6	10.53	MR, LO	8.0	LO
H5N2	23.68	MR	8.0	MR
H5N3	–		4.0	LO
H5N9	–		4.0	TR
H6N1	7.89	MR	–	
H6N2	2.63	MR	–	
H6N5	2.63	MR	–	
H6N8	18.42	LU, LO, TR	–	
H7N7	–		36.0	MR, LI, LO
H10N1	18.42	LO	–	
H10N9	2.63	LO	–	
H11N1	2.63	LU	–	
H11N9	7.89	MR, LO	8.0	TR

LO, Lake Ogawara; LI, Lake Izunuma; MR, Minase river; LU, Lake Uchinuma; TR, Tsubo river.

[a] Proportional prevalence was calculated based on the number of specific subtype to total isolated subtypes per each season and expressed in percentage.

[b] November 2006–March 2007 [*Source*: Jahangir et al. (2008)].

[c] November 2007–March 2008 (this report).

[d] Markings indicates location/s of isolation of that specific subtype into each season.

[e] No isolate.

Table 4
Inter-annual comparison of prevalence of NDV and other haemagglutinating viruses isolated from northern pintails in Tohoku district, Japan during two successive winter seasons: 2006–2007 and 2007–2008.

Sampling site	Haemagglutinating viruses					
	Season 1[a]			Season 2[b]		
	Total sample	NDV[c]	Others[d]	Total sample	NDV	Others
Minase river	452	2	4	959	4	9
Lake Izunuma	540	3	6	409	–[e]	2
Lake Uchinuma	434	5	8	166	–	–
Lake Ogawara	432	–	3	507	4	–
Tsubo river	208	1	2	340	3	–
Total	2066	11	23	2381	11	11

[a] November 2006–March 2007.

[b] November 2007–March 2008.

[c] Newcastle disease virus.

[d] Viruses were not identified but caused haemagglutination with 0.5% chicken red blood cells.

[e] No isolate.

subtype was 23.68%, which decreases to 8.0% in 2007–2008. However, in the later season two more H5 subtypes with different NA subtype (N3 and N9) combinations were isolated. We isolated H7N7 subtype, in later season with highest proportional rate (36.0%). The most common HA and NA subtypes of first season were H6 and N1, respectively. However, in the following season H7 and N7 subtypes were more prevalent (Fig. 1). Interestingly, we could not found a single H7 and H6 subtype in 2006–2007 and 2007–2008, respectively. Furthermore, N7 subtype was not found in the former season.

According to the temporal data relating to the first season (Jahangir et al., 2008), most of the viruses were isolated from November to January, while in the following season maximal number of AIV subtypes isolated occurred in March (Table 2). Different combinations of various HA and NA subtypes of AIVs were detected during November–January of the first season. In contrast, only four subtypes of AIVs were isolated in the same period of the following season. However, six different AIV subtypes were isolated in March of the second season, whereas in the former season we could not isolate any AIV in March. Spatial variation is also evident in Table 3. For example, except Lake Izumuna, AIVs were isolated from all sampling sites in the former season. In contrast, in the following season virus was isolated from all sampling sites except Uchinuma.

3.5.1. Inter-annual prevalence variations of NDV and other haemagglutinating viruses

As is the case with AIV, prevalence variations of NDV and other haemagglutinating viruses were also observed during the study period (Table 4). Briefly, in the former season we could isolate maximal NDV from Lake Uchinuma. In contrast, not a single virus was found later on in that lake. Similarly, four NDVs were isolated from Lake Ogawara in the second season, whereas in the first season it was nil.

4. Discussion

4.1. AIV prevalence in northern pintail (2007–2008)

As a part of continuing surveillance of AIVs, we here report the findings of the second winter (November 2007–March 2008) investigation, based on samples derived from northern pintails, a widespread migratory waterfowl wintering in the sampling sites we chose. In addition, findings regarding NDV that we isolated from those samples, as well as from previous samples not yet published, are presented. The results indicate that different AIVs and APMVs, including NDV, are regularly, though infrequently, harbored by healthy northern pintails while wintering in Japan, and in part apparently persist from year to year. Viral circulation is likely sustained by ongoing fecal-oral transmission, as is the case with various enteric waterborne viruses.

On the whole, we isolated 47 haemagglutinating viruses from 2381 composite samples of freshly defecated fecal materials of pintails, collected throughout the winter season of 2007–2008. The overall prevalence rate of haemagglutinating viruses found in the 2381 composite samples was 1.97%. Prevalence of AIV was found to be 1.05% (25/2381) (Table 1). In our previous study (2006–2007), we also found a low prevalence rate – 1.84% (Jahangir et al., 2008). Our findings comply, though, with the findings of Stallknecht and Shane (1988). They reported that AIV prevalence becomes higher especially in breeding areas during summer and fall, in particular during premigratory congregation, but may decrease during birds' migration by 20 times. During wintertime, thus, a minimal rate of infectedness is foreseeable, and is consistent with the sampling locations of our study, which include wintering sites probed from November to March. Great variance regarding infectedness rate within aquatic birds, ranging from 0.6% to 26%, has been evidenced in previous studies reported in various publications (Hinshaw et al., 1980; Nettles et al., 1985; Otsuki et al., 1987; Slemons et al., 1991; Turek et al., 1983). Still, low level of infectedness is apparently sufficient for perpetuating both AIVs and APMVs, including NDV, as pointed at by our results.

4.2. Subtype variation and inter-annual comparison of AIV during 2006–2008

The findings obtained throughout two successive winter seasons (2006–2007 and 2007–2008) study strongly suggest that northern pintails carry different subtypes of AIVs every year. Although H5 and H7 HA subtypes are included among the viruses we isolated, all isolates were shown to be LPAI viruses.

In the present study (2007–2008) we isolated 25 AIVs with 9 different antigenic combinations, the most frequent one being H7N7 subtype ($n = 9$) (Table 3 and Fig. 1). This finding is inconsistent with some previous reports (Stallknecht and Shane, 1988; Krauss et al., 2004) where it is documented that generally viruses representing the H5, H7 and H9 are of low prevalence in wild ducks. On the other hand, our findings fit with the observations of Hanson et al. (2003) and Munster et al. (2005). They outlined these subtypes can be more common at specific locations and times. The subtype H7N7 was found from three out of five different sampling sites (Table 2). Moreover, from one of these three locations (Lake Ogawara) H7N7 subtype was isolated during two separate sampling periods (January and March).

Out of the nine subtypes detected throughout the second winter season, four subtypes were found in the first winter season as well (Table 3). During the later season we could not find any subtype belonging to H6, whereas it was found to be the most prevalent in the previous season. Also, N1 subtype was considerably more prevalent in the first winter than the following one. With regard to the LPAI H5 and H7 viruses we isolated, H5 was appreciably prevalent in both seasons, whereas H7 was found only during the second season, though most prevalently. These findings are compatible with the findings of Krauss et al. (2004) who reported that the diversity of subtypes in wild birds is not constant and varies between years. In one study Runstadler et al. (2007) documented that isolation and prevalence of AIV may vary year to year, season to season, month to month, week to week, and even day to day, and these variations may be due to immune status of the birds, landscape ecology (water levels, vegetation, etc.) and the changing distribution of species congregate. Normally, a subclinically infected individual duck would shed AIV for less than 2 weeks (Brown et al., 2006; Kida et al., 1980), so the prevalence of virus-positive ratio will be affected by the size of flock. This means that viruses will gradually disappear from a given flock, may be due to increasing herd immunity, as long as other susceptible host species are not collocated. Radio marking of pintails in Japan shows that pintails move around ranging only several kilometers from the wintering site once they set at winter (Alaska Science Center, 2007). This movement may provide, but not necessarily, a way of introduction of new virus at a given wintering site throughout the entire winter period as we found during two successive winter seasons. Further more, early onset of spring migration in second season, bringing about the arrival of new pintail flocks from southerly areas, could possibly have been the reason for the remarkably high relative infectedness we found in March 2008 (Tables 2 and 3) (Jahangir et al., 2008).

In another study, Stallknecht and Brown (2007) reported that AIV prevalence in wild birds may vary within populations of species, between hosts, locations and years, while various factors, including migratory behaviors, habitat preferences, and geographical ranges contribute to such variations. Our findings on subtype variations within two study periods are compatible with the earlier reports. Our previous (Jahangir et al., 2008) and present findings also com-

ply with the avowal that AIV prevalence varies location to location and year to year (Table 3). For example we isolated 38 AIVs during 2006–2007, but none of the viruses were isolated from samples collected from Lake Izunuma ($n = 540$) (Jahangir et al., 2008) (Table 3). Also, 68% (17/25) AIVs isolated during 2007–2008 were derived from samples collected in March 2008 ($n = 580$) (Table 1). In contrast, none of the samples collected in March 2007 ($n = 281$) were found positive for AIV.

Likewise, in winter 2007–2008 we detected three different NA subtype combinations with H1 subtype (H1N1, H1N2 and H1N3), while only one combination – H1N1 – was found in the preceding winter (Table 3). Interestingly, the primer sets used to identify subtype H1 in the first winter (Lee et al., 2001) failed to amplify specific gene of H1 subtypes isolated later on. To subtype these AIV isolates (H1N1, H1N2 and H1N3) we cloned the entire HA gene into pCR2.1 vector. Plasmid was purified after transformation to and culturing of competent *E. coli*. Finally, sequencing was performed by means of purified plasmid with vector specific primers, and through BLAST search of obtained nucleotide it was confirmed that the isolated subtype was H1. The failure of primer indicates that changes of nucleotides occurred at least at the target region of these primers (HA1). Failure of amplification of H1 gene segments of viruses isolated during November 2006–March 2007 in Japan with Lee's primer sets (Lee et al., 2001) also reported (Tsukamoto et al., 2008). Notably, it has been reported that H8 subtype is extremely rare in ducks (Hanson et al., 2005; Krauss et al., 2004; Stallknecht et al., 1990) and in the present study we did not find any H8 subtype, as was the case in our previous report (Jahangir et al., 2008).

All in all, then, the spatial and temporal variations we found during the two seasons regarding antigenic subtypes do not follow a specific pattern, as reported elsewhere (Runstadler et al., 2007); yet the multiplicity of subtypes allover, in just one host species, is notable (Table 3). We observed, however, that during both seasons there was indirect evidence for genetic reassortment events, taking into account the following antigenic combinations detected in the same sampling sites:

In the former season:

HA subtype H6 in different combinations with NA subtypes: N1, N2, and N5 (Minase river).
NA subtype N1 in different combinations with HA subtypes: H1 and H10 (Lake Ogawara).
NA subtype N9 in different combinations with HA subtypes: H10 and H11 (Lake Ogawara).

In the later season:

NA subtype N2 in different combinations with HA subtypes: H5 and H1 (Minase river).

While no physical connection can be pointed at between the pintail flocks of the two seasons, or of the different sampling sites mentioned above (Minase river and Lake Ogawara), the detection of the detailed antigenic combinations in certain sampling sites during the same season is likely indicative of the occurrence of reassortment events.

4.3. NDV and other haemagglutinating virus isolates

In the present study we isolated 22 non-AIV haemagglutinating viruses, and of these 11 (50%) were identified as NDV (Table 4). Somewhat differently, we isolated a total of 72 haemagglutinating viruses previously, and of these 38 viruses were AIV (Jahangir et al., 2008) (Table 4). Thus, the rest 34 viruses were non-AIV haemagglutinating viruses, namely APMVs or EDS virus. Out of these 34 isolates, 11 viruses (about a third) were identified as NDV at that time (data not published). Wild ducks are not only the natural reservoirs of almost all possible combinations of 16 HA and 9 NA subtypes of AIVs (Stallknecht, 1998; Swayne and Halvorson, 2003) but also carry and spread APMVs. In spite of various reports dealing with the isolation of APMVs from free living ducks, the epizootiology of these viruses in these diverse populations has received minute attention. This is partly because most isolation of APMVs from waterfowl has been adjunct to surveillance of these populations for AIV (Stallknecht et al., 1991). Another cause could be lessened public health significance, due to the mostly non-zoonotic nature of NDV. However, NDV is economically considerably important with regard to poultry industry throughout the world. To prevent incursions of these viruses along with others into poultry industry, solid concept about the epidemiology of these viruses is primary. As a part of AIV surveillance study APMV-6 along with other members of this group virus was first isolated from duck in Hong Kong by Shortridge et al. (1980). Later Stallknecht et al. (1991) isolated four serotypes of NDV, APMV-4, APMV-6 and APMV-8 from wild ducks, with NDV being the dominant one. Here we also examined but did not find any APMV-6 in pintail whereas we did isolate NDV. Based on present and previous findings (Jahangir et al., 2008) it is quite clear that in addition to AIV, NDV plus other APMV and/or egg drop syndrome virus are cocirculating every year within pintail populations wintering in Japan. The relative occurrence of NDV among the isolates we obtained allover was found to be 11 (NDV) 38 (AIV) 23 (other haemagglutinating viruses) for the first winter; and 11 (NDV) 25 (AIV) 11 (other haemagglutinating viruses) for the second winter. Just like AIV, then, NDV might be distributed over vast areas by pintails, along their migration routes.

4.4. Virulence of isolated H5 and H7 subtypes

Among 16 different HA subtypes of influenza viruses (Fouchier et al., 2005), infections with viruses of the H5 and H7 subtypes are of the most distress as these subtypes of viruses may mutate (or transform through genetic recombination) into highly pathogenic strains. It is reported that if low pathogenic H5 and H7 subtype viruses allowed for an extended period of time to circulate in poultry, they can undergo mutational changes that might result in the emergence of highly pathogenic strains of the virus (Banks et al., 2001; Hirst et al., 2004; Horimoto et al., 1995; Suarez et al., 2004). Notably, the antigenic subtype H7N7, which is the most prevalent one found in our study represents, as well, the most frequent antigenic subtypes among the various strains known as HPAI strains from 1959 until 2006 (regardless of the present enzootic HPAI H5N1) (Harder and Werner, 2006). Although virulence of AIV is polygenic, the HA protein plays a pivotal role in determining pathogenicity (Garten and Klnek, 1999; Steinhauer, 1999). Post-translational proteolytic cleavage of the precursor HA molecule into HA1 and HA2 subunits is essential for virus infectivity and spread of the virus (Klnek et al., 1975). The amino acid sequence at the cleavage site and glycosylation near the cleavage site are two chief structural features known to determine the HA cleavability. The amino acid motifs of HA cleavage site deduced from nucleotide sequences in our study signify the absence of multiple basic amino acids at the cleavage site. Thus the isolates belonging to H5 and H7 subtypes reported in this study were low pathogenic.

Sampling of healthy pintails for surveillance of HPAI viruses is highly relevant, taking into account their tolerance towards such viral strains. Pintails infected experimentally by the HPAI H5N1 virus were not affected clinically (Brown et al., 2006) thereby showing their capacity as potential carriers and spreaders of HPAI. At the same time, presumably there are HPAI strains that do affect pintails. The monitoring of pintails in terms of both LPAI and HPAI viruses is hence important. This attribute of pintails, together with their

extensively ramified intercontinental flyways, have thus significant implications with respect to both host and virus ecology.

5. Conclusions

Based on the results obtained, the perpetuation of AIV, NDV and other unidentified haemagglutinating viruses in – and possibly through – fecal materials of pintails is evident, as well as that pintail populations constitute an important source of these viruses for poultry and other avian species. Every year migratory pintails carry prevailing and apparently new variants of these viruses, thereby bringing about intra- and inter-species viral transmissions in their breeding grounds and stopover locations. The northern pintail might constitute a host and a spreader of prime importance, because it is in general a typically migrant species comprising huge populations that cover vast areas from the arctic to the tropics across the entire Northern Hemisphere. Furthermore, it is apparently one of very few species that migrate regularly from East Asia to North America. Its last wintering station along that route is Japan (Koehler et al., 2008; EPSN outdoors News). The relatively low infectedness rate of pintails found in this and our previous study is still significant, particularly considering that it apparently pertains to a population which is probably much more immune in comparison to summer and fall populations, within which appreciable portions of juveniles are still sensitive to infection (FAO/OIE/WHO). Early onset of spring migration in second season, bringing about the arrival of new pintail flocks from southerly areas, could possibly have been the reason for the remarkably high relative infectedness we found in March 2008.

In summary, we investigated the northern pintail, a widespread migratory duck, as a significant carrier of AIVs, with special reference to inter-annual comparisons of prevalence of AIVs and of their antigenic diversity. Some of the HA subtypes and NA subtypes detected during the first season reoccurred in the second season, as well as some of their combinations; yet, several new subtypes and combinations appeared during the second season. We also provide evidence of appreciable infectedness by NDV and other haemagglutinating viruses in this host species. Our data indicate that northern pintails are subclinically infected by, perpetuate, and distribute different subtypes of AIVs as well as NDV and other viruses of family paramyxoviridae and/or EDS virus, during their winter roaming in Japan and presumably later on, during their migrations across vast areas over the Northern Hemisphere.

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Appendix A

A.1. Identifying pintail feces

All of the investigated wintering sites are commonly occupied by northern pintails and whooper swans. In addition, yet much less prevalently, other waterfowl species roam (particularly at Lake Izunuma and Uchinuma area) and include spot-billed duck, pochard, tufted duck, geese and mallard (Paul Flint et al., Assessment of virus movement across continents: using northern pintail (*Anas acuta*) as a test, Trip Report, Capture and Marking of Northern Pintails in Japan, 11–21 February 2007, http://alaska.usgs.gov/science/biology/avian_influenza/projects.html, accessed 12th November, 2007). Only few mallard ducks and tufted ducks were observed at our sampling spots of these two lakes. At Lake Ogwara sea gulls were very common.

Another unique point to be taken into account is in that in all the sampling locations hand feeding is common. This practice made some of these birds populations accustomed to near humans whenever visitors reach the site. But most interestingly northern pintails became much more habituated with such feeding practice and very closely approach the visitors, in contrast to other collocated species.

Out of five wintering sites only at one wintering site (Lake Uchinuma) swans sometime approach visitors but not commonly, and during our visits we were never approached by them for feeding. Such behavior adopted by pintails creates an opportunity to easily and specifically obtain feces of pintails in the study area without direct catching them. Based on architecture, quantity and coarseness, it was not difficult to discriminate feces of swan origin. Although mallards' feces are similar, they never approach during sampling at Lake Izunuma. Other species also behaved as mallards. Generally, sea gulls' feces are yellowish or creamy and very small, fairly different from that of pintails (personal observation). Fecal materials were collected immediately after defecation by the pintail was observed. Throughout the winter (November–March) except November, the surrounding land of all the sampling sites was covered with snow which facilitated collection of uncontaminated feces. However, for sampling in the month of November more caution was used to avoid contamination with any surrounding material from the ground or grass. Based on all of these facts we pledge that collected fecal materials were of pintail origin.

A.2. Composite fecal sampling

Collecting fecal samples in the field can support comparative analyses and cannot yield findings in terms of absolute population infectedness rates. This being the case, because on the one hand sampling each fecal material separately may still include two or more fecal materials defecated by the same individual (during the same day, hence regarded as fresh feces); on the other hand, pooling two or more fecal samples as a composite sample may probably represent two or more individuals, rather than just one individual. Therefore, whenever fecal sampling is done in the field, pooling two or more samples into composite samples is rather expedient; it enables qualitative monitoring as well as comparative analyses (both qualitative and quantitative, relying all on the same methodological principal of sampling), though not precise quantitative monitoring.

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