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NOTE

Antibodies to H5 subtype avian influenza virus and Japanese encephalitis virus in northern pintails (*Anas acuta*) sampled in Japan

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Abstract

Blood samples from 105 northern pintails (*Anas acuta*) captured on Hokkaido, Japan were tested for antibodies to avian influenza virus (AIV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) to assess possible involvement of this species in the spread of economically important and potentially zoonotic pathogens. Antibodies to AIV were detected in 64 of 105 samples (61%). Of the 64 positives, 95% and 81% inhibited agglutination of two different H5 AIV antigens (H5N1 and H5N9), respectively. Antibodies to JEV and WNV were detected in five (5%) and none of the samples, respectively. Results provide evidence for prior exposure of migrating northern pintails to H5 AIV which could have implications for viral shedding and disease occurrence. Results also provide evidence for limited involvement of this species in the transmission and spread of flaviviruses during spring migration.

Key Words: avian influenza virus, Japanese encephalitis virus, West Nile virus

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Northern pintail (*Anas acuta*) ducks are commonly infected by avian influenza viruses (AIVs)[8,9,10], migrate between East Asia and North America at low rates[7, 16], and have been implicated in the intercontinental gene flow of AIVs[12, 20]. Serologic evidence supports prior infection of northern pintails sampled in East Asia with Japanese encephalitis virus (JEV) and West Nile virus (WNV) which raises the possibility that this species could be involved in the spread of these flaviviruses[27]. In this study, we assessed prevalence of antibodies to AIV, JEV, and WNV from northern pintail blood samples collected in Japan to better understand the possible involvement of this species in the transmission and spread of economically important and potentially zoonotic pathogens along migration routes.

Northern pintails were captured between 21 April and 9 May 2012 at the Waterfowl Observatory at Kutcharo Lake, Hokkaido, Japan (Fig. 1) using a traditional Japanese muso-ami (clap-style) net with permission of the Japanese Ministry of the Environment as part of ongoing efforts to assess the potential spread of infectious agents by migratory birds. Whole blood from 105 northern pintails was collected on eight replicate Nobuto filter paper strips (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) per individual, air-dried, and stored at ambient room temperature. Samples were then apportioned among laboratories for tests to detect antibodies to AIVs and flaviviruses. All methods were approved by the USGS Alaska Science Center's Animal Care and Use Committee (#2012-3).

For AIV tests, samples were prepared by cutting the blood absorbed portion of each filter paper strip into three or four pieces, placing in 400 µl phosphate buffered saline (PBS), and incubating for 60 min at room temperature according to the manufacturer's recommendations resulting in an approximate 1 : 10 serum dilution.

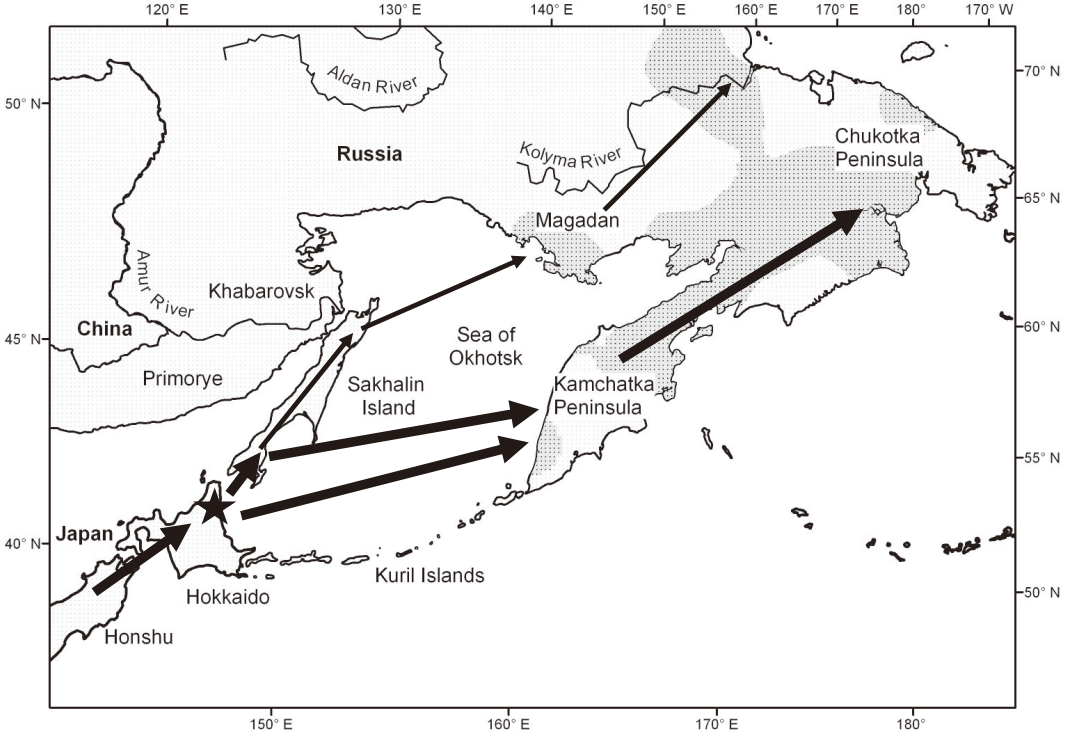

Fig. 1. Main migration routes of northern pintails marked in Japan to breeding areas in eastern Russia during spring as determined from satellite telemetry, 2007–2009. Adapted from Hupp *et al.*[7]. Arrow width is proportional to the number of marked individuals that followed each route. Shaded area represents the breeding distribution of marked pintails in eastern Russia. The approximate location of Kutcharo Lake is indicated by a star.

Samples were then screened for antibodies using a commercially available blocking enzyme-linked immunosorbent assay (bELISA; IDEXX Laboratories, Westbrook, USA) as reported in Dusek *et al.*[4]. Positive samples were subsequently tested by hemagglutination inhibition (HI) assay as described by Senne[23] using A/bar-headed goose/Mongolia/X53/2009 (H5N1) clade 2.3.2.1 for antigen. For HI assays, the 1 : 10 serum to PBS elution was treated with 10% chicken red blood cells (CRBCs) at ambient temperature for 30 minutes to remove non-specific agglutinins. Sample elutions were diluted to 2^{10} (titer of 1024) and any sample with a titer of 2^5 (titer of 32) or greater was considered positive. To confirm H5 specificity and help rule out steric inhibition associated with neuraminidase, all bELISA positive sera also were tested by HI using A/turkey/WI/1968 (H5N9). A subset of 16 bELISA positive samples also were tested by HI using a North American H9N2 poultry isolate, A/turkey/WI/1966, to rule out non-specific reaction. Fifteen HI positive samples were treated with restriction destroying enzyme (RDE) and re-tested to assess possible effects of non-specific inhibitors. Finally, 16 bELISA negative samples were tested by HI using A/bar-headed goose/Mongolia/X53/2009 (H5N1) clade 2.3.2.1 to serve as negative controls.

For flavivirus tests, filter paper strips were eluted in 1 ml of stock solution consisting of Dulbecco's PBS with CaCl and MgCl (100 mg/L each; Invitrogen, Burlington, Canada) and an antibiotic mixture of 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). Fluid was incubated at room temperature for 60 min, 4°C for 16 hours, and then eluate was transferred to a sterile tube and stored at −20°C until testing. Resultant eluate for each sample was estimated to be 1 : 10 (whole blood) sample concentration. Samples were tested for neutralization of JEV and WNV using plaque-reduction neutralization tests as reported in Yeh *et al.*[27]. The NY385-99 strain of WNV (American Type Culture Collection, Manassas, VA, USA) and Anyang300 strain of JEV (Animal and Plant Quarantine Agency, Anyang, Republic of Korea) were used in this study.

Elutions from 64 of 105 northern pintail samples tested positive for antibodies to AIV using bELISA (61%). Sixty-one of these 64 bELISA positive samples were also positive for HI using A/bar-headed goose/Mongolia/X53/2009 (H5N1) clade 2.3.2.1 antigen (95%; Table 1) and 52 of 64 were positive for HI of A/turkey/WI/1968 (H5N9) (81%; Table 1). All 16 bELISA positive samples were negative for HI of A/turkey/WI/1966 (H9N2) (Table 1). All fifteen samples treated with RDE were positive for HI of A/bar-headed goose/Mongolia/X53/2009 (H5N1) clade 2.3.2.1 and all 16 bELISA negative samples were negative for HI of this antigen. Five of the 105 (5%) northern pintail samples contained neutralizing antibodies to JEV (5%) while none tested positive for WNV.

The percentage of AIV positive birds detected in our sample of northern pintails from

Table 1. Hemagglutination inhibition assay titers of type A influenza bELISA positive samples from northern pintail ducks against A/bar-headed goose/Mongolia/X53/2009 H5N1 clade 2.3.2.1, A/turkey/WI/1968 H5N9 classical clade, and A/turkey/WI/1966 H9N2. Samples with a Log_2 titer ≥ 5 were considered positive.

Antigen	Log_2 titer								
	<2	3	4	5	6	7	8	9	10
A/bar-headed goose/Mongolia/X53/2009 H5N1	0/64[a]	2/64	1/64	6/64	42/64	4/64	7/64	2/64	0/64
A/turkey/WI/1968 H5N9	1/64	4/64	7/64	8/64	44/64[b]	NT[c]	NT	NT	NT
A/turkey/WI/1966 H9N2	16/16	0/16	0/16	0/16	0/16	NT	NT	NT	NT

[a]Number positive at/total tested
[b]Sera was only diluted to 2^6 when tested against the A/turkey/WI/1968 antigen, therefore the results may be interpreted as >6.
[c]NT = Not tested

Hokkaido, Japan in spring (61%) is consistent with seroprevalence reported in northern pintails in Alaska (57%)[25] and mallards (*Anas platyrhynchos*) sampled in Europe (62–68%)[3,24]. Prevalence estimates for H5 antibodies in northern pintails sampled in this study using two antigens (58% and 50% as reacting to H5N1 and H5N9 strains, respectively) were higher than those reported in mallards sampled at the Moselle wetlands, France (28%)[18] and wild ducks sampled in Pennsylvania, USA (25%)[17] and Alberta, Canada (6%)[14]. H5 subtype AIVs were isolated from 4% of mallards at the Moselle wetlands[18] and $<$1% of wild duck samples from Pennsylvania[17] and Alberta[14] during surveillance sampling associated with serum collection. Thus, it remains unclear if waterfowl sampled as part of these studies were previously exposed to H5 AIV at local wetlands or at other geographic locations prior to sampling.

During the autumn and winter of 2010–2011, highly pathogenic H5N1 was isolated from 63 wild bird samples collected from at least 17 different locations throughout Japan including H5N1 clade 2.3.2.1 isolates originating from a northern pintail, whooper swans (*Cygnus cygnus*), a greater scaup (*Aythya marila*), and 'duck' fecal samples collected at Hokkaido wetlands[22]. However, no outbreaks of highly pathogenic H5N1 were documented in Japan during the autumn and winter of 2011–2012 just prior to sample collection for this study[19]. Since 2001, low pathogenic H5 AIVs have been isolated at low rates ($<$1%) during winter surveillance from fecal samples of northern pintails[9,10] and other wild bird species in Japan[6], including samples collected at locations in Hokkaido[1,26]. It is not possible to determine if the detected antibodies resulted from previous exposure to H5 viruses recently isolated in Japan, but the high observed prevalence suggests either recent or recurring infections with AIVs of this subtype. With regard to recurring infections or exposures, northern pintails sampled for this study are highly migratory[7] (Fig. 1) and were all sampled after hatch year. Thus, it is possible that northern pintails have been exposed to multiple AIVs including H5 subtype viruses over their lifetime. Based on previous isolations of HPAI H5N1 in waterfowl in Japan[22] and other parts of East Asia[11, 15] we cannot discount that previous exposure to this virus may have occurred and contributed to the high H5 antibody prevalence observed in this study.

HI titers may be a function of affinity of antibodies to antigens, timing of sampling relative to exposure, and frequency of exposure. The geometric mean titer (GMT) for the 64 northern pintail samples that reacted to A/bar-headed goose/Mongolia/X53/2009 (H5N1) was 1 : 708 using a ten times conversion of observed titers (to correct for dilution through elution with PBS). These titers are higher than has been observed in mallards experimentally infected by A/duck/Vietnam/TG24-01/2005 (H5N1) calculated 24 days post inoculation (GMTs 1 : 104–338 depending on whether or not they had been primed with low pathogenic AIVs prior to inoculation)[5]. Antibodies to both homo- and heterosubtypic antigens have been shown to increase in wood ducks after subsequent challenge with A/whooper swan/Mongolia/244/2005 (H5N1)[2]. A similar effect may occur in northern pintails that have been exposed to multiple AIVs prior to spring migration. As prior AIV exposure may protect against overt disease and reduce shedding in waterfowl[2, 5], previous exposure of northern pintails to H5 AIV could influence transmission risks, disease occurrence, and viral shedding dynamics for migratory birds in East Asia.

The relatively low proportion of samples that tested positive for prior exposure to JEV and lack of samples positive for WNV provides evidence for limited involvement of northern pintails in the transmission and spread of flaviviruses during spring migration. Low rates of serologic exposure to flaviviruses have similarly been found in northern pintails sampled in Japan[21] and the Republic of Korea[27] during fall

migration. Explanations for the low detection of antibodies in northern pintail samples to flaviviruses include: lower levels of viral circulation prior to sampling periods, short duration of antibody persistence, poor viral replication in waterfowl, resistant to infection, or lack of exposure. WNV reservoir competence index values derived from susceptibility, mean daily infectiousness, and duration of infectiousness values for two waterfowl species, Canada goose (*Branta canadensis*) and mallard, were relatively low (0.03 and 0.48, respectively) among 25 avian species tested (range 0.00–2.55; mean 0.73) in experimental infections[13]. Given the sample size and limited spatiotemporal scope of the current investigation, additional sampling of northern pintails to test for prior exposure to AIV, JEV, and WNV may be warranted. Additionally, experimental laboratory inoculation studies that incorporate serum sampling using northern pintails would be useful for interpreting data on antibody presence and titers.

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