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Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005

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Abstract

Summary—This study describes surveillance for avian influenza viruses (AIV) in the Minto Flats State Game Refuge, high-density waterfowl breeding grounds in Alaska. Five hundred paired cloacal samples from dabbling ducks (Northern Pintail, Mallard, Green Wing Teal, and Widgeon) were placed into ethanol and viral transport medium (VTM). Additional ethanol-preserved samples were taken. Of the ethanol-preserved samples, 25.6% were AIV RNApositive by real-time RT-PCR. The hemagglutinin (HA) and neuraminidase (NA) subtypes were determined for 38 of the first-passage isolates, and four first-passage isolates could not be definitively subtyped. Five influenza Avirus HA–NA combinations were identified: H3N6, H3N8, H4N6, H8N4, and H12N5. Differences in the prevalence of AIV infections by sex and by age classes of Northern Pintail and Mallard ducks were detected, but the significance of these differences is undefined. In the 500 paired samples, molecular screening detected positive birds at a higher rate than viral isolation ($\chi^2 = 8.35$, $p = 0.0035$, df = 1); however, 20 AIV isolates were recovered from PCR-negative ducks. Further research is warranted to compare the two screening protocols' potential for estimating true prevalence in wild birds. Our success during 2005 indicates Minto Flats will be a valuable study site for a longitudinal research project designed to gain further insight into the natural history, evolution, and ecology of AIV in wild birds.

Introduction

Migratory birds from six continents pass through Alaska, where they form interspecies assemblagess which provide opportunities for the transfer and evolution of pathogens. The stimuli for this study were the rapid spread of the Asian lineages of highpathogenic (HP) H5N1

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avian influenza viruses (AIV) in Eurasia and Africa and reports of wild bird involvement in the epidemic, which raised concerns that wild birds might contribute to the introduction of the viruses into the Western Hemisphere. AIV is a particular concern because of the perceived risk for introduction and emergence of highly pathogenic strains of the virus into agricultural and human populations [3,4,8,41]. Birds migrating from Southeast Asia, China, Japan, or the Russian Far East could introduce the Asian lineages of HP H5N1 AIV into Alaskan wildbird nesting and breeding grounds where the viruses could pass to birds which later migrate to the lower latitudes using the North American migratory bird flyways [3,22].

Ducks were first shown to be natural hosts for AIVs in the Pacific (CA), Atlantic (DE), Mississippi (WI), and Central (NE) flyways during the early 1970s [29]. Waterfowl, shorebirds, and gulls are currently considered to be natural maintenance hosts for AIV, adapted to carry and transmit a wide range of influenza strains in nature [29,39,40]. Influenza strains that wild birds carry have been implicated in the three major influenza pandemics of the last century [36,40]. Prior to 2002, the majority of wild birds testing positive for AIV were apparently healthy, and patent disease was rare [3,13,35]. AIV can be found in many bird taxa but most consistently in waterfowl, gulls and shorebirds [16,22,32]. Prevalence of the virus in cloacal swabs taken from waterfowl and shorebirds or from samples of mud and water varies widely between 0 and 75% (R. Slemons and D. Halvorson, personal communication). Prevalence is affected by host species, host age, and season, and by many environmental factors. At any given location, the prevalence of different viral strains may fluctuate from year to year [9], but AIV is more frequently detected in birds migrating south from breeding grounds in the Northern Hemisphere in late summer than in birds migrating north from overwintering areas in the spring (R. Slemons, personal communication).

If public health authorities are to develop models for predicting human influenza pandemics, they must have a solid understanding of AIV natural history and epizootiology in the wild bird reservoirs. Few field studies have examined multiyear variability of AIV prevalence in single sampling locations and none have done so in northern latitudes. Furthermore, no studies have examined the dynamics of viral infection temporally along migratory routes. Many of the species showing high AIV prevalence are widely dispersed across multiple major flyways while most field studies have been restricted to Mid-Atlantic states, the Great Lakes region, or western Canada below 55° N latitude. The study of Arctic regions, where continental and intercontinental flyways merge, has been largely neglected but is likely to play a key role in the ecology of AIV strains. Information from entire flyways is required before we can understand and predict the occurrence, persistence, emergence, movement, and transmission of potentially pathogenic influenza viruses.

At our 2005 study location in Minto Flats, Alaska, Ito and colleagues collected feces between 1991 and 1994 to screen for avian influenza for their seminal study. They found an overall viral prevalence of 3–7% and recovered several subtypes and HA–NA combinations from “Minto Lake” [14]. The Minto Flats State Game Refuge and surrounding area have long been recognized as a major habitat for waterfowl populations and support some of the highest breeding densities on the continent [5]. This wetland complex is a nesting, brood-rearing, and molting habitat as well as a migration stopover site for 21 species of waterfowl including fifteen species of ducks, 2 species of geese, and Trumpeter Swans [2,12,24,26]. These waterfowl populations are not only substantially representative of the continental population of waterfowl but also an important subsistence and recreational resource in Alaska. Duck species breeding at Minto include Northern Pintail, Green-winged Teal, Mallard, Northern Shoveler, and Greater Scaup. These species are of specific concern for influenza dispersal because of their Holarctic distribution. Multiyear data on viral prevalence from this site should provide a major contribution to understanding viral movement in the major North American flyways.

There is an urgent need to expand surveillance for AIV in wild birds, and the development of rapid, efficient, and inexpensive surveillance methods that will facilitate these efforts is a priority [1]. In a study of respiratory virus surveillance in humans using ethanol-fixed nasal swabs, Krafft et al. used rapid, sensitive, molecular screening assays that did not require the culture and recovery of a viable specimen [15]. We reasoned that this approach could be applied to AIV surveillance in wild birds. The present study is the first to utilize ethanol-fixed cloacal swabs for AIV detection by molecular methods.

In this study, we examined the prevalence and antigenic diversity of avian influenza viruses circulating in wild dabbling ducks in the Minto Flats region of interior Alaska. We confirmed from cloacal swabs of individual ducks that several influenza subtypes and HA–NA combinations were causing infections in local populations of wild nesting ducks. In this report, we present baseline data for continuing studies of the evolution, dispersal, and ecology of avian influenza on a model breeding site for wild migratory ducks in Alaska. We also examine the differences observed between molecular and viral isolation methods for assessing viral prevalence.

Methods

Sample collection

Samples were collected from ducks live-trapped at 11 different trap locations in the Minto Flats State Game Refuge, an expanse of remote shallow ponds and waterways drained by the Chatinika and Tolvana Rivers in interior Alaska, near the Alaska Native village of Minto (GIS coordinates near 64° 53′ N, 148° 46′ W). Ducks were trapped and swabbed at the 11 sites on each day, twice a day – morning and evening – in cooperation with the Alaska Department of Fish and Game waterfowl banding operations from August 8-21, 2005. This period is after fledging of recently-hatched birds and premigration and corresponds to the molting time for waterfowl in the area.

A single Dacron swab with polyester shaft (Fisher Scientific, #14-959-90) was used to acquire a cloacal swab from each duck. All cloacal swabs were preserved in 1.2 ml 100% ethanol for later screening by real-time RT-PCR (RRT-PCR) analysis to detect influenza A virus matrix gene and screening for the AIV H5 gene. Whenever samples could be maintained in a strict cold chain (500 samples), matching swabs were placed into viral transport media (VTM) (M4RT from Remel Inc.) for later virus isolation attempts. The cold chain was maintained by keeping the cloacal swabs in VTM out of direct sunlight and then placing them on ice immediately after sampling. Within 3 hours, the VTM samples were placed in a cryogenic shipping container (−70 °C) and maintained at (−70 °C until processing. Ethanol-preserved samples were maintained in the dark at <30 °C in the field and at 4 °C in the laboratory until processing. The ethanol-preserved samples were shipped to co-authors JKT, ZMS, and DLE for molecular studies. VTM samples were shipped in cryogenic shipping containers to co-authors RDS and JN for viral isolation and further processing.

RNA preparation from ethanol-preserved samples

The total volume of each ethanol sample (1.2ml) was used for RNA preparation. Samples were vortexed for 10 sec and the swab removed using an aseptic technique. The ethanol cell suspension was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 14,000 rpm for 10min to pellet cells. After removing the ethanol supernatant, the remaining pellet was resuspended in 50 μl TE (pH 6.5). RNA was purified from swab samples with the MagMAX-96 viral isolation kit (Ambion, Inc.) according to the manufacturer's instructions. RNA from a positive control and a negative control (water) were included on each 96-well plate.

cDNA synthesis and RT-PCR

First-strand cDNA was produced using 2.0 μl of 0.5μg/μl random hexamer primers, 1.0 μl 10mM dNTP, 4.0 μl RNA and DEPC-water to 13 μl total volume. This reaction was incubated for 10 min at 65 °C and placed on ice. 4.0 μl of this reaction was added to 4.0 μl of 5× first-strand buffer, 2.0 μl 0.1M DTT, 1μl RNAse inhibitor and 1.0 μl MMLV reverse transcriptase (Invitrogen) to a total volume of 21 μl. The reaction was incubated at 25 °C for 10 min, 37 °C for 50min, and 65 °C for 10 min and placed on ice. Our technique is a two-step RRT-PCR in which we isolate the first-strand cDNA product and then utilize an aliquot of it for real-time PCR. In contrast, Spackman et al. used the entire first strand reaction mix for the follow-on Taqman™ assay [31]. We chose this two-step approach so as to preserve the primary RT-PCR product for as many Taqman™ assays as possible. First-strand cDNA was used in a screen for the matrix gene segment of AIV using a Taqman™ assay designed for this study using the following primers: Avian Influenza A Matrix Forward 5′-aratgagtcttc traccgaggtcg-3′; Avian Influenza A Matrix Reverse 5′-tgcaaagacatcytcaagyytctg-3′; and the internal probe 6-FAM-tcag gccccctcaaagccga-TAMRA. Degeneracy was incorporated into the primers to account for the diversity of matrix gene segment sequences currently available in GenBank; other- wise our primers are identical, and the cDNA primary product is predicted to be of the same length as those from Spackman et al. [31]. Primers and probes were synthesized by Integrated DNA Technologies, Coralville, IA. Each reaction utilized 12.5 μl 2× Universal master mix (ABI), 1 μl 20mM forward primer, 20 μM reverse primer, 5 μM FAM probe, 3 μl cDNA and 6.5 μl ddH_2O. Cycling conditions in an ABI PRISM Sequence Detection System 7700 were as follows: Stage 1–50 °C for 2min, Stage 2–95 °C for 10 min, and Stage 3–40 cycles of 95 °C for 15min and 60 °C for 1min. A threshold cycle (C_T) cutoff of <40 was used.

RT-PCR for cDNA templates larger than 200 bp was not observed in the ethanol-fixed samples. To control for unsuccessful RNA recovery in the ethanol-fixed swabs and for inappropriate amplification from DNA template in the extracted sample, we developed an RNA control assay using the cDNA sequence of the glyceraldehyde-3-phosphate dehydrogenase- like protein (GADPH, GenBank accession AY436595) of the mallard (*Anas platyrynchus*). The primers span the intron between exons 4 and 5 of the gene (as aligned to the chicken GADPH genomic DNA sequence, GenBank accession M11213). The primers are: forward 5′-cgggaatgcc atcaccat-3′; reverse 5′-agcatctgcccacttgatgtt-3′ giving a 60-bp product. A band that could only have arisen from the processed RNA transcript was only seen in samples after reverse transcription, demonstrating that the PCR product could not be generated from residual genomic DNA. We tested the assay on 100 randomly selected samples, representing all four bird species sampled in this study, and all gave a positive result. The control PCR product was ampli- fied in a reaction containing 5 μl 10× PCR buffer, 5 μl 25mM $MgCl_2$, 1μl 10mM dNTPs, 1μl of each primer, 0.5 μl Taq Gold (Applied Biosystems), and 2 μl of the cDNA. Each reaction was brought up to 50 μl total with ddH_2O. Cycling conditions for the GADPH positive control are as follows: 95 °C for 10min followed by 40 cycles of 96 °C for 30 sec/63 °C for 30 sec/72 °C for 30 sec and a final extension of 72 °C for 5min before cooling to 4 °C. Positive PCR bands were detected by ethidium bromide staining on a 1.5% agarose gel.

All RT-PCR matrix-gene-positive cases were subsequently tested for the H5 hemagglutinin subtype by RT-PCR using two different primer sets as described by Ng et al. [21].

Virus isolation; identification of type A influenza virus and HA and NA subtyping

Virus isolation attempts from the cloacal swabs placed in VTM were conducted using well established protocols [37]. Basically, antibiotics were added to each vial following thawing of the samples, and the vials were vortexed and centrifuged at 1200 rpm for 10min. Then, 0.15 ml of supernatant was inoculated into the allantoic cavity of each of four 10-day-old, specific-pathogen-free embryonating chicken eggs. The eggs were incubated for 48 hours and chilled

overnight, and the allantoic fluid was tested for the presence of hemagglutinating agents using chicken red blood cells. All hemagglutinating agents were analyzed for the presences of type-specific antigen of type A influenza viruses using the Directigen® Flu A test (Becton Dickenson Diagnostic Systems). All AIV isolates were sent to the U.S. Department of Agriculture National Veterinary Services Laboratory in Ames, Iowa, for HA and NA subtyping using the hemagglutination and neuraminidase inhibition tests [37]. HA molecular subtyping was also performed on viral RNA from second egg-passage stocks of these AIV isolates using the primer set described by Phipps et al. [25].

Results

Sample population—A total of 880 ducks (2 American Wigeons (*Anas americana*), 19 Green Winged Teal (*Anas crecca carolinensis*), 121 Mallards (*Anas platyrynchos*), and 738 Northern Pintails (*Anas acuta*)) captured in baited, live-traps were included in the study set. Within these groups, almost 90% of the birds sampled had hatched in 2005 and were classified as “hatch-year” (HY) ducks, and the remainder were classified as “after-hatch-year” (AHY) ducks. Pintail females were captured about twice as frequently as male Pintails, while the sexes sampled for Mallards and Green Winged Teals were captured in roughly equal proportions.

RT-PCR screen—A total of 880 cloacal swab samples were collected in ethanol and analyzed by RRT-PCR for the presence of the influenza A virus matrix protein gene, and 225 (25.6%) were positive. The percent of PCR positives from the 738 Pintail and 121 Mallard cloacal swabs were 24.1 and 37.2%, respectively, while 5.9% of the 19 Green Winged Teal samples and one of the two American Widgeon samples were positive (Table 1).

When all PCR data were analyzed for all species combined, there were no significant differences in viral prevalence correlated with sex or age (χ^2 0.020, $p = 0.89$, df = 1; $\chi^2 = 2.81$, $p = 0.094$, df = 1, respectively). Within the Mallards, there was a significantly higher prevalence of AIV infections in HY ducks ($\chi^2 = 4.17$, $p = 0.041$) compared to AHY ducks. The prevalence of AIV infections in female HY Mallards was 52.2% compared to a 31.7% prevalence in HY male Mallards ($\chi^2 = 4.54$, $p = 0.033$).

Viral isolation and subtyping—A total of 94 hemagglutinating agents were recovered from the 500 paired cloacal swabs placed in VTM. Forty-two of the hemagglutinating agents tested positive for type A influenza virus with the Directagen® Flu A test resulting in an AIV recovery rate of 8.4% (Table 1). The AIV isolates were recovered from 8.2 and 11.1% of Pintail and Mallard species, respectively. No AIVs were recovered from the 11 Green Winged Teal samples or the two American Widgeons. All of the isolates were from HY ducks.

Antigenic subtyping was completed on 38 of the 42 AIV isolates (Table 2). Antigenic subtypes included four HA subtypes (H3, H4, H8, and H12) and four NA subtypes (N4, N5, N6, and N8) found in five HA–NA combinations including H3N6 (3 Pintails), H3N8 (10 Pintails, 2 Mallards), H4N6 (9 Pintails, 2 Mallards), H8N4 (1 Pintail, 2 Mallards), and H12N5 (7 Pintails, 2 Mallards). Antigenic subtyping on the remaining four AIV isolates was inconclusive, which was most likely due to mixed AIV infections. The 38 samples serologically subtyped were subsequently analyzed by RT-PCR for a portion of the HA gene [25]. Blast search of the DNA sequence of the PCR products matched the serotype in each case.

All samples that tested positive for the matrix segment by RT-PCR were screened for the H5 subtype using two different H5 primer sets and none was H5 positive. No further subtyping of the positive samples collected in ethanol was possible in the term of this study. The published molecular subtyping assay results in a 640-bp product [25]. None of the tested ethanol-fixed samples yielded PCR products of this size.

Viral isolation vs. RT-PCR screen—Of the 880 ducks, a total of 245 (27.8%) were positive for AIV by viral isolation and/or RRT-PCR analysis. For the 500 samples assayed by both viral isolation and RRT-PCR analysis, 22 ducks were positive by both assays. However, 20 ducks were negative by the RRT-PCR screening and positive by viral isolation. In contrast, 52 ducks were positive by RRT-PCR screening and negative by viral isolation. Molecular screening detected positive birds at a higher rate (74/500, 14.8%) than viral isolation (42/500, 8.4%) ($\chi^2 = 8.35, p = 0.0038$, df = 1) (Table 1). Some possible reasons for these differences are discussed below.

Over the 14 days of sampling, spikes in the prevalence of AIV infections were detected by RRTPCR analysis between the 5^{th} and 10^{th} days of sampling (Table 3). These peaks occurred at a time when forest fires grounded float plane support, and we could not maintain the cold chain of custody required by VTM media to screen by egg inoculation. We continued to sample in ethanol during this time.

Discussion—We report several subtypes and HA/NA combinations in avian influenza viruses isolated directly from cohabitating Alaskan duck species. We believe that this relatively large data set creates a strong foundation for future studies of AIV prevalence, evolution, and ecology in Alaska environments. In addition, our use of viral isolation methods in parallel (and not subsequent to) molecular methods yielded striking differences between these two methods to screen for virus. These results indicate a clear need for critical analysis and caution in comparing studies of field prevalences of AIV using different methods.

Alaskan AIV—Viral subtypes differ among the three surveys done in Alaska in 1976 (R. Slemons, personal communication), 1991–94 [14], and 2005 (the present study). In a pioneering surveillance study 30 years ago, shortly after AIV had been discovered to be enteric in wild birds, Barney Easterday and his colleagues looked for AIV in wild birds in Alaska (R. Slemons, personal communication). Study sites were in the northernmost part of Alaska near Point Barrow on St Paul Island in the Bering Sea 100 miles west of the Alaska mainland. AIV isolates that were recovered from 11 species included at least seven hemagglutinin subtypes (H1, H2, H5, H6, H9, H10, and H11) and three neuraminidase subtypes (N2, N6, and N9). Other hemagglutinin subtypes may have not been detected by the then available reference antisera.

In the early 1990s, Ito and colleagues found AIV at a prevalence of 3.5% (108/3120) [14]. They isolated four HA subtypes in Minto Lake with H3, H4, and H10 being common (6–11 fecal samples) and H7 found in one sample (Table 2). They also found one H2 isolate elsewhere in Alaska. They reported six NA subtypes; N6, N7, and N8 were common, each found in 6–11 fecal samples, while N2, N3, N9 were each found in only one fecal sample (Table 2).

In our 38 ducks subtyped from Minto Flats collections in 2005, we found four hemagglutinin subtypes (H3, H4, H8, and H12) and four neuramidase subtypes (N4, N5, N6, and N8). Of the five HA/NA combinations we detected, three (H3N8, H4N6, and H2N5) were found more commonly (9–13 ducks each) while H3N6 and H8N4 were each isolated from three individuals.

H3 and H4 are widespread in wild birds across the eastern and western hemispheres, while H8 and H12 subtypes have been reported only rarely [16,22]. Both H8N4 and H12N5 were found at low frequencies (0.3%and 0.5%, respectively, among the viral isolates) in migrating ducks sampled in Alberta [28]. H12N5 was much more common (7.5%) in shorebirds. In addition, Slemons and coworkers have isolated multiple subtypes, including H12, from non-migratory ducks in eastern Maryland [30].

When birds migrating south in the fall are captured in such locations as Lake Erie or Chesapeake Bay on the east coast of North America, a significant proportion carry virus and the overwhelming majority of these infected birds are young of the year [22,34] (Slemons, unpublished). It may be that incomplete maturation of their immune systems and the physiological stress imposed by the many days of flying render the younger migrating birds more susceptible to infection when they are sampled at middle latitudes. In contrast, our prevalence data from Alaska shows no significant differences (at $p<0.05$) between young of the year and older birds on the nesting grounds at Minto Flats. The birds we trapped in 2005 are likely to be a mixture of those hatched in the immediate vicinity and those from other breeding areas. The relative frequencies of viral subtypes across seasons, years, and geographic sites, the differences in genotypes and phenotypes among host species, the immunological responses of each wild bird species to infection by each subtype, and the roles of abiotic factors in viral survival outside the host all deserve continuing systematic study over diverse geographical and ecological sites before one can construct computational models and predict the global ecology of AIV.

Full sequence data from the viral subtypes identified in this study may tell us something about the diversity within each of the subtypes present and about the frequency and potential for reassortment and selection in this population of viruses. There is a clear need for a surveillance sampling effort that has greater continuity over the span of several years to identify variation in viral prevalence and collect the appropriate collateral data to develop hypotheses to explain such changes. Our continuing AIV surveillance, in collaboration with the Alaska State Department of Fish and Game is a strong step in this direction.

VI vs. RT-PCR—Molecular screening for the presence of influenza virus detected roughly twice as many positive samples as did classical viral isolation in embryonating chicken eggs. Despite this, nearly half of the viruses grown (20 of 42) were not identified by the initial RT-PCR screen, although all viral isolates could be subsequently amplified by RT-PCR on the cultured sample. There is strong sequence conservation of the 5′ end of the matrix gene (sense orientation) in avian strains, making it unlikely that samples which grew a virus failed to amplify virus by RT-PCR due to poorly matched PCR primers. It is possible that PCR inhibitors in the cloacal samples may have accounted for this discrepancy in the case of very low viral titres.

The higher rate of detection in RT-PCR samples might also represent amplification from nonviable viral particles, but not due to live virus shedding [15]. Alternatively, the decreased sensitivity of viral isolation may be due to the inherent technical difficulty of sample storage, handling, and growth in embryonating eggs after the sample is taken. Ethanol fixation preserves viral RNA, whereas the VTM sample can potentially undergo bacterial overgrowth, and thus the virus might have been degraded into unamplifiable viral RNA and/or unculturable virus despite the best efforts at maintaining a strict cold chain until isolation. For the first 20 pairs of samples that were positive by RT-PCR from the ethanol-fixed sample and negative after we attempted viral isolation from the VTM sample, we did RNA extraction and molecular screening for the matrix protein gene on the VTM member of the pair. All these samples tested RT-PCR negative, suggesting that there was a difference between the VTM and ethanol samples at the time of processing.

Ecology of AIV—From our dataset, we can draw no definitive conclusions about differences in prevalence of different strains of AI across different sexes, ages, or species. Minto Flats is only one locality, albeit an unusually rich breeding ground for several important host species which we sampled repeatedly over only 10% of the season that the birds are in Alaska. We caught mostly young birds hatched in 2005 that were primarily Northern Pintail and Mallards.

In spite of these shortcomings, we believe that our results suggest several conclusions that deserve further investigation in subsequent years and in other localities:

- First, species appear to be differentially susceptible to avian influenza infection (Mallards > Pintails > Green Winged Teal) in our 2005 samples at Minto Flats. The earlier work of Ito and colleagues is consistent with this suggestion [14]. Like our results, those of the Ito group suggest that the distribution of viral subtypes across host species is non-random.
- Second, female birds, particular females hatched in the year of sampling, may be more likely to carry virus. A multiyear study of viral prevalence over different life history stages of ducks should help assess this possibility.
- Third, there are striking differences in prevalence seen over a two-week period of sampling from 4 to 68% on a given day.
- Fourth, although differences in AIV prevalence were not observed with the age of the birds when looked at as a group, a significant difference was observed within the Mallard group.

Prevalence peaks—It is unfortunate that the peak in forest fires which restricted access to Minto Flats coincided with the peak in viral prevalence seen after molecular screening (Table 3). There is no indication from the molecular data that the increases in prevalence on those days were due to contamination; in fact, the sampling protocol was designed to prevent contamination. A single aseptic swab was used for each sample and the sample tube was never opened in the field except to insert the swab. We believe that the most likely explanation of changes in prevalence is an influx of infected birds from outside Minto Flats in mid-August. Records of field biologists from earlier years show that some ducks banded in other areas of Interior Alaska are recaptured in Minto Flats weeks later (M. Petrula, personal communication). Such movements of birds could introduce different AIV strains originating from many different locations, thus altering prevalence and allowing potential mixing of different strains. The exchanges of birds among the localities could be studied by increased banding and recapture data, but other techniques may be even more powerful. Stable isotopes have been used to define the origin and movement of birds using feather samples and may identify the origins of birds that are sampled [10,11,17,18,20,27,38]. Additionally, improved understanding of the population genetics of the species sampled might allow a broader definition of breeding subgroups and AIV origin for a given sample and bird [6,7,19,23,33].

Although the use of alcohol-stored samples can increase the number of samples examined due to the ease of storage and transport, we would caution that the exclusive use of alcohol-stored samples will eliminate the availability of virus isolates for further molecular and pathogenic characterization of virus that can provide additional information on the ecology and biology of these viruses.

Concluding remarks—There is a clear need for an increased understanding and collection of data concerning AIV ecology. Many factors could contribute to the detection and prevalence of influenza viruses from year to year, season to season, week to week, and even day to day. These include the immune status of the birds, landscape ecology (water levels, vegetation, etc.), and the changing distribution of species assemblages.

The debate over the importance of wild bird transmission of highly pathogenic H5N1 strains of avian influenza virus became intense in 2006 and 2007. Worldwide, governments are spending many millions of dollars for surveillance in wild bird species while the H5N1 virus continues to spread into new regions in Europe and Africa and reemerge inparts of Asia [3]. In the agricultural arena, the black market and smuggling of infected birds remain highly

suspect as a cause of continuing outbreaks in domestic poultry. However, the development of wild bird surveillance plans has depended in part on the fear of increased human contact and opportunities for transmission that are due to contact with infected wild birds directly. To make a valid and meaningful assessment of the role that wild birds play in the origin, movement, and transmission of pathological forms of the influenza virus, we must understand the natural ecology and epidemiology of AIV.

In Minto Flats, we can carry out field studies to complement lab-based work to understand the ecology and biology that affect transmission, persistence, and evolution of the influenza virus. The ability to conduct collection of samples and associated metadata from large numbers of ducks of several species at the junction of key flyways makes Minto Flats a uniquely favorable location for multiyear studies of the ecology, evolution, and epizootiology of AIV.

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References

1. Proceedings of the 5th International Symposium on Avian Influenza. Athens, Georgia, USA. April 14–17, 2002. Avian Dis 2003;47(Suppl 3):783–1267. [PubMed: 14655685]
2. Bellrose, FC. Ducks, geese and swans of North America. 3rd. Stackpole Books; Harrisburg, PA: 1980.
3. Chen H, Smith GJ, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JS, Guan Y. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 2005;436:191–192. [PubMed: 16007072]
4. Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, Guan Y. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci USA 2006;103:2845–2850. [PubMed: 16473931]
5. Conant, B.; Hodges, JI. Minto Flats waterfowl resources. Juneau; Alaska: 1985. p. 1-4.Unpublished Report, Waterfowl Investigations U.S. Fish and Wildlife Service
6. Dawson RJ, Gibbs HL, Hobson KA, Yezerinac SM. Isolation of microsatellite DNA markers from a passerine bird, Dendroica petechia (the yellow warbler), and their use in population studies. Heredity 1997;79(Pt 5):506–514. [PubMed: 9369012]
7. Gibbs HL, Dawson RJ, Hobson KA. Limited differentiation in microsatellite DNA variation among northern populations of the yellow warbler: evidence for male-biased gene flow? Mol Ecol 2000;9:2137–2147. [PubMed: 11123625]
8. Guan Y, Poon LL, Cheung CY, Ellis TM, Lim W, Lipatov AS, Chan KH, Sturm-Ramirez KM, Cheung CL, Leung YH, Yuen KY, Webster RG, Peiris JS. H5N1 influenza: a protean pandemic threat. Proc Natl Acad Sci USA 2004;101:8156–8161. [PubMed: 15148370]
9. Hatchette TF, Walker D, Johnson C, Baker A, Pryor SP, Webster RG. Influenza A viruses in feral Canadian ducks: extensive reassortment in nature. J Gen Virol 2004;85:2327–2337. [PubMed: 15269374]
10. Herrera LG, Hobson KA, Rodriguez M, Hernandez P. Trophic partitioning in tropical rain forest birds: insights from stable isotope analysis. Oecologia 2003;136:439–444. [PubMed: 12802673]
11. Hobson KA, Bowen GJ, Wassenaar LI, Ferrand Y, Lormee H. Using stable hydrogen and oxygen isotope measurements of feathers to infer geographical origins of migrating European birds. Oecologia 2004;141:477–488. [PubMed: 15375691]
12. Hooper, DC. Waterfowl investigations at Minto Lakes. University of Alaska Fairbanks; Fairbanks, AK: 1952. p. 62

13. Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, Scholtissek C, Puthavathana P, Buranathai C, Nguyen TD, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci USA 2005;102:10682–10687. [PubMed: 16030144]

14. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 1995;140:1163–1172. [PubMed: 7646350]

15. Krafft AE, Russell KL, Hawksworth AW, McCall S, Irvine M, Daum LT, Connoly JL, Reid AH, Gaydos JC, Taubenberger JK. Evaluation of PCR testing of ethanol-fixed nasal swab specimens as an augmented surveillance strategy for influenza virus and adenovirus identification. J Clin Microbiol 2005;43:1768–1775. [PubMed: 15814997]

16. Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG. Influenza Aviruses of migrating wild aquatic birds in North America. Vector Borne Zoonotic Dis 2004;4:177–189. [PubMed: 15631061]

17. Marra PP, Hobson KA, Holmes RT. Linking winter and summer events in a migratory bird by using stable-carbon isotopes. Science 1998;282:1884–1886. [PubMed: 9836637]

18. Mazerolle DF, Hobson KA, Wassenaar LI. Stable isotope and band-encounter analyses delineate migratory patterns and catchment areas of white-throated sparrows at a migration monitoring station. Oecologia 2005;144:541–549. [PubMed: 15800746]

19. Milot E, Gibbs HL, Hobson KA. Phylogeography and genetic structure of northern populations of the yellow warbler (*Dendroica petechia*). Mol Ecol 2000;9:667–681. [PubMed: 10849283]

20. Moller AP, Hobson KA. Heterogeneity in stable isotope profiles predicts coexistence of populations of barn swallows Hirundo rustica differing in morphology and reproductive performance. Proc Biol Sci 2004;271:1355–1362. [PubMed: 15306333]

21. Ng EK, Cheng PK, Ng AY, Hoang TL, Lim WW. Influenza A H5N1 detection. Emerg Infect Dis 2005;11:1303–1305. [PubMed: 16102326]

22. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science 2006;312:384–388. [PubMed: 16627734]

23. Peters JL, McCracken KG, Zhuravlev YN, Lu Y, Wilson RE, Johnson KP, Omland KE. Phylogenetics of wigeons and allies (Anatidae: Anas): the importance of sampling multiple loci and multiple individuals. Mol Phylogenet Evol 2005;35:209–224. [PubMed: 15737592]

24. Petrula, MJ. Nesting Ecology of Ducks in Interior Alaska Department of Biology and Wildlife. University of Alaska Fairbanks; Fairbanks, AK.: 1994.

25. Phipps LP, Essen SC, Brown IH. Genetic subtyping of influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. J Virol Methods 2004;122:119–122. [PubMed: 15488629]

26. Rowinski, LJ. A review of waterfowl investigations and a comparison of aerial and ground censusing of waterfowl at Minto Flats. University of Alaska Fairbanks; MS.: 1958.

27. Rubenstein DR, Hobson KA. From birds to butterflies: animal movement patterns and stable isotopes. Trends Ecol Evol 2004;19:256–263. [PubMed: 16701265]

28. Sharp GB, Kawaoka Y, Wright SM, Turner B, Hinshaw V, Webster RG. Wild ducks are the reservoir for only a limited number of influenza A subtypes. Epidemiol Infect 1993;110:161–176. [PubMed: 8381747]

29. Slemons RD, Johnson DC, Osborn JS, Hayes F. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis 1974;18:119–124. [PubMed: 4205344]

30. Slemons RD, Hansen WR, Converse KA, Senne DA. Type A influenza virus surveillance in freeflying, nonmigratory ducks residing on the eastern shore of Maryland. Avian Dis 2003;47:1107–1110. [PubMed: 14575122]

31. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 2002;40:3256–3260. [PubMed: 12202562]

32. Spackman E, Stallknecht DE, Slemons RD, Winker K, Suarez DL, Scott M, Swayne DE. Phylogenetic analyses of type A influenza genes in natural reservoir species in North America reveals genetic variation. Virus Res 2005;114:89–100. [PubMed: 16039745]
33. Stai SM, Hughes CR. Characterization of microsatellite loci in wild and domestic Muscovy ducks (*Cairina moschata*). Anim Genet 2003;34:387–389. [PubMed: 14510679]
34. Stallknecht DE, Shane SM. Host range of avian influenza virus in free-living birds. Vet Res Commun 1988;12:125–141. [PubMed: 3055662]
35. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol 2005;79:11269–11279. [PubMed: 16103179]
36. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature 2005;437:889–893. [PubMed: 16208372]
37. Thayer, SG.; Beard, CW. Isolation and identification of avian pathogens. Swayne, DE.; Glisson, JR.; Jackwood, MW.; Person, JE.; Reed, WM., editors. American Association of Avian Pathologists; Kennett Square, PA: 1998. p. 255-256.
38. Wassenaar LI, Hobson KA. A stable-isotope approach to delineate geographical catchment areas of avian migration monitoring stations in North America. Environ Sci Technol 2001;35:1845–1850. [PubMed: 11355202]
39. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179. [PubMed: 1579108]
40. Webster RG, Hulse DJ. Microbial adaptation and change: avian influenza. Rev Sci Tech 2004;23:453–465. [PubMed: 15702713]
41. Xu X, Subbarao K, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 1999;261:15–19. [PubMed: 10484749]

Table 1

Summary of influenza viral isolation screening and a comparison and Chi-square tests of samples isolated by viral isolation versus the same subset of samples screened by RT-PCR

	Viral isolation			Matrix segment RT-PCR			VI vs. RT-PCR	
	Total #	+[a]	%	Total #	+	%	χ^2	p
Pintail	417	34	8.2	417	54	12.9	4.29	0.038
Mallard	72	8	11.1	72	19	26.4	4.31	0.038
Green Wing Teal	11	0	0	11	1	1	1.00	0.32
Total	500	42	8.4	500	74	14.8	8.35	0.0038

[a] + = number of positive samples.

Table 2

Summary of viral subtypes isolated in 2005 compared to previous studies

Subtype	1991–94 (Ito et al. [14]) Minto lake	2005 Minto flats
H3N6		3
H3N8	11	12
H4N6	6	11
H7N3	1	
H8N4		3
H10N2	1	
H10N7	7	
H10N9	1	
H12N5		9

Table 3

Matrix segment RT-PCR and viral isolation screening results by date

	Matrix segment RT-PCR			Viral isolation		
Date	**+ samples**	**# sampled**	**% positive**	**+ samples**	**# sampled**	**% positive**
8/8/2005	2	51	3.9	3	51	5.9
8/9/2005	8	118	6.8	11	118	9.3
8/10/2005	16	76	21.1	5	76	6.6
8/11/2005	12	89	13.5	10	75	13.3
8/12/2005	35	63	55.6	–	–	–
8/13/2005	3	36	8.3	–	–	–
8/14/2005	11	37	29.7	–	–	–
8/15/2005	10	44	22.7	–	–	–
8/16/2005	32	60	53.3	–	–	–
8/17/2005	40	59	67.8	0	1	0
8/18/2005	14	54	25.9	–	–	–
8/19/2005	14	67	20.9	5	53	9.4
8/20/2005	24	90	26.7	5	90	5.6
8/21/2005	4	36	11.1	3	36	8.3
Total	225	880		42	500	