An Early Detection System for Highly Pathogenic H5N1 Avian Influenza in Wild Migratory Birds

U.S. Interagency Strategic Plan

Introduction

Avian influenza (AI) is a type A influenza virus that is naturally found in certain species of waterfowl and shorebirds. However, the occurrence of highly pathogenic avian influenza (HPAI) subtype highly pathogenic H5N1 avian influenza has raised concern regarding the potential impact on wild birds, domestic poultry, and human health should it be introduced into the United States (U.S.). Numerous potential routes for introduction of the virus into the U.S. exist including illegal movement of domestic or wild birds, contaminated products, via an infected traveler, as a bioterrorism event, and the migration of infected wild birds. This plan focuses primarily on the detection of a potential introduction of highly pathogenic H5N1 avian influenza virus by migratory birds.

Avian influenza viruses are classified on the basis of two proteins, hemagglutinin (H) and neuraminidase (N), found on the surface of the virus. Specific viral subtypes have one of 16 different H proteins and one of 9 different N proteins, resulting in 144 possible combinations or subtypes based on this classification scheme. Within each subtype, there are numerous combinations of genetic sequences that determine the pathogenicity of the subtype to an infected host.

Wild birds, in particular certain species of waterfowl and shorebirds, are considered to be the natural reservoirs for all 144 subtypes. These subtypes are adapted to survive in these wild species and usually cause little or no disease. However, gradual genetic drift (i.e., mutation) can occur and a particular subtype can become adapted to infect other species of wild birds and domestic birds. Although this slight genetic change in the virus allows it to infect new species, it usually does not cause disease in the new host. The virus can also change if a host is simultaneously infected with another type A influenza virus. In such situations, mixing of the genetic material from the two virus strains (genetic shift) can occur, resulting in the formation of a new strain. The combination of gradual drifts and rapid shifts results in the production of a strain that now causes morbidity and mortality in susceptible hosts. If the morbidity and mortality is significant, the virus is classified as a highly pathogenic avian influenza (HPAI) virus.

During 1995-96, it is thought that antigenic drift occurred in an AI virus of wild birds, allowing the virus to infect chickens in China. This was followed by reassortment into the HPAI virus subtype highly pathogenic H5N1 avian influenza. Since that time, this highly pathogenic H5N1 has been circulating in Asian poultry and domestic fowl resulting in significant mortality to these species. Highly pathogenic H5N1 avian influenza likely underwent further antigenic drift and

shift allowing infection in additional species of birds, mammals, and humans. More recently, this virus moved back into wild birds resulting in significant mortality of species such as bar-headed geese, brown-headed gulls, black-headed gulls, ruddy shelducks, and great cormorants in China during April 2005.

Although the spread of H5N1 in Asia has been primarily due to movement of domestic birds, the movement of this virus into wild birds raised the possibility that these species may also spread the virus. This was thought to be the case in August 2005, when bar-headed geese and whooper swans died on Erkhel Lake, Mongolia, in an area not known to have domestic poultry or fowl nearby.

Given the adaptation of highly pathogenic H5N1 avian influenza to wild birds, increasing concern has developed over the potential for migrating species to introduce the virus into new regions of the world such as North America. Therefore, at the request of the Homeland Security Council's Policy Coordinating Committee for Pandemic Influenza Preparedness, the U.S. Departments of Agriculture (USDA) and Interior (DOI) were asked to develop a coordinated National Strategic Plan for early detection of HPAI introduction into North America by wild birds. Dr. Tom DeLiberto (USDA-APHIS Wildlife Services) and Rick Kearney (USGS Biological Resources Division) convened an interagency Working Group, which consists of representatives from USDA, DOI, U.S. Department of Health and Human Services (HHS), the International Association of Fish and Wildlife Agencies IAFWA), and the state of Alaska (Attachment 1).

On 10 August 2005, the Working Group met by teleconference to initiate development of a "Plan For the Detection of HPAI Virus in Migratory Birds in the United States". After some discussion among the participants it was decided that while the immediate concern was the introduction of highly pathogenic H5N1 avian influenza virus via migratory birds into Alaska and the Pacific Flyway (including Hawaii and other Pacific Islands), the group would also begin to address detection of the virus in all the North American flyways.

Goal of the Strategic Plan

The goal of this plan is to describe the essential components of a unified national system for the early detection of HPAI, specifically highly pathogenic H5N1 avian influenza, in migratory birds. While the immediate concern is a potential introduction of highly pathogenic H5N1 avian influenza into the U.S., the development of a system that is capable of detecting the introduction of all HPAI viruses through migratory birds would significantly improve the biosecurity of the Nation. This document provides guidance to Federal, State, university, and non-governmental organizations for conducting HPAI monitoring and surveillance of migratory birds in the U.S. It is expected that this document will be used by agencies and organizations to develop regional and/or state-specific implementation plans for HPAI surveillance.

Data collected in accordance with the guidelines presented in this document will be assimilated into a National database for use by all agencies, organizations, and policy makers. Furthermore, although the original charge of the Working Group was to monitor migratory birds as a potential route of entry into the U.S., the standardized methodologies and procedures identified in this

document are applicable to other wild birds as well. Agencies and organizations conducting monitoring and surveillance in non-migratory birds are encouraged to follow these guidelines so that their data can be incorporated into and tracked via the National Early Detection System. This system for highly pathogenic H5N1 avian influenza detection will provide early warning for potentially catastrophic mortality events in North American wild birds and poultry, and minimize the potential for human exposures. Agencies and organizations are encouraged to participate in this system by following the guidelines presented in this document when conducting AI sampling in wild birds.

While this plan focuses on detection of highly pathogenic H5N1 avian influenza virus, the Working Group fully supports efforts to characterize all AI viruses in wild birds. Such information is critical to our understanding of the ecology of AI viruses and their transmission among wildlife, livestock, and humans. Birds will be sampled in conjunction with existing studies when possible, and additional bird captures will be initiated as necessary to provide a broad species and geographic surveillance effort.

A National Early Detection System for Highly pathogenic H5N1 avian influenza in Migratory Birds

The ability to efficiently control the spread of a highly infectious, exotic disease such as highly pathogenic H5N1 avian influenza, is dependent upon the capacity to rapidly detect the pathogen if introduced. For this reason, a National Early Detection System for Highly pathogenic H5N1 avian influenza in Wild Migratory Birds is not only prudent, it is necessary. Effective implementation of this National Detection System will require decentralized planning and execution at regional and state levels, combined with centralized coordination to ensure national level analysis of surveillance data for risk assessment. It also must involve a partnership between public and private interests and include efforts by Federal, State, and local governments as well as nongovernmental organizations, universities, and other interest groups. Lastly, it requires flexibility and commitment by all groups for successful implementation.

Decentralized Planning and Execution

Wild migratory birds, by their very nature, are not subject to disease containment controls as are domestic birds and people. While their movements are generally uncontrollable, these movements are largely predictable on both a daily and seasonal basis. Local movements within or between breeding, feeding, and roosting areas are frequently well known by State and local wildlife management authorities and others familiar with local bird populations. Long range movements associated with seasonal migration are also well known for many species, especially those waterfowl and shorebird species of particular interest in highly pathogenic H5N1 avian influenza detection and surveillance.

Coordinating groups such as the four Flyway Councils already exist to deal with issues related to migratory bird management on a broad geographic scale. These Councils include representation from each of the States in their respective bird flyways as well as the U.S. Fish and Wildlife Service. Therefore, the planning and execution of local and regional highly pathogenic H5N1

avian influenza early detection efforts will best be accomplished by the States in collaboration with Federal agencies.

Centralized Coordination

States and flyways are exposed to varying degrees of threat from highly pathogenic H5N1 avian influenza. Each has unique circumstances that will shape the direction and intensity of its early detection efforts. Consequently, gaps among regional programs may emerge over time. Centralized coordination will evaluate the effectiveness of state and regional efforts, allowing for prioritization of available federal resources.

Integration of this National Early Detection System with similar influenza surveillance systems in other species (e.g., domestic, feral, zoo) as well as humans will also require centralized coordination. Surveillance data from all of these systems will be incorporated into national risk assessments, and preparedness and response planning efforts.

Geographic Prioritization of Sampling Efforts

This Strategic Plan targets bird species in North America that have the highest risk of being exposed to or infected with the highly pathogenic H5N1 avian influenza subtype because of their migratory movement patterns. Currently, these include birds that migrate directly between Asia and North America, birds that may be in contact with species from areas in Asia with reported outbreaks, or birds that are known to be reservoirs of AI. However, should highly pathogenic H5N1 avian influenza virus be detected in domestic birds in the U.S., sampling of wild birds within the affected flyway may become a high priority as well.

In general, bird flyways represent migration corridors within continental landmasses. However, Alaska and areas in Eastern Siberia represent a unique situation where major flyway systems cross continental boundaries (Attachment 2, fig. 2-1). Two major Asian flyways (the East Asian-Australasian and East Asian) include both Southeast Asia and the Arctic regions of Siberia, the Russian Far East, and Alaska. The East Asian-Australasian Flyway, defined primarily in the context of shorebird use, extends across 20 countries from the Siberian and Alaskan Arctic through North and Southeast Asia including U.S. trust territories in the Pacific to Australia and New Zealand.

Similarly, in North America, the Pacific Flyway extends from Arctic Canada, Alaska, and Eastern Siberia through coastal and western regions of Canada, the United States and Mexico, and on to Central and South America (Attachment 2, Fig. 2-2). Many migratory species that nest in Arctic Siberia, Alaska, and Canada follow the Pacific Flyway to wintering areas. Although not considered a major pathway, birds from both Eastern Siberia and Alaska intermingle in both the Pacific and Central Flyways. The overlap at the northern ends of these flyways and in Hawaii and Oceania establishes a path for potential disease transmission across continents and for mixing, re-assortment, and exchange of genetic material among strains from Eurasia and North America.

If highly pathogenic H5N1 avian influenza virus spreads to North America via migratory birds, the above analysis of the major flyways suggests that the virus would most likely arrive first in Alaska. Such a scenario is reasonable, as the contribution of Eurasian AI viruses to the genetic composition of viruses in North American migratory birds has already been demonstrated. Given the current knowledge on highly pathogenic H5N1 avian influenza distribution, the Working Group developed a prioritized sampling approach based on Alaska and the major North American flyways.

This approach prioritized the following regions in decreasing order of importance:

1. Alaska, the Pacific Flyway, and Oceania
2. Central Flyway
3 Mississippi Flyway
4. Atlantic Flyway

Agencies participating in the development of this plan are committed to efforts that ensure adequate sampling based on the above prioritization. However experiences with previous introductions of exotic diseases into North America (e.g., West Nile Virus) have demonstrated that detection and surveillance systems must be adaptable to changes in pathogens and risk factors associated with their potential introduction. If changes in the relative risks of highly pathogenic H5N1 avian influenza introduction into the US result in regional reprioritization, agencies must be prepared to redistribute resources accordingly.

Sampling Strategies

This strategic plan recommends decentralized planning and execution of highly pathogenic H5N1 avian influenza early detection efforts. To provide a uniform structure for the development of local plans, it recommends the consideration of five strategies for collecting monitoring and surveillance data on highly pathogenic H5N1 avian influenza virus in wild birds. Agencies and organizations are encouraged to use one or more of these strategies when designing AI surveys in wild birds. These strategies are:

Investigation of Morbidity/Mortality Events (Attachment 3):

Over 40 species of wild birds have been shown to be susceptible to infection with highly pathogenic H5N1 avian influenza virus. While not all species infected necessarily exhibit disease, the current strain(s) of H5N1 circulating in Asia have been shown to cause morbidity and mortality in a wide variety of these species. The systematic investigation of morbidity and mortality events in wild birds to determine if highly pathogenic H5N1 avian influenza is playing a role in causing illness and death offers the highest and earliest probability of detecting the virus if it is introduced by migratory birds into the United States. State natural resource agencies and Federal refuges and parks, primarily within the DOI's U.S. Fish and Wildlife Service National Wildlife Refuge System and the National Park Service, are the principal authorities in a position to detect and respond to mortality events involving wild birds. Morbidity and mortality events

involving wildlife are often detected by, or reported to, these agencies and entities. This strategy capitalizes on an existing morbidity/mortality program being conducted by DOI and its partners.

Surveillance in Live Wild Birds (Attachment 4):

This strategy incorporates sampling of live-captured, apparently healthy wild birds to detect the presence of highly pathogenic H5N1 avian influenza virus. This effort will select bird species in North America that represent the highest risk of being exposed to, or infected with, Highly pathogenic H5N1 avian influenza virus because of their migratory movement patterns, which include birds that migrate directly between Asia and North America, or birds that may be in contact with species from areas in Asia with reported outbreaks. Should highly pathogenic H5N1 avian influenza virus be detected in domestic birds in the U.S., sampling of wild birds in the flyway in the affected area may become a high priority as well. Data collected by organizations currently conducting research and monitoring for avian influenza in Alaska will be incorporated with additional bird captures as necessary to provide a broad species and geographic surveillance effort. This strategy capitalizes on research activities currently being conducted by DOI, USDA and their partners.

Surveillance in Hunter-killed Birds (Attachment 5)

Check stations for waterfowl hunting are operated by the US Fish and Wildlife Service and state natural resource agencies. Hunter check stations provide an opportunity to collect additional samples to determine the presence of HPAI and other subtypes of avian influenza viruses and supplement data collected during surveillance of live wild birds. As with surveillance of live wild birds, sampling of hunter-killed birds will focus on hunted species that are most likely to be exposed to HPAI in Asia; have relatively direct migratory pathways from those areas to the U.S. via Alaska or directly to the Pacific Coast; mix in Alaska staging areas with species that could bring the virus from Asia; or should HPAI be detected in domestic birds in the U.S., may mix with wild birds in the flyway of the affected area. Collection of samples from these species will occur at hunter check stations in the lower 48 states during hunting seasons in areas where these birds stage during migration or over-wintering.

Sentinel Species (Attachment 6):

Waterfowl, exhibition gamefowl, and poultry flocks reared on backyard premises have been used as sentinels for active surveillance for avian diseases of interest to the commercial poultry industry and regulatory agencies. Currently in Alaska, the State veterinarian uses targeted surveillance of domestic flocks at concentration points due to remote location of villages and lack of resources; enthusiasts travel to poultry exhibitions with birds from distant locations; and. surveillance effectively covers a large geographic area. Enhancement of this approach would be valuable. However, placement of sentinel ducks in strategic locations may also prove useful. Placement of sentinel ducks has been used successfully for surveillance of diseases of importance to the poultry industry, including influenza A. Also, sentinel ducks in wild pelagic bird colonies improved virus detection rates fivefold, suggesting that this approach is advantageous in ecological studies.

Environmental Sampling (Attachment 7):

Avian influenza viruses are generally released by waterfowl through the intestinal tract and viable virus can be detected in both feces and the water in which the birds swim, defecate and feed. This is the principal means of virus spread to new avian hosts and potentially to poultry, and other susceptible livestock. Analysis of both water and fecal material from waterfowl habitat can provide evidence of AI virus circulating in wild bird populations, the specific AI subtypes, levels of pathogenicity, and possible risks to poultry and susceptible livestock. Monitoring of water and/or fecal samples gathered from waterfowl habitat is a reasonably cost effective, technologically achievable means to assess risks to poultry.

Sample Collection

Samples collected for AI surveillance may include carcasses, tracheal and cloacal swabs, feces, and environmental samples (e.g., water). Prior to initiating a surveillance activity, it is important to identify the laboratory in which the samples will be submitted. Sample handling and transportation procedures may differ among laboratories. It is recommended that samples collected for inclusion into the National Early Detection System be submitted to a laboratory that uses standardized procedures identified in the Laboratory Diagnosis section of this document or by using the attached detailed descriptions of sampling methodologies.

If birds are found morbid or dead, it is important to use proper personal protection techniques (http://www.nwhc.usgs.gov/research/WHB/WHB_05_03.html, http://www.cdc.gov/flu/avian/professional/protect-guid.htm) and to submit the entire carcass to a veterinary diagnostic laboratory for necropsy (Attachment 8). Field biologists should contact the specific laboratory that they will be working with well in advance of any specimen collection and shipping to receive specific instructions for specimen submissions to that laboratory. Laboratories should always be notified ahead of time when a shipment is being made to their facility.

When collecting samples from live or hunter-killed birds, tracheal and cloacal swabs are preferred. Most AI strains tend to replicate more efficiently in the intestinal tract than in the respiratory tract of natural host species (i.e., waterfowl and shorebirds). Consequently, cloacal swabs are generally preferred. However, recent isolations of highly pathogenic H5N1 avian influenza virus in wild birds have documented higher levels of virus in tracheal samples. Therefore, it is recommended that both samples be collected from birds when possible. While the collection of cloacal swabs is a relatively easy procedure, obtaining proper tracheal swabs can be problematic and requires personnel trained in the sampling technique. Examples of tracheal/cloacal swab collection protocols can be found in Attachment 9. Tracheal and cloacal swabs should be placed in separate tubes, and swabs should not be pooled across individuals.

Monitoring of water and/or fecal samples gathered from waterfowl habitat is a reasonably cost effective, technologically achievable means to detect the presence of HPAI and alert decision makers to the risks to poultry in the Western Hemisphere from new, potentially highly pathogenic subtypes of AI (Attachment 7). A surveillance system based on water sampling is not ready to implement at the present. However, the validation of this method could come on-

line in a short period of time and would represent considerable cost savings without loss of sensitivity. Fecal sampling is an established technique and is ready for use in surveillance with the establishment of sampling guidelines. Both approaches yield advantages where individual bird sampling is too costly or logistically impractical. Either approach could yield a spatial and habitat risk assessment for site contamination with highly pathogenic H5N1 avian influenza virus. The main considerations are where and when to get the samples, ensuring proper storage and transport, and the capacities and capabilities of the laboratories doing the analyses. Real-time reporting and the infrastructure to support such reporting is a serious constraint on any surveillance system. The ability to integrate, analyze, and responsibly disseminate these data is critical and needs to be addressed.

Sample Size Determination

Prior to initiating a surveillance program, it is important to determine the sample size necessary to make statistically valid inferences concerning the presence of highly pathogenic H5N1 avian influenza virus in a sample population. In the context of this plan, the population of interest is not defined because this definition will vary by geographic location, time of year, species of interest, and sampling method employed. For example, sampling a breeding population versus a wintering population, for a single species, may result in very different interpretations of the geographic distribution of the population of interest. If water samples are being collected, then the population may consist of several water bodies. Therefore, it is crucial that prior to collections beginning, statistically valid sample size estimations be incorporated into regional and state implementation plans.

Laboratory Diagnostics

All samples collected for inclusion in the National Early Detection System should be analyzed in accordance with the standard procedures included in this document. A list of laboratories certified to conduct testing for highly pathogenic H5N1 avian influenza virus is included in Attachment 11. Samples will be analyzed as soon as possible after collection. Tracheal/cloacal swabs and fecal samples will be analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using the matrix gene RT-PCR assay (Attachment 12). The matrix gene RT-PCR assay is capable of detecting all 16 hemagglutinin and nine neuraminidase subtypes. Matrix gene RT-PCR-positive samples would indicate the presence of avian influenza and they should be further characterized by the H5- and H7-specific RT-PCR assays of Spackman et al. (2002) as modified in Attachment 11. The H5 RT-PCR test is known to detect the current Highly pathogenic H5N1 avian influenza viruses.

Positive H5 and H7 RT-PCR tests would indicate the presence of AI viruses with the potential of causing pathology in domestic poultry. Therefore, all samples positive for H5 and H7 by RT-PCR will be submitted for virus isolation for verification. Samples positive for live virus in virus isolation and positive for H5 or H7 by RT-PCR will be submitted to the USDA APHIS National Veterinary Services Laboratory (NVSL) for confirmation. The NVSL is capable of performing the intracranial chicken pathogenicity index (ICPI) test on the resultant virus to determine directly the pathogenicity of the virus in chickens. Identification of a highly pathogenic H5 or H7 virus is a reportable disease and immediate notification to the agency submitting the sample,

the state veterinarian, the area veterinarian in charge (AVIC), the state public health official and the CDC/USDA Select Agent program. Samples will be immediately secured as required by the Select Agent Programs.

All positive H5 and H7 samples will also be sent to the USDA Agriculture Research Service Southeastern Poultry Research Laboratory in Athens, GA, for complete molecular sequencing. This will provide for complete typing of the virus and allow for phylogenetic analysis.

Data Management

Real-time reporting and the infrastructure to support such reporting is a serious constraint on any surveillance system. The ability to integrate, analyze, and responsibly disseminate these data is critical. In addition, the data collected for this National Surveillance System will consist of samples submitted by many agencies and organizations. This will require a system to manage the input of animal and sample collection data through multiple routes, the ability to easily match, compare, and transfer laboratory data about these samples, and provide a platform in which all data is secure, accessible, and able to be mapped and used for spatial modeling.

The National Biological Information Infrastructure Wildlife Disease Information Node (WDIN) managed by the U.S. Geological Survey's National Wildlife Health Center has created a prototype web-enabled HPAI data management system, which will serve as a template for data collected from live and hunter-killed wild birds. (See http://wildlifedisease.nbii.gov/ai). The WDIN has developed comparable systems for the management of data from multiagency wildlife disease surveillance efforts such as Chronic Wasting Disease, and for USDA APHIS Wildlife Services Plague and Tularemia. General aspects of the proposed WDIN Interagency HPAI Data Management System are described in Appendix 13.

Sentinel bird data will be incorporated into a web-enabled, national data management system for backyard and small-flock poultry developed by the USDA APHIS Veterinary Services' Application Information Management Team at the Centers for Epidemiology and Animal Health. This database system was developed to reduce the number of data-collection problems experienced by field personnel and to generally improve the quality and efficiency of data collection. The initial testing of the design occurred in October 2005, with actual deployment scheduled for December 2005. If the project is successful in the pilot state of California, the system will likely be expanded for national implementation in 2006. This system will allow all necessary data collected in the field to be shared among all approved organizations without the need for manual data entry, and will provide greater chain-of-custody assurance from a legal and diagnostic perspective. Field personnel will be equipped with computer hardware and software which will facilitate the rapid and accurate collection of samples and data. These devices will share the collected information as needed with the diagnostic lab (National Veterinary Services Laboratories), and will send the data to primary information systems within USDA. To assist in data entry and to further improve data quality, bar-coding will be implemented as key identifiers for samples collected and for cases submitted.

Recommendations

Given the current state of knowledge of the epidemiology of highly pathogenic H5N1 avian influenza virus it is recommended that a coordinated interagency/organization early detection system be implemented in the U.S. An analysis of risk factors, including current worldwide distribution of the virus and the migratory patterns of wild birds, indicated that this system should primarily focus sampling efforts in Alaska, Oceania, and the Pacific and Central flyways. However, if adequate resources become available, the system should be expanded to include surveillance of migratory birds in the Mississippi and Atlantic Flyways as well.

State and Federal agencies should immediately begin developing implementation plans based on the guidance provided in this Strategic Plan. Development of these plans should be conducted with the participation of all relevant management agencies and organizations such that sampling designs are produced that allow for statistically sound inference of the presence or absence of highly pathogenic H5N1 avian influenza virus in wild birds. Furthermore, it is recommended that such coordination be conducted through the Flyway Councils, so that regionally based sampling designs can be implemented. Each Flyway (Atlantic, Mississippi, Central, and Pacific) has a Flyway Council, a formal organization generally composed of one member from each State and Province in that Flyway. The Flyway Councils, established through the International Association of Fish and Wildlife Agencies (IAFWA), also assist in researching and providing management techniques for Federal, State, and Provincial Governments, as well as private groups and the public.

State and Federal agencies also should develop communication plans in the event that a HPAI is detected in wild birds. For example, highly pathogenic H5N1 avian influenza virus is a reportable disease that requires notification of the State Veterinarian, and the Area Veterinarian in Charge (AVIC). Highly pathogenic H5N1 avian influenza is also a CDC/USDA Select Agent, requiring notification of the CDC/USDA Select Agent Programs and adherence to Select Agent guidelines is required.

Finally, it is recommended that a Steering Committee, consisting of one representative each from USGS, FWS, USDA APHIS, IAFWA, HHS, the National Flyway Council, and the State of Alaska be formed to coordinate wild bird AI surveillance in the United States. Specific roles of this Committee should include:

- Facilitate communication between state and federal agencies, and organizations involved in AI surveillance for wild birds.
- Coordinate implementation and data analysis of AI surveillance programs nationally.
- Provide periodic summaries of AI surveillance for wild birds in the United States.
- Provide periodic recommendations for AI surveillance in wild birds based on previous sampling efforts and changes in virus epidemiology.
- Facilitate communication and coordination among state and federal agencies for contingency planning and other preparations for the appearance of highly pathogenic H5N1 avian influenza virus in wild birds in North America.

Sampling strategies to detect highly pathogenic H5N1 avian influenza virus in wild bird populations will change depending upon the risk assessment and management goals and prevailing status of the pathogen in North America. For early detection of highly pathogenic

H5N1 avian influenza virus, efforts should focus on likely cross-over routes of birds from Asia to North America (e.g., Alaska). Efforts should focus on areas of high aggregations of waterfowl intersecting with logistical sampling support such as the National Wildlife Refuge System and state waterfowl management areas.

If highly pathogenic H5N1 avian influenza virus gains a foothold in North America, the surveillance network should be placed along known waterfowl movement paths from the point of origin (i.e., point of detection). These paths can be inferred from known migration routes of specific species. However, practically, and given the patterns emerging in Eurasia, if highly pathogenic H5N1 avian influenza virus becomes established in North America the likelihood of rapid and diffusive spread across the continent is high. At this point local waterfowl and environmental sampling should target areas of strategic value, e.g., human population centers and areas of high density of poultry production. In the former case, such areas would be represented by urban zoo-parks and lakes. These areas would represent the highest level of risk of human contact with contaminated water and/or waterfowl. In the latter case, ponds, lakes and waterfowl management areas around high density poultry production areas would provide the best ability to assess risk of transmission to humans and poultry. Surveillance efforts patterned on these areas are most amenable to local and state efforts for first detection and subsequent risk assessment if H5N1 subtype(s) achieve enzootic status in North America.

ATTACHMENT 1

Interagency highly pathogenic H5N1 avian influenza Early Detection Working Group

Working Group Members

Chairpersons

Tom DeLiberto
USDA APHIS WS
(970) 266-6088
Thomas.J.DeLiberto@aphis.usda.gov

Rick Kearney
USGS-BRD
(703) 648-4019
rkearney@usgs.gov

Members

US Department of Interior

Leslie Dierauf
USGS BRD NWHC
(608) 270-2401,
ldierauf@usgs.gov

Dirk Derksen
USGS BRD ASC
(907) 786-3531
dirk_derksen@usgs.gov

Chris Brand
USGS BRD NWHC
(608) 270-2440
christopher_brand@usgs.gov

Mike Higgins
USFWS
(410) 573-4520
Mike_J_Higgins@fws.gov

Hon S. Ip
USGS BRD NWHC
(608) 270-2464
hip@usgs.gov

Gary Frazer
USFWS
(703) 648-4059
Gary_Frazer@fws.gov

Paul Padding
USFWS
(301) 497-5982
paul_padding@fws.gov

Bob Leedy
USFWS
(907) 786-3446
robert_leedy@fws.gov

Margaret Wild
NPS

(970) 225-3593
Margaret_Wild@nps.gov

US Department of Agriculture

J. Todd Weaver
USDA APHIS VS CEAH
(970) 494-7213
todd.weaver@aphis.usda.gov

Thomas Gomez
USDA APHIS VS @ CDC
(404) 639-3233
tmg1@cdc.gov

Larry Clark
USDA APHIS WS NWRC
(970) 266-6036
Larry.Clark@aphis.usda.gov

Seth Swafford
USDA APHIS WS
(301) 734-3570
seth.swafford@aphis.usda.gov

Tracy DuVernoy
USDA APHIS VS
(301) 734-7781
Tracy.S.Duvernoy@aphis.usda.gov

International Association of Fish and Wildlife Agencies

Gary Taylor
IAFWA
(202) 624-7890
gtaylor@iafwa.org

Dan Yparraguirre
CA Dept. of Fish and Game
(916) 445-3685
dyparraguirre@dfg.ca.gov

Russ Mason
Nevada Dept. of Wildlife
(775) 688-1520
rmason@ndow.org

John Fischer
SCWDS
(706) 542-1741
jfischer@vet.uga.edu

Bruce Morrison
Nebraska Game and Parks Commission
(402) 471-5430
bruce.morrison@ngpc.ne.gov

State of Alaska

Matt Robus
AK Dept. of Fish and Game
(907) 465-4190
matt_robus@fishgame.state.ak.us

Louisa Castrodale
AK Division of Public Health
(907) 269-8002
louisa_castrodale@health.state.ak.us

Kimberlee Beckmen
AK Dept. of Fish and Game
(907) 459-7257
kimberlee_beckmen@fishgame.state.ak.us

Bob Gerlach
AK Division of Agriculture
(907) 269-7635
bob_gerlach@dec.state.ak.us

Tom Rothe
AK Dept. of Fish and Game
(907) 267-2206
tom_rothe@fishgame.state.ak.us

US Department of Health and Human Services

Leigh A. Sawyer
Office of the Secretary, DHHS
(202) 205-0065
Leigh.Sawyer@hhs.gov

Nicholas Komar
CDC Arbovirus Diseases Branch
(970) 221-6496
nck6@cdc.gov

Nina Marano
CDC
(404) 639-3831
NBM8@CDC.GOV

National Association of State Public Health Veterinarians

Carina Blackmore
Florida Dept. of Health
(850) 245-4732
Carina_Blackmore@doh.state.fl.us

Support Staff

Mary Kimball
USDA APHIS WS
(970) 494-7940
Mary.L.Kimall@aphis.usda.gov

ATTACHMENT 2

Migratory Bird Flyways in Asia and North America

Figure 2-1: Asian Migratory Bird Flyways

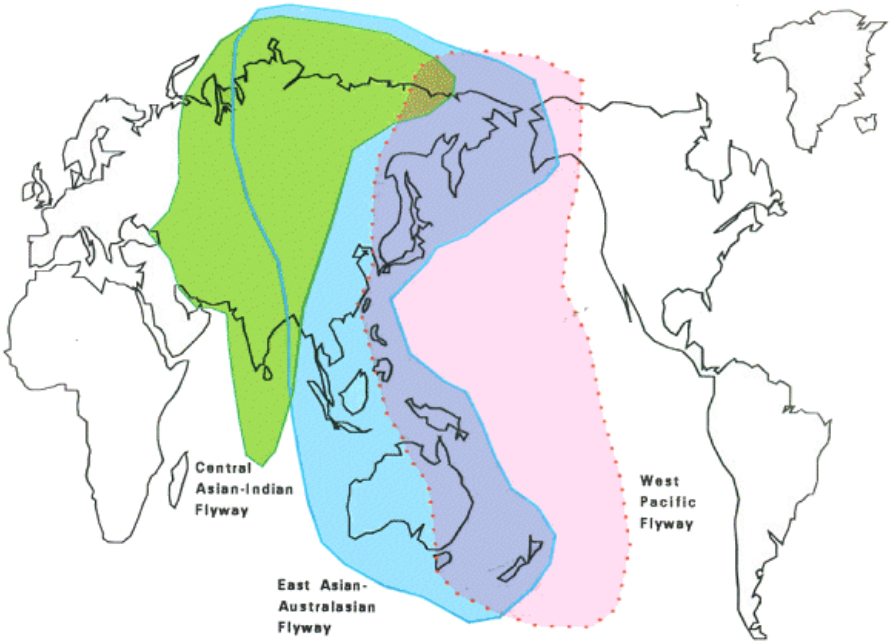
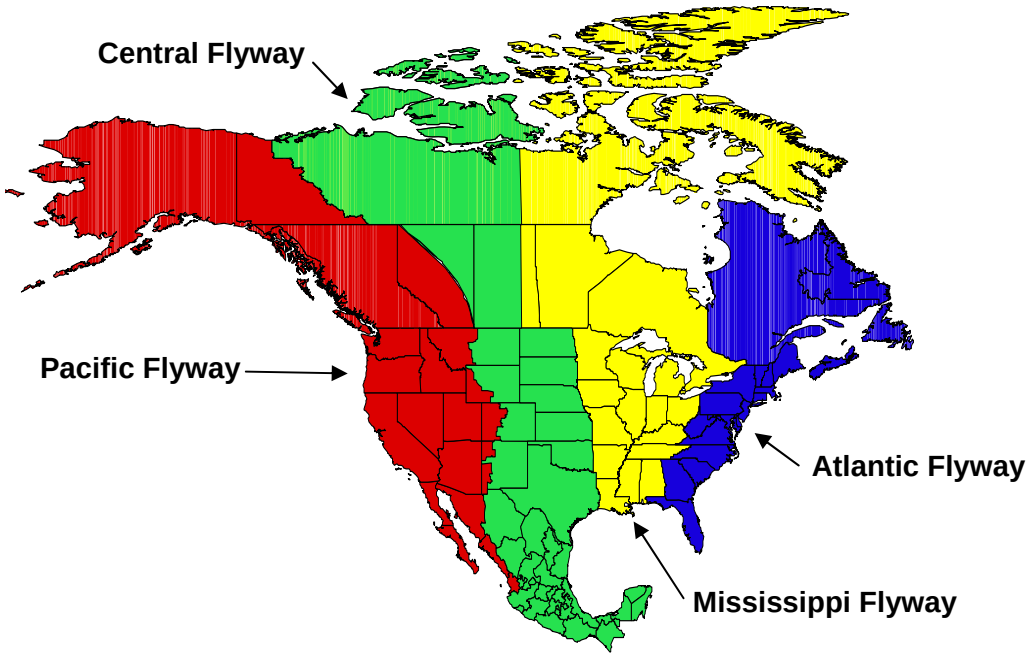


Figure 2-2. North American Migratory Bird Flyways



ATTACHMENT 3

Investigation of Morbidity and Mortality Events in Wild Birds

Overview

The systematic investigation of morbidity and mortality events in wild birds to determine if the highly pathogenic H5N1 avian influenza subtype of avian influenza (AI) is playing a role in causing illness and death offers the highest and earliest probability of detecting the virus if it is introduced by migratory birds into the United States. There is increasing evidence that highly pathogenic H5N1 avian influenza virus is capable of killing wild birds which is not the usual characteristic of AI viruses. As such, the documentation of the movement of the virus through Asia and into Europe has been discovered in part, through the investigation of mortality events of wild migratory birds. Benefits gained from conducting disease investigations of wildlife mortality events are not unique to AI. Many other important diseases have been discovered and described after initial detection through the wildlife disease investigation process (e.g. West Nile Virus). The investigation of wildlife diseases operates with consistent procedures while maintaining enough flexibility to accommodate the unique characteristics of specific disease agents involved. The initial detection of a mortality event is strongly dependent upon well-trained and observant field personnel. These people in turn communicate with an experienced staff of disease investigation specialists that obtain the maximum amount of information surrounding the event. Depending upon the significance and intensity of the mortality event, these highly trained investigators may visit the site of the mortality event to conduct field investigations so as to obtain further information first hand. In addition to determining a cause of death, disease investigation specialists provide useful management recommendations to potentially reduce further morbidity and mortality.

In the event that highly pathogenic H5N1 avian influenza is detected in wild birds, it will be important to investigate the proximity of domestic poultry and swine operations in order to initiate activities to minimize contact between the wild birds and these other animals. Morbidity and mortality of wild birds is most likely to occur in areas where migratory birds infected with highly pathogenic H5N1 avian influenza mingle with other wild bird species, particularly in wetland habitats. Likewise, early outbreaks of highly pathogenic H5N1 avian influenza would most likely occur in Alaska and along the Pacific Flyway of the United States and Canada, where migratory birds from Asia stage in the summer and early fall and subsequently migrate within North America. However, given that migrants also move from Alaska to other parts of North America, surveillance strategies should include other flyways as well. In this surveillance plan, participating state, Federal and tribal agencies, and cooperators will conduct targeted surveillance for highly pathogenic avian influenza (HPAI) both in response to disease outbreaks in wild

birds focusing intensively in Alaska, the Pacific Flyway, and Oceania, and in response to mortality events in high-priority (i.e., most likely) species throughout the United States.

Methodology

The key to success of this surveillance strategy involves: 1) early detection of morbidity and mortality, 2) rapid reporting and submission of appropriate biological specimens to qualified diagnostic facilities, 3) immediate assessment of the field event (descriptive epidemiology), 4) rapid, accurate, and consistent diagnosis and confirmation, 5) immediate reporting of diagnostic results once confirmed, and 6) pre-planned contingency and response training for the occurrence of HPAI.

Specific steps necessary to orchestrate the early detection of highly pathogenic H5N1 avian influenza include:

1) State, Federal, and tribal resource personnel will be instructed to increase vigilance and to establish routine and systematic monitoring of wild bird populations for morbidity and mortality. Standard guidelines will be prepared with the assistance of the U.S. Geological Survey (USGS) - National Wildlife Health Center (NWHC) and the U.S. Department of Agriculture (USDA) - Wildlife Services (WS) to increase uniformity of effort. The most intensive monitoring will occur in Alaska and selected areas of high risk in the Pacific Flyway and Oceania where migratory birds from affected countries are likely to interact with North American bird species.

2) A uniform protocol for reporting mortality events and instructions for the safe handling and shipment of specimens to identified diagnostic facilities will be developed. Field and response personnel will be trained. Reporting of mortality events will be through appropriate channels within each state, Federal, or tribal entity to the NWHC, where a centralized database (WDIN) will be maintained, made available to contributors, and summarized in modified form for public dissemination.

3) Field personnel or teams designated by respective land management agencies will respond to mortality events by conducting field investigations to determine onset, course, duration, distribution, species, and other environmental conditions associated with mortality events. The NWHC and USDA-WS will assist in developing guidelines and training. In certain circumstances, NWHC and USDA-WS personnel will conduct field investigations or assist other agencies.

4) Representative and suitable carcasses and other biological samples and specimens will be submitted to one or more identified diagnostic facilities capable of conducting immediate necropsy and laboratory analyses. Guidelines will be developed to assure that the appropriate number and types of samples are collected to ensure that there is a statistically-based confidence

in the sample size analyzed in response to a mortality event. Necropsies, histology, and laboratory investigations will be utilized to substantiate a diagnosis of highly pathogenic H5N1 avian influenza virus. Virus isolation, hemagglutination inhibition tests, and molecular testing specifically for H5N1 will be performed to detect the presence of the virus in specimens.

5) Reporting of results to submitters will be done as early as possible, including preliminary results that may refute or support the presence of HPAI. Highly pathogenic H5N1 avian influenza virus is a CDC/USDA Select Agent, thus the CDC/USDA Select Agent Programs will be notified immediately upon identification of the virus and all Select Agent guidelines will be followed as required. Final results of HPAI tests will be reported immediately to the submitter. As highly pathogenic H5N1 avian influenza is a reportable disease, the State Veterinarian, and the Area Veterinarian in Charge (AVIC) will be informed simultaneously of the discovery. A final report will also be provided to the WDIN. Public release of information will occur only after these final results are thus reported.

6) Wildlife disease contingency plans will be established at an appropriate landscape scale to enable rapid deployment of personnel and resources to take action. Disease contingency plans can be developed for general response to a mortality event, with special reference and consideration for highly pathogenic H5N1 avian influenza virus. The NWHC and USDA-WS will assist in providing guidelines and training in the establishment of contingency plans.

To increase early detection and response capabilities to the extent needed to protect the United States from highly pathogenic H5N1 avian influenza virus, enhancements to current activities will need to include:

- Personnel and resources in the field to intensively monitor for mortality events,
- Systematic methods to detect mortality early in the field,
- Resources to fully investigate all such events, and
- Surge capacity at wildlife disease diagnostic facilities

Wildlife professionals employed by state natural resource agencies and by the U.S. Department of Interior (DOI) Fish and Wildlife Service and National Park Service are the principal authorities positioned to detect and respond to morbidity and mortality events involving wild birds. The DOI Bureau of Land Management, Tribal Nations, and several other state, Federal, and local agencies (including the U.S. Department of Defense) also have authority over lands that they administer and manage. Morbidity and mortality events involving wildlife are often detected by, or reported to these agencies and entities.

Investigations into the causes of wildlife mortality events are dependent on the perceived significance of the event and on the knowledge or availability of disease diagnostic facilities capable of providing assistance. The USGS - NWHC, located in Madison,

Wisconsin, is a full-service wildlife diagnostic and research laboratory that assists Federal, state, and tribal agencies in responding to wildlife disease outbreaks. Together with its Honolulu Field Station, which serves Hawaii and Pacific Trust Territories, the NWHC is the principal facility relied upon by the DOI, as well as by most states, to investigate and diagnose wildlife diseases, including those of migratory birds. Numerous state natural resource agencies in the Pacific Flyway also have established wildlife disease laboratories and programs with staff that respond to wildlife disease outbreaks in their respective states. USDA, state and university diagnostic laboratories, and regional entities such as the Southeastern Cooperative Wildlife Disease Study are also involved in wildlife disease investigations. The NWHC maintains an extensive database on wildlife mortality events across the United States and Canada to which Federal, state, provincial, and tribal agencies contribute. The NWHC also supports and houses the Wildlife Disease Information Node (WDIN), a part of the USGS - National Biological Information Infrastructure (NBII).

Discussion

The primary strength of the strategy of targeted investigations of avian mortality events is based upon the observation that highly pathogenic H5N1 avian influenza kills some species of wild birds. As such, a wild bird die-off serves as a “trigger event” that immediately focuses the investigation to a given area and species. Further, because the current form of the virus circulating in Asia will be new to North America, the hypothesis is that highly pathogenic H5N1 avian influenza will be detected if it is the cause of an observed mortality event. Therefore concentrating on recovering carcasses and samples from wild bird die-offs affords a timely opportunity to detect HPAI. Conversely, live bird surveillance provides the opportunity to detect birds that may shed the virus without ill-effects and offers the possibility of early detection of the arrival and especially, the spread of highly pathogenic H5N1 avian influenza. Due to the size of the country and the number of species of wild birds involved, careful consideration will be needed to identify relevant species and sampling locations for live bird surveillance. Hunter-harvested birds will provide an opportunity to augment live bird surveillance by providing large numbers of birds using a reduced level of field resources. However, as a limited number of species are targeted for hunting, sound scientific judgment should be exercised in choosing species and locations for analysis. All of the strategies described above will require considerably more resources in personnel to be effective, and the greater number of submissions (surge capacity) will require laboratories to be prepared in advance.

Recommendations

Because the primary goal of the process outlined in this plan is the earliest possible detection of highly pathogenic H5N1 avian influenza in wild birds, all of the strategies described are important, but not all strategies are practical to conduct in all areas of the country. The live wild bird surveillance strategy would be a most effective tool to determine the pattern of virus spread subsequent to a die-off attributed to highly pathogenic H5N1 avian influenza. Wild animal die-offs are important to investigate for multiple reasons, however it should be noted that highly pathogenic H5N1 avian

influenza will not be the cause of most of the mortality events investigated through a targeted surveillance strategy. Rather, other bacterial and viral diseases that are either zoonotic or important to agriculture may be detected through these surveillance programs. Mortality event investigation provides the opportunity to obtain the greatest amount of information about health and disease in wild birds without an a priori bias. Supplemental wildlife disease information will be prioritized and gathered as funding and personnel allow. Surveillance for Highly pathogenic H5N1 avian influenza will remain the top priority.

Appendix

Field Personal Safety:
In an area where highly pathogenic H5N1 avian influenza has not been detected, field personnel should follow the recommendations provided in the NWHC Guidelines for Handling Birds (http://www.nwhc.usgs.gov/research/WHB/WHB_05_03.html). Personal protective equipment (PPE) should include boots, coveralls, and gloves. In addition, the use of goggles and N95 masks are recommended.

In areas where highly pathogenic H5N1 avian influenza has been detected, especially during a mass mortality event, field personnel should follow the latest guidelines of the CDC (http://www.cdc.gov/flu/avian/professional/protect-guid.htm). PPE should include complete coveralls, gloves, and boot covers that are either disposable or that can be disinfected. Goggles, N95 masks (NIOSH respirator preferred) as well as a health monitoring plan are required.

Attachment 4

Surveillance for Highly pathogenic H5N1 avian influenza virus in Live Wild Birds

Overview

This surveillance strategy incorporates sampling of live-captured, apparently healthy migratory birds to detect the presence of highly pathogenic avian influenza (HPAI) H5N1 virus. Virus isolation from tracheal and cloacal samples is a common method for detecting avian influenza (AI) viruses and has been used before in various geographic regions, including Alaska (Ito et al., 1995; Hanson et al., 2003; Slemons et al., 2003; Krauss et al., 2004). This effort focuses on bird species in North America that represent the highest risk of being exposed to or infected with highly pathogenic H5N1 avian influenza virus because of their migratory movement patterns, which include birds that migrate directly between Asia and North America, or birds that may be in contact with species from areas in Asia with reported outbreaks.

In general, bird flyways represent migration corridors within continental land masses. However, Alaska and corresponding areas in the Russian Far East represent a unique case where major flyway systems cross continental boundaries. Two major Asian flyways (the East Asian-Australasian and East Asian) include both Southeast Asia, Oceania, and the arctic regions of Siberia, the Russian Far East, and Alaska. The East Asian-Australasian Flyway, defined primarily in the context of shorebird use, extends from the Siberian and Alaskan arctic through North and Southeast Asia including U.S. trust territories in the Pacific to Australia and New Zealand, covering 20 countries.

Similarly, in North America, the Pacific Flyway extends from Arctic Canada, Alaska, and Eastern Siberia through coastal and western regions of Canada, the United States and Mexico, and on to Central and South America. Many migratory species that nest in Arctic Siberia, Alaska, and Canada follow the Pacific Flyway to wintering areas. Although not considered a major pathway, birds from both Eastern Siberia and Alaska intermingle in both the Pacific and Central Flyways. The overlap at the northern ends of these flyways and in Hawaii and Oceania establishes a path for potential disease transmission across continents and for mixing, re-assortment, and exchange of genetic material among strains from Eurasia and North America.

There is concern about the spread of HPAI westward from Asia to Europe. However, there is comparatively little movement of wild birds between Europe and North America. Consequently, if highly pathogenic H5N1 avian influenza virus arrives in the U.S. or a U.S. territory in migratory birds, it would most likely arrive first in Alaska or one of the Pacific Islands. Such an event is not unreasonable, as the contribution of Eurasian AI viruses to the genetic composition of viruses in North American wild birds has already been shown.

Methodology

Identification of Priority Species

Birds should be sampled in conjunction with existing studies when possible, and additional bird captures should be initiated as necessary to provide a broad species and geographic surveillance effort. Initial efforts should focus on one or more species in each of the following three groups that could potentially bring highly pathogenic H5N1 avian influenza virus to the US Pacific Islands and trust territories and/or Alaska and, subsequently, southward through the Pacific and potentially the other North American flyways:

1. Species that travel directly to Alaska or Oceania from Southeast Asia or Australasia. Some of these birds winter in Southeast Asia while others migrate along coastal Southeast Asia to and from wintering areas in Australasia. Based on what is known about the geographic distribution of the current highly pathogenic H5N1 avian influenza virus outbreaks, this is the group most likely to bring the virus to Alaska or the U.S. Islands and trust territories in Oceania. Before any species in this group can be a source of infection for birds in other areas of North America, inter-specific transmission of the virus to temperate migrants must occur in Alaska. Examples include the bar-tailed godwit (Fig. 4-1), dunlin, and red knot.

2. Species that breed in Alaska, with some fraction of the population known to winter in Asia. Although the portion of the population that winters in Asia may be small, some of these species are highly gregarious at other times of the year, particularly during molting, staging, and on their primary wintering grounds. Because the primary wintering grounds of several of these species are in the North American Pacific Flyway, carriers arriving in Alaska from Asia could potentially transmit the virus to a large portion of the North American population. This scenario for highly gregarious species requires only intraspecific transmission in Alaska. The course of events for less gregarious species and those that tend to winter in more northerly latitudes is more likely to require interspecies transmission. Examples include the black brant, northern pintail (Fig. 4-1), long-tailed duck (Fig. 4-2), yellow-billed loon, and red-breasted merganser.

3. Species that intermingle across Siberia, the Russian Far East, and Alaska. This group has become more important with the confirmation of HPAI in poultry near Novosibirsk in Siberia. However, unless highly pathogenic H5N1 avian influenza virus spreads further north and east in this region, the most likely way for this group to become infected would be contact with species that winter in southern Asia and breed in northern Asia. Under such circumstances, inter-specific transmission would be required on both sides of the Bering Strait before the virus could be carried from Alaska to temperate regions of North America. Examples include the Steller's eider, spectacled eider, emperor goose (Fig. 4-3), sharp-tailed sandpiper, sandhill crane.

Figure 4-1. Migratory routes of two species that illustrate movements of birds between Asia and North America

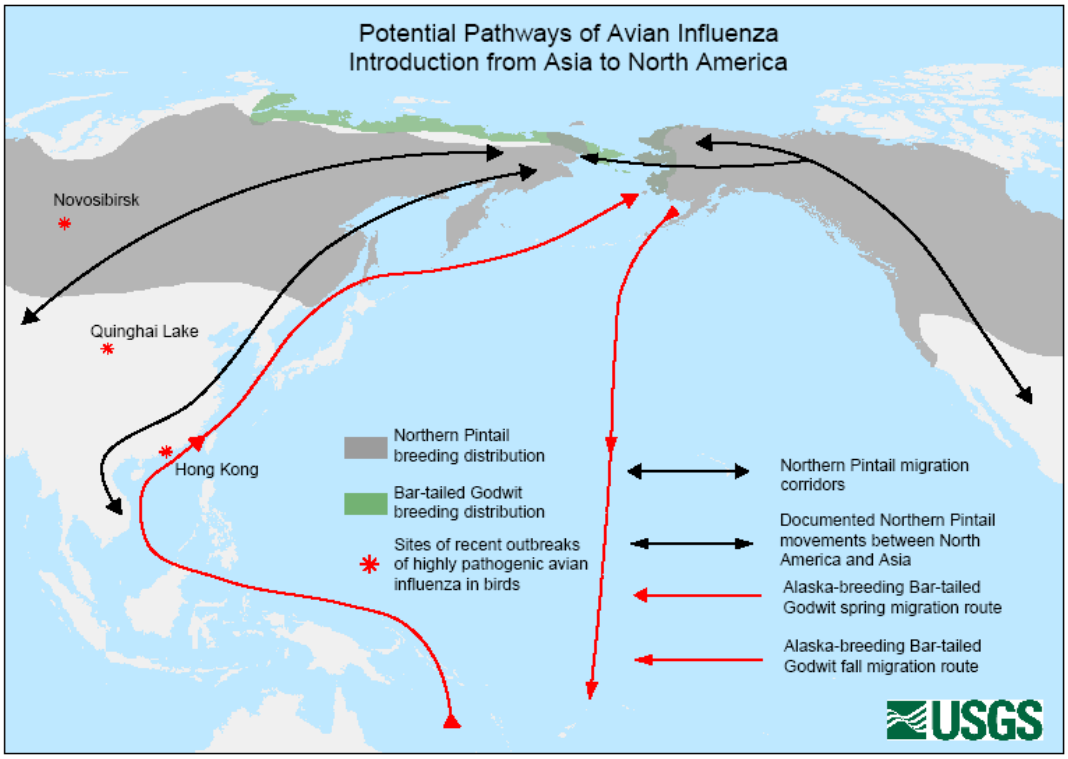


Figure 4-2. Migratory routes of long-tailed ducks between Alaska and Asia

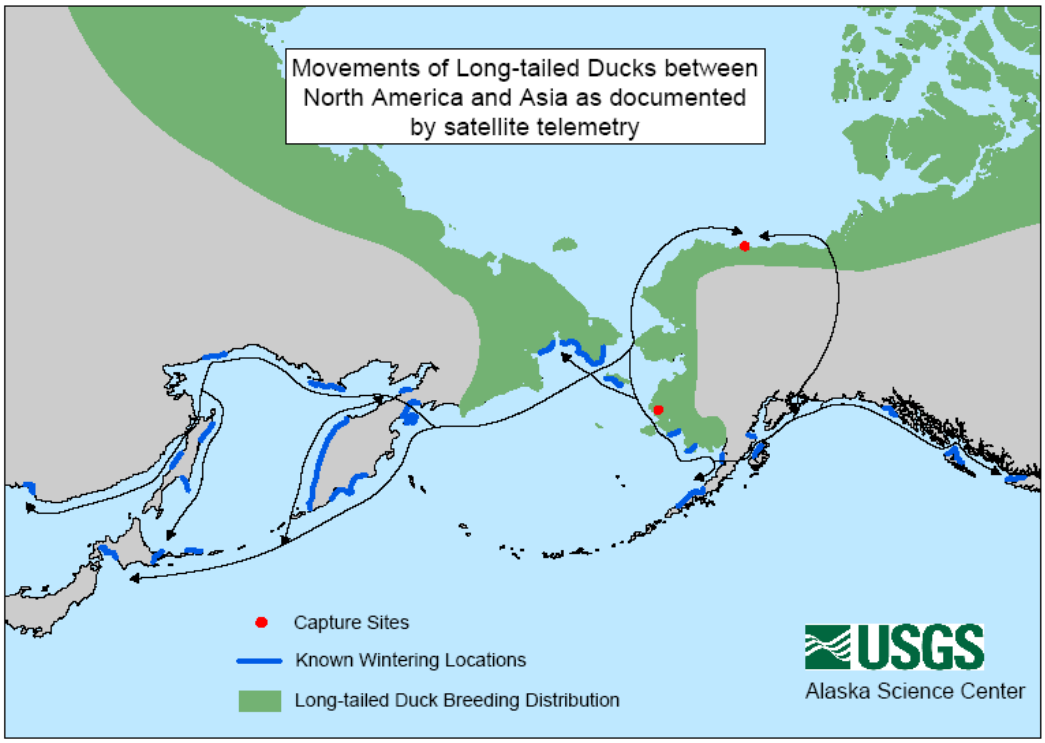
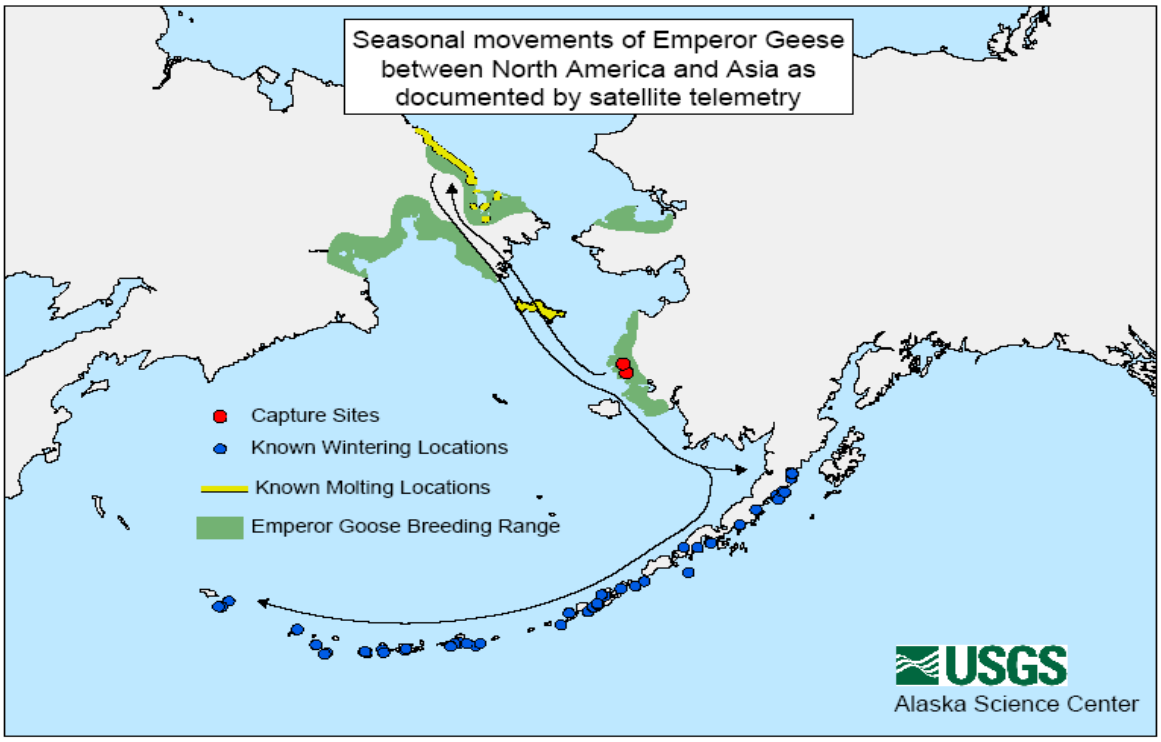


Figure 4-3. Migratory pathways of emperor geese between Alaska and Russia.



The strategy for selection of species to be sampled should initially focus on migrants that have the greatest likelihood of making contact with wild migratory birds, domestic flocks, and geographic areas in Asia where highly pathogenic H5N1 avian influenza virus has been documented. Members of three taxonomic groups of waterbirds—loons, waterfowl, and shorebirds—offer the most immediate potential for meeting the selection criteria and being carriers of the virus (see Tables 4-1to 4-3).

Table 4-1. Loon species as potential carriers of the highly pathogenic H5N1 avian influenza subtype to North America.

	Asian Contact				
Species	**Direct**	**Indirect**	**Timing**	**Specifics**[1]	**Point of Contact**
Yellow-billed Loon	Yes	Yes	Oct-Apr	Birds breeding on Alaskan north slope winter off of Japan, Korea, China.	Coastal Japan, Korea, China
Red-throated Loon	Yes	Yes	Oct-Apr	Birds breeding on Alaskan north slope winter off of Chukotka and Kamchatka.	Coastal Chukotka and Kamchatka
Pacific/Arctic Loon				Little data is available but probably follow similar patterns to yellow-billed and red-throated loons.	

[1] For the most part, loon species mix with other waterbirds along migration routes and at staging and wintering areas.

Table 4-2. Waterfowl species as potential carriers of the Highly pathogenic H5N1 avian influenza subtype to North America.

	Asian Contact				
Species	**Direct**	**Indirect**	**Timing**	**Specifics[1]**	**Point of Contact**
Tundra Swan	Yes	Yes	Jun-Sep	Portion of breeding range in Chukotka. From there birds migrate to staging and wintering areas throughout Pacific Flyway.	Chukotka
Whooper Swan	Yes	Yes	Nov - Apr	Eastern Asian population breeds in E. Russia and NW China. Small numbers wintering in Aleutian and Pribilof islands.	E. Russia and NW China; Aleutian and Pribilof islands
Emperor Geese	Yes	Yes	Year round	Portion of breeding range in Chukotka and some AK breeders molt in Chukotka. From there birds migrate to staging and wintering areas in the W. AK and Aleutian Islands. Small segment of population stage and winter in Commander Islands and Kamchatka.	Chukotka and W. Alaska
Black Brant	Yes	Yes	Jun-Sep	Portion of breeding range in Chukotka, some AK breeders molt on Wrangel Island (Russia), and Siberian breeders molt on Alaskan North Slope (Teshekpuk). Most of population migrates to staging areas in W.AK and winters along Pacific coast to Mexico. Birds breeding west of Kolyma (Russia) winter in Korea, China, and Japan (mixing in fall and to some degree in spring).	Chukotka, and N. and W. Alaska, W. coast of N.A.
Aleutian Canada Geese	Yes	Yes	Jun-Sep	Portion of population breeds on Commander Island (Russia), then migrate through Alaska into Oregon and California.	Commander Island
Snow Geese	Yes	Yes	Jun-Sep	Portion of population breeds on Wrangel Island (Russia), then migrates through Alaska into Pacific Flyway states (mixing with other waterbird species).	Wrangel Island
Eurasian Wigeon	Yes	No		Regular vagrant along west coast of North America, especially Aleutian Islands.	W. coast of North America, especially Aleutian Is.
Northern Pintail	Yes	Yes	May-Sep	Some Siberian breeders winter in W. US (California). Also birds banded in North America have been recovered over large areas of E. Siberia and Kamchatka.	Siberia
Baikal Teal	Yes	No		Occasional vagrant to North America chiefly in Aleutians & extreme Western Alaska.	Aleutians & extreme Western Alaska
Common Pochard	Yes	No		Vagrant in W. Alaska (Aleutian and Pribilof Islands).	W. Alaska (Aleutian and Pribilof Islands)
Steller's Eiders	Yes	Yes	Nov-Sep	Most of the population breeds E. Siberian arctic, these birds molt on Alaska Peninsula (Izembek and Nelson Lagoons). Birds winter in largest numbers in Commander and Kuril Islands (Russia) and in smaller numbers in Northern Japan, along Alaska Peninsula, and Aleutian Islands.	Alaska Peninsula, and Aleutian Islands; E. Siberian arctic, Commander and Kuril Islands (Russia), Northern Japan
	Asian Contact				

Species	Direct	Indirect	Timing	Species	Point of Contact
Common Eiders	Yes	Yes	Year round	Portion of AK and Canada breeders molting and wintering in Chukotka. Portion of E. Siberian breeding population winters in Bering Sea (Aleutian Islands) mixing with AK and Canada breeders.	Chukotka and Aleutian Is.
King Eiders	Yes	Yes	Year round	Portion of AK and Canada breeding population molt and winter in Kamchatka and Kuril islands (Russia). Portion of E. Siberian breeding population winters in Bering Sea (Aleutian Islands) mixing with AK and Canada breeders.	Kamchatka and Kuril islands (Russia) and E. Siberian arctic
Spectacled Eiders	No	Yes	Nov-Apr	E. Siberian arctic and Alaska breeders mix during winter in Bering Sea.	Bering Sea
Long-tailed Ducks	**Yes**	**Yes**	**Oct-Apr**	**Yukon Kuskokwim Delta breeders and North Slope molters molt and winter along Chukotka, Kamchatka, south along the Russian coast, and Kuril and Sakhalin islands.**	**Chukotka and Kamchatka**
Tufted Duck	Yes	No		Regular vagrant along west coast of North America.	W. coast of North America

Other Possibilities

Species	Specifics
Greater White-fronted Geese	Circumpolar distribution.
Green-winged Teal	Breed throughout middle latitude Northern Hemisphere.
Mallard	Holarctic distribution.
Northern Shoveler	Holarctic distribution.
Gadwall	Breed on Alaskan peninsula, Kamchatka, China, Russia.
Greater Scaup	Holarctic distribution. AK breeders winter on Atlantic coast
Harlequin Ducks	Pacific population breeds from E. Siberia through Alaska to W. Canada.
Black Scoters	Pacific population breeds in Siberia and Kamchatka, into western Alaska and sparsely across Canada.
Common Goldeneye	Circumpolar distribution.
Red-breasted Merganser	Holarctic distribution.

[1] For the most part, all the waterfowl species mix with other waterbirds along migration routes and at staging and wintering areas.

Table 4-3. Shorebird species as potential carriers of the Highly pathogenic H5N1 avian influenza subtype to North America.

	Asian Contact				
Species	**Direct**	**Indirect**	**Timing**	**Specifics**	**Point of Contact**
Pacific Golden-Plover	Yes	Yes	Jun-Sep	Pop. nesting in Siberia/Chukotka returns to North America; during passage through AK mixes with local nesting Pacific Golden-Plovers that winter in Hawaii and central Oceania. Breeding birds from Siberia thought to also migrate overland to Southeast Asia and Oceania. Birds wintering in the Marshall and Mariana Islands migrate through Southeast Asia whereas birds wintering in the Hawaiian Islands thought to migrate through Alaska.	Russian Far East and w. Alaska; Oceania, main Hawaiian Islands, Northwestern Hawaiian Islands, Marshall Islands, Guam and the Northern Marianas
Black- bellied Plover	Yes	Yes	Jun-Sep	Pop. nesting in Siberia/Chukotka returns to North America, mixing with birds in w. Alaska before both migrate to nonbreeding areas in North and Central America.	Russian Far East and w. Alaska
Semipalmated Plover	Yes	Yes	Jun-Sep	North American breeding and North and South American nonbreeding species with small breeding pop. recently established in Chukotka. Chukotka pop. mixes with other species from the East Asian flyway (EAF) before returning to the Americas.	Chukotka, w. Alaska
Bar-tailed Godwit	Yes	Yes	Apr-Nov	About 90,000 birds migrate along the coast of E. Asia en-route to breeding grounds in n. and w. Alaska. Coastal w. Alaska principal autumn staging area where birds mix with 15-20 spp. of shorebirds and equal number of waterfowl spp. that migrate to the Americas.	Birds spending nonbreeding season in Australia with potential to also mix with other pop. of godwits (*L. l. menzberi*) that are restricted to coastal E. Asia
Marbled Godwit	No	Yes	Aug-Oct	Mixes with Bar-tailed Godwits at staging sites on AK Pen. Migrates to Pacific NW.	AK Peninsula
Whimbrel	No	Yes	Jul-Aug	Contact occurs with Bar-tailed Godwits and plovers (Black-bellied and Pacific Golden) in w. AK & on AK Pen. Estuaries.	AK Peninsula
Bristle-thighed Curlew	Yes	Yes	May-Sep	Possible direct contact on nonbreeding grounds in Oceania; indirect through contact with Bar-tailed Godwits on w. AK breeding and YKD staging grounds	Seward Pen., Andreafsky Wilderness, Yukon Delta NW Hawaiian Is. and the Marshall Is.
Greater Yellowlegs	No	Yes	Aug-Sep	Mixes with Bar-tailed Godwits at staging sites on YKD & AK Pen.	W. Alaska
Wandering Tattler	Yes	Likely	May-Sep	Birds breeding in AK migrate to Hawaii and likely elsewhere in Oceania. Bird in Australia during nonbreeding season may be from Alaska and/or part of breeding range in Chukotka.	Chukotka, W. Alaska
Ruddy Turnstone	Possibly	Likely	May-Sep	Not known if birds nesting on Chukotka come to AK postbreeding. However, birds nesting in AK known to migrate to sites in EAF & Oceania. Birds from Eastern Siberia and Western Alaska also migrate along the East Asian coast, and a portion winter in the Mariana and Marshall Islands.	W. Alaska; Oceania, NW Hawaiian Islands, Marshall Island, Guam and Mariana Islands
	Asian Contact				

Species	Direct	Indirect	Timing	Specifics	Point of Contact
Black Turnstone	No	Yes	Jul-Sep	On YKD mingles with species (Bar-tailed Godwit, Sharp-tailed Sandpiper, and *C. a. arcticola* Dunlin) that pass along EAF during migration.	W. Alaska
Long-billed Dowitcher	Yes	Yes	Jun-Oct	Birds nesting in Chukotka/Siberia return to AK and then migrate to nonbreeding areas in temp. NA. When in Russia there exists potential to have contact with numerous species that migrate along the EAF.	Siberia, W. Alaska
Surfbird	No	Yes	Jul-Sep	At AK Peninsula estuaries mixes with flocks of Bar-tailed Godwits that migrate along the EAF.	SW Alaska
Red Knot	Yes	Yes	May-Oct	Subspecies *C. c. roselaari* breeds in w. and n. Alaska and on Wrangel I. and spends nonbreeding season along Pacific coast of N. & C. America. Birds staging on YKD in spring possibly mix with *C. c. rogersi* pop. that reaches Alaska via Australia & EAF. If no mixing with *C. c. rogersi*, then birds on Wrangel I. are in direct contact with other spp. of waders and waterfowl from EAF.	Wrangel Is., W. Alaska
Sanderling	?	Likely	Sep-Oct	Birds in autumn in W. Alaska likely from Asian nesting areas where direct contact likely. Nonbreeding areas of birds in w. AK in autumn unknown.	W. Alaska
Semipalmated Sandpiper	No	Yes	Jun-Aug	Widespread nesting species in n. Alaska where in direct contact with Dunlin (*C. a. arcticola*) that migrate through and winter in EAF.	N. and W. Alaska
Western Sandpiper	Possibly	Yes	Jun-Sep	Breeds in Chukotka with birds returning to nonbreeding areas in NA; also has contact with *C. a. arcticola* Dunlin in N. AK.	N. and W. Alaska
Red-necked Stint	Yes	Yes	Jun-Sep	Old World species that occasionally nest in w. Alaska with Western Sandpipers.	N. and W. Alaska
Pectoral Sandpiper	Yes	Yes	Jun-Aug	Birds nesting in Siberia/Russian Far East return to nonbreeding areas in SA via passage through N. America. In Siberia the species is in direct contact with numerous spp. of waders and waterfowl from the EAF.	W. & N Alaska
Sharp-tailed Sandpiper	Yes	Yes	Aug-Oct	Possibly the entire annual cohort of juveniles comes to w. AK from Siberian nesting grounds where they have had contact with several pop. of birds that have migrated along the EAF.	Mostly W. Alaska
Buff-breasted Sandpiper	Yes	Yes	Jun-Aug	Breeds in Canada, AK but pop. also nests on Wrangel Is. and migrates through AK & N.A. to reach nonbreeding areas in S.A.	Wrangel Is., W. Alaska
	Asian contact				
Species	**Direct**	**Indirect**	**Timing**	**Specifics**	**Point of Contact**

Rock Sandpiper	Yes	Yes	Jun-Oct	*C. p. tschuktschorum* subspecies has portion of breeding range in Chukotka. Birds from there migrate to nonbreeding areas in the Pacific NW via w. AK staging sites where they mix with Dunlin (C. *a. pacifica)* & Rock Sandpipers (C. *p. ptilocnemis*).	W. & SW Alaska
Dunlin	Yes	Yes	May-Oct	*C. a. arcticola* nests in n. AK and migrates to nonbreeding areas in central EAF (Japan, Korea, Taiwan). While in AK it has contact with numerous spp. of waders and waterfowl that migrate to N, C. & S. America.	N. & W. Alaska
Buff-breasted Sandpiper	Yes	Yes	Jun-Aug	Breeds in Canada, AK but pop. also nests on Wrangel Is. and migrates through AK & N.A. to reach nonbreeding areas in S.A.	Wrangel Is., W. Alaska

To further focus sampling, five criteria were employed to rank these migratory waterbirds and other migrants that are potential carriers of highly pathogenic H5N1 avian influenza virus (see Tables 4-4 to 4-6 below). These ranking criteria include 1) proportion of the population occurring in Asia, 2) contact with a known area of highly pathogenic H5N1 avian influenza virus, 3) habitats used in Asia, 4) population size in Alaska, and 5) likelihood of obtaining a representative sample of sufficient size. Table 4-7 is a summary of primary and secondary species that should be considered as sampling targets for highly pathogenic H5N1 avian influenza virus in the four major flyways (see also Attachment 5).

Table 4-4. Ranking matrix for populations of waterfowl and cranes to be sampled for HPAI during the 2006 field season in Alaska.

Taxon	**Total or partial contact with Asia**	**Contact with known "hot spot"**	**Habitat used in Asia**	**Pop. in Alaska**	**Can samples be obtained?**	**Score**
Steller's Eider	**4**	**1**	**4**	**3**	**3**	**15**
	Most (>90%) of the Pacific-wintering population (250,000) breeds in northeastern Asia	No known use of AI-infected areas	Uses estuarine and freshwater habitats	Winter pop approx 80,000 Breeding population <1,000	Relatively easy to trap during fall molting period	
Northern Pintail	**2**	**2**	**4**	**4**	**3**	**15**
	Unknown number of Siberian-breeding birds migrate through Alaska to winter in North America	Asian summer range overlaps with known AI-infected areas	Freshwater marshes, ephemeral wetlands	Summer population approximately 1 million	Easy to capture in Alaska in autumn	
Taxon	**Total or partial contact with Asia**	**Contact with known "hot**	**Habitat used in Asia**	**Pop. in Alaska**	**Can samples be obtained?**	**Score**

		spot"				
Lesser Snow Goose	**5**	**1**	**4**	**3**	**2**	**14**
	The Wrangel Island colony of 110,000 breeding birds is managed as a discreet population	No known use of AI-infected areas	Freshwater marshes, ephemeral wetlands	Entire breeding population of 110,000 breeding birds plus young of the year migrate through Alaska en-route to the west coast states	Could be difficult to obtain target number depending on timing and route of migration.	
Emperor Goose	**2**	**1**	**4**	**3**	**3**	**13**
	Approximately 20,000 birds molt in Chukotka, several thousand breed in the Anadyr lowlands	No known use of AI-infected areas	Breeds moist tundra meadows and near wetlands	approximately 90% of the population winters in Alaska and approximately 60% summers in Alaska	Relatively easy to trap during summer and fall molting period	
Black Brant	**1**	**1**	**4**	**3**	**3**	**12**
	Several thousand birds nest in the Anadyr lowlands and on Wrangel Island	No known use of AI-infected areas	Breeds in moist sedge coastal tundra areas	Near entire Pacific population of 130,000 birds stage at Izembek Lag prior to fall migration to winter from B.C. to Mexico	Samples could be obtained easily from fall birds	
Spectacled Eider	**4**	**1**	**4**	**2**	**1**	**12**
	Over 90% of the world population (approx 300,000) nests in Arctic Russia	No known use of AI-infected areas	Breeds moist tundra meadows and near wetlands	Approximately 9,000 birds breed on the Arctic Slope, and 8,000 on the Yukon-Kuskokwim Delta	Could be difficult to obtain target number	
Aleutian Cackling Goose	**1**	**1**	**4**	**3**	**2**	**11**
	Small numbers breed on Commander Islands and winter in Asia	No known use of AI-infected areas	Breeds on Aleutian Islands in wet, grassy freshwater meadows	Approximately 70,000 birds in fall population	Could be difficult to obtain target number	
Long-tailed Duck	**2**	**1**	**2**	**3**	**3**	**11**
	Approx 250,000 breed in northeastern Russia, unknown numbers cross to North America	No known use of AI-infected areas	Nests coastal tundra; postbreeding use estuarine areas	Approx 80,000 summer in western Alaska, 600,000 in northern Alaska and western Canada	Samples could be obtained easily from fall birds	

Taxon	Total or partial contact with Asia	Contact with known "hot spot"	Habitat used in Asia	Pop. in Alaska	Can samples be obtained?	Score
Tundra Swan	**1**	**1**	**4**	**3**	**2**	**11**
	Unknown numbers breed in eastern Chukotka; may be associated with Pacific Flyway	No known use of AI-infected areas	Nests coastal tundra; migration and non-breeding in coastal habitats	Approximately 150,000 summer in Alaska	Could be difficult to obtain target number	
Common Eider	**2**	**1**	**2**	**3**	**2**	**10**
	Approx 30,000 breed in northeastern Russia	No known use of AI-infected areas	Breeds in wet or moist tundra meadows near wetlands or on barrier islands	Alaska population believed to be 25,000 western Alaska plus 120,000 in northern Alaska plus western Canada	Could be difficult to obtain target number in most locations	
King Eider	**2**	**1**	**2**	**3**	**2**	**10**
	Approx 150,000 breed in northeastern Russia	No known use of AI-infected areas	Breeds in moist and upland tundra	Approx 360,000 breed in northern Alaska and western Canada	Could be difficult to obtain target number in most locations	
Lesser Sandhill Crane	**2**	**1**	**3.5**	**3**	**2**	**11.5**
	unknown numbers of mid-continent population breed in Siberia	No known use of AI-infected areas	Breeds in wet or moist tundra meadows near wetlands or on barrier islands, often feeds in agricultural areas where available	Alaska population believed to be in the low tens of thousands	Could be difficult to obtain target number	
Ranking criteria:						
	1. Proportion of the population occurring in Asia. Score as 1-5 where 5=100%	2. Contact with a known 'hotspot' or source. Score as 1=no contact, 2=contact	3. Habitats used in context of likelihood of exposure 1=Offshore marine, 2=Estuary, 3=Terrestrial, 4=Freshwater.	4. Population size in Alaska during 2006. Score to the closest number 1=1,000, 2=10,000, 3=100,000, 4=1,000,000	5. Can we obtain a representative sample of sufficient size (n=200)? Score 1=no, 2=maybe, 3=yes.	

Table 4-5. Ranking matrix for populations of shorebirds to be sampled for avian influenza during the 2006 field season in Alaska.

Taxon	Total or partial contact with Asia	Contact with known "hot spot"	Habitat used in Asia	Pop. in Alaska	Can samples be obtained	Score
Dunlin (*C. a. arcticola*)	**5**	**2**	**3**	**4**	**3**	**17**
	Entire pop. winters from Taiwan north to Yellow Sea and n. Japan	Winters throughout areas where H5N1 identified	Estuarine and freshwater habitats; also ephemeral inland lakes where domestic waterfowl raised	Est. at 650,000	Relatively. easy to trap on nest and during post-breeding when in flocks	
Sharp-tailed Sandpiper	**5**	**1**	**3.5**	**2**	**3**	**14.5**
	Breeding restricted to n. central Siberia with annual cohort of immatures coming to Alaska; adults move through EAA flyway	Migrating adults pass through known "hot spots" in central E. Asia. Species of concern if adults can pass virus to offspring on breeding grounds	Freshwater marshes, brackish wetlands, salt ponds, sewage farms, ephemeral wetlands	Between 10,000 and 40,000 depending on annual production	Easy to capture in Alaska in autumn	
Bar-tailed Godwit (*L. l. baueri*)	**5**	**2**	**2**	**3**	**2**	**14**
	Entire pop. nests w. and n. Alaska & stages central E. Asia (Yellow Sea, Korea, Japan) in spring; southward migration direct across Pacific	On migration stops in central E. Asia (Yellow Sea, Japan, Korea)	Estuarine	Est. at 120,000, but 2005 census efforts accounted for <50,000	Could be difficult to obtain target number	
Ruddy Turnstone (*A. i. interpres*)	**3**	**2**	**2.5**	**2.5**	**3**	**13**
	Portion of W. Alaska nesting pop. migrates to SE and E Asia; pop. nesting Chukotka moves to W. Alaska in fall before returning to E and SE Asia. Eastern Siberia and West Alaska breeding birds also migrate down the East Asian coast, with some birds wintering in the Mariana and Marshall Islands	On migration stops in central E. Asia (Yellow Sea, Japan, Korea)	Breeds upland tundra; migration and non-breeding coastal (rocky intertidal, sand beaches, & mudflats)	>35% of North American pop. (= ~20,000 birds) in Alaska, plus historically large numbers visit (>20,000 on Pribilof Is.) from Chukotka	Unless post-breeding concentrations found (e.g., Pribilof Is.) could be difficult to meet target sample. 200 turnstones can be captured in either the Marshall or Mariana Islands or both	

Taxon	Total or partial contact with Asia	Contact with known "hot spot"	Habitat used in Asia	Pop. in Alaska	Can samples be obtained?	Score
Pectoral Sandpiper	**3**	**1**	**4**	**3**	**2**	**13**
	Greater than 50% of pop. nests in Russia west to Eastern Taimyr Peninsular.	To date no known use of "hot spots"	Breeds marshy/grassy tundra; post-breeding uses brackish ponds freshwater marshes	200,000-300,000	Could be difficult to obtain target number	
Red Knot (*C. c. rogersi & roselaari*)	**4**	**2**	**2**	**2.5**	**2**	**12.5**
	C.c. roselaari pop. nests Wrangel I. and w. Alaska and winters Pacific coast of the Americas. *C. c. rogersi* nests Chukotka/New Siberian Isl. & winters Aust./New Zealand, passing through c. E. Asia	On migration *C. c. rogersi* passes through areas where H5N1 identified	Estuarine	*C. c. roselaari* <50,000; C. c. *rogersi* 220,000. *C. c. rogersi* thought to stop in Alaska in spring but numbers unknown (possibly several 10,000s)	Could be difficult to obtain target number	
Long-billed Dowitcher	**3**	**1**	**3**	**3**	**2**	**12**
	>30% of pop. breeds in Russia where range expanding w. to Taimyr Pen.; >95% of entire pop. winters in North and Central America. Unknown numbers winter in Asia (Japan)	To date no known use of "hot spots"	Breeds coastal lowlands in wet, grassy freshwater meadows; uses estuarine and managed wetlands during migration & winter	North American pop. = 450,000 (>90% of this in Alaska during migration)	Could be difficult to obtain target number	
Rock Sandpiper (*C. p. tschuktschorum*)	**3**	**1**	**2.5**	**2**	**3**	**11.5**
	~ 20-30% of pop. nests in Chukotka	To date no known use of "hot spots"	Nests upland tundra; post-breeding use estuarine areas	Total pop. 50,000. ~20K nest Chukotka but all return to AK en route to non-breeding areas in Pacific NW	Easy to trap on nest and during post-breeding flocking	

Taxon	Total or partial contact with Asia	Contact with known "hot spot"	Habitat used in Asia	Pop. in Alaska	Can samples be obtained?	Score
Pacific Golden-Plover	**3**	**2**	**2.5**	**2**	**2**	**11.5**
	Nesting occurs w. & sw Alaska and over large portion of n. Siberia and Chukotka. Interchange known between Asia and Alaska but not quantified. Alaska-nesting birds disperse to Oceania and Pacific coast of N & C America. Birds wintering in the Marshall or Mariana Islands are believed to be birds that have migrated overland from Siberia to Southeast Asia and Oceania.	Likely in c. East Asia	Nests upland tundra; migration and nonbreeding in coastal habitats	16,000	Could be difficult to obtain target number in Alaska; however, 200 plovers can be captured in either the Marshall or Mariana Islands or both	
Buff-breasted Sandpiper	**2**	**1**	**3**	**2**	**2**	**10**
	Small portion of pop. nests Wrangel I. & Chukotka then returns to non-breeding area in southern S. America	To date no known use of "hot spots"	Variable but generally dry upland tundra	3,000, including ~1000 birds from Chukotka/Wrangel I. stopping on southward migration	Could be difficult to obtain target number	

Ranking criteria:

1. Proportion of the population occurring in Asia. Score as 1-5 where 5=100%
2. Contact with a known 'hotspot' or source. Score as 1=no contact, 2=contact
3. Habitats used in context of likelihood of exposure 1=Offshore marine, 2=Estuary, 3=Terrestrial, 4=Freshwater.
4. Population size in Alaska during 2006. Score to the closest number 1=1,000, 2=10,000, 3=100,000, 4=1,000,000
5. Can we obtain a representative sample of sufficient size (n=200)? Score 1=no, 2=maybe, 3=yes.

Table 4-6. Ranking matrix for populations of passerines and a larid to be sampled for HPAI during the 2006 field season in Alaska.

Taxon	Proportion of population in Alaska	Contact with known "hot spot"	Habitat used in Asia	Population. in Alaska	Can samples be obtained	Score
Arctic Warbler (*Phylloscopus borealis kennicotti*)	**5**	**2**	**3**	**4**	**3**	**17**
	Endemic subspecies to Alaska	Winters in Myanmar, Thailand, se. China, Taiwan, Philippines south to Andaman Is., Malay Peninsula, and Indonesia east to Moluccas	Terrestrial. Wooded habitats, cultivated areas, grasslands, gardens, and mangroves	Est. at 2,700,000	Many locations where the most abundant breeding bird. Easy to capture during breed and migration	
Eastern Yellow Wagtail (*Motacilla tschutschensis*)	**5**	**2**	**3.5**	**4**	**3**	**17.5**
	Endemic species to Alaska	Taiwan, Indonesia, Sunda Isles, and Moluccas	Terrestrial. Open areas with water, sugarcane fields, rice fields, sparse grasslands, cassava plots; usually in association with wild and domestic grazing mammals	Est. 1,400,000	Easy to capture and areas with known concentration of breeding birds already identified	
Gray-cheeked Thrush (*Catharus minimus*)	**3**	**2**	**3**	**4**	**3**	**15**
	48% of global population in Alaska	Breeds in E. Siberia	Terrestrial. Shrubs often in riparian habitats	Est. 5,000,000	Most abundant bird in many locations. Already captured at many banding sites.	
Glaucous Gull (*Larus hyperboreus)*	**2**	**2**	**3**	**2**	**3**	**12**
	40,000 (100%) of US breeding population in AK	Contact with humans and garbage dumps	Terrestrial/coastal	Approx 40,000	Samples easily obtainable	

Table 4-7. Suggested migratory bird species for highly pathogenic H5N1 avian influenza surveillance in the four North American flyways.

Pacific Flyway	
Taxon	**Ranking**
Tundra Swan (Western Population)	Primary
Lesser Snow Goose (Wrangel Island Population)	Primary
Northern Pintail	Primary
Long-billed Dowitcher	Primary
Red Knot (small numbers)	Primary
Pacific Golden Plover (small numbers)	Primary
Ruddy Turnstone (very small numbers)	Primary
Black Brant (Pacific Population)	Secondary
Cackling Goose	Secondary
Pacific Greater White-fronted Goose	Secondary
Mallard	Secondary
American Wigeon	Secondary
American Green-winged Teal	Secondary
Northern Shoveler	Secondary
Central Flyway	
Taxon	**Ranking**
Lesser Sandhill Crane (Mid-continent)	Primary
Tundra Swan (Eastern Population)	Primary
Northern Pintail (low percentage from Alaska)	Primary
Pectoral Sandpiper	Primary
Buff-breasted Sandpiper	Primary
Long-billed Dowitcher	Primary
Greater White-fronted Goose (Mid-continent)	Secondary
Lesser Snow Goose (Western Central Flyway)	Secondary
Mallard	Secondary
American Wigeon	Secondary
American Green-winged Teal	Secondary
Northern Shoveler	Secondary

Mississippi Flyway	
Taxon	**Ranking**
Pectoral Sandpiper	Primary
Dunlin	Primary
Long-billed Dowitcher	Primary
Greater White-fronted Goose	Secondary
Northern Pintail	Secondary
Mallard	Secondary
American Wigeon	Secondary
American Green-winged Teal	Secondary
Northern Shoveler	Secondary
Lesser Scaup	Secondary
Greater Yellow-legs	Secondary
Lesser Yellow-legs	Secondary
Ruddy Turnstone	Secondary
Gray-cheeked Thrush	Secondary
Atlantic Flyway	
Taxon	**Ranking**
Tundra Swan (Eastern Population)	Primary
Greater Scaup	Primary
Horned Grebe (possibly Europe/Greenland breeders)	Primary
Lesser Scaup	Secondary
Canvasback	Secondary
Long-tailed Duck (unknown east-west interchange)	Secondary
Western Sandpiper	Secondary
Least Sandpiper (do not breed in Asia)	Secondary
Greater Yellow-legs (do not breed in Asia)	Secondary
Black-bellied Plover	Secondary

Sample Size

When sampling for highly pathogenic H5N1 avian influenza virus it is critical that an appropriate sample size for each species or species group in each designated sample population is obtained. Equation 1 provides a method for calculating the recommended sample size:

$$n = \log(1-c) / \log(1-p) \qquad \text{(eq. 1)}$$

where n is the sample size, c is the desired level of confidence, and p is the prevalence of positive samples in the population. An adequate sample size should allow for >95%

confidence that AI is detected at $\leq$ 1.5% prevalence. These criteria result in an estimated sample size of 200:

$$n = \log (1-.95) / \log (1-0.015) = 200$$

Thus, a minimum of 200 samples should be collected from the population of interest based on an assumed prevalence of 1.5% of highly pathogenic H5N1 avian influenza. We caution that this calculation is very sensitive to the assumed prevalence, which we can not know a priori. For example, if prevalence of the disease at the time of sampling is 0.1% (i.e., 1 in 1000 birds is infected) the necessary sample size is 3000. As prevalence decreases the likelihood of detecting the disease in an individual bird also decreases due to the low probability of detection and practical limitations on laboratory processing capability. We also caution that this formula is weakened here because it is based on assumptions that may not apply to H5N1 virus in wild birds, namely that the agent is homogeneously distributed within a host population that also is homogeneously distributed.

Sample Collection

Tracheal and cloacal swabs should be collected from individuals of each species at each location using the procedures identified in Attachment 9.

Discussion

Wild birds, particularly waterfowl and other waterbirds, are natural hosts of avian influenza viruses and are believed to play an important role in the epizootiology of these viruses. All hemagglutinin and neuraminidase subtypes have been found in waterfowl and shorebirds (Webster et al., 1992; Krauss et al., 2004; Widjaja et al., 2004). This proposed sampling effort provides the best opportunity for detection of highly pathogenic H5N1 avian influenza virus in live migratory birds that may bring the disease from Asia to Alaska, the Pacific Islands and the west coast of the U.S. The primary advantage to this approach is that species will be sampled that travel directly to Alaska or the US Pacific Islands and trust territories from Southeast Asia or Australasia, have some fraction of the population known to winter in Asia or Pacific trust territories, or intermingle with other species across Siberia, the Russian Far East, and Alaska. The primary disadvantage is the logistical considerations in live capture of the birds in remote areas.

Recommendations

Sampling live birds will allow us to determine if they are currently infected with highly pathogenic H5N1 avian influenza virus or other AI viruses. When collecting samples from live birds, tracheal and cloacal swabs are preferred. Most AI strains tend to replicate more efficiently in the intestinal tract than in the respiratory tract of natural host species (i.e., waterfowl and shorebirds). Consequently, cloacal swabs are generally

preferred. However, recent isolations of highly pathogenic H5N1 avian influenza virus in wild birds have documented higher levels of virus in tracheal samples. Therefore, it is recommended that both samples be collected from birds when possible. While, the collection of cloacal swabs is a relatively easy procedure, obtaining proper tracheal swabs can be problematic and requires personnel trained in the sampling technique. Examples of tracheal/cloacal swab collection protocols can be found in Attachment 9. Tracheal and cloacal swabs should be placed in separate tubes, and swabs should not be pooled across individuals.

Specific implementation plans should be developed for each state/flyway. It is strongly advised that agencies and organizations coordinate their sampling efforts to assure that adequate sample sizes are obtained from each species within each state/flyway. Coordination can be achieved through the existing migratory bird flyway councils.

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ATTACHMENT 5

Surveillance for Highly pathogenic H5N1 avian influenza in Hunter-killed Birds

Overview

Check stations for waterfowl hunting are operated by some state natural resource agencies and National Wildlife Refuges to collect information on local waterfowl harvest. Hunter check stations provide an opportunity to collect additional samples to monitor for the presence of Highly pathogenic H5N1 avian influenza virus and other avian influenza (AI) virus subtypes. This sampling would supplement live bird surveillance (Attachment 4) by increasing the number of selected species, geographic locations, and time periods represented in surveillance efforts. Previous and current studies that have sampled hunter-killed waterfowl to detect AI include an ongoing 20-year study in Ohio by Dr. Richard Slemons of Ohio State University, current work in North Carolina by Dr. David Stallknecht of the Southeastern Cooperative Wildlife Disease Study (the North Carolina Wildlife Resources Commission has been collecting samples from hunter-killed ducks and tundra swans for Dr. Stallknecht), and sampling in New Mexico, Texas, and Maryland by state wildlife agencies and/or university researchers. A number of the state natural resource agencies and other researchers are developing plans to sample hunter-killed birds for AI in 2005 and/or 2006.

This conceptual surveillance strategy includes (1) sampling birds killed in fall by sport hunters and in summer by subsistence hunters in Alaska: (2) sampling birds killed in fall in the lower 48 states. As with surveillance of live-captured birds, sampling hunter-killed birds in Alaska will focus on hunted species that are most likely to be exposed to HPAI in Asia, and that have relatively direct migratory pathways from those areas to Alaska (primary species). Additional samples collected on the wintering grounds in the lower 48 states will include both primary species and species that mix with the primary species in Alaska staging areas (secondary species). Currently, the probability of highly pathogenic H5N1 avian influenza virus transmission from primary species to secondary species is poorly understood, but AI viruses are known to remain viable for months in cold freshwater. From a surveillance standpoint, if secondary transmission proves to be potent and extensive, a very large number of species could be involved. However, this conceptual program is focused on early detection, with adaptation to more intensive efforts as needed. Thus, it is recommended that sampling efforts involving hunter-killed birds in the lower 48 states should concentrate on the species/populations and wintering areas in which the presence of highly pathogenic H5N1 avian influenza virus is most likely to be detected. Some research indicates that susceptibility may vary among game birds. The complete design, implementation and development of an operational plan and the funding necessary for this strategy requires closer coordination with states through the Flyway Council system.

A similar approach to investigate the possible movement of Highly pathogenic H5N1 avian influenza virus from Europe could be developed. There are few data on migratory bird movement rates between North America and Europe, but the band recovery data that are available suggest very low exchange rates. Presently we only have data on movement from North America to Europe, but if we make the tenuous assumption that movement rates are similar in both directions, then only 3 species emerge as likely primary species: Eurasian wigeon, northern pintail, and green-winged teal. Of American wigeon banded in the Atlantic Flyway, only 6 of 2,211 recoveries (0.27%) were from Europe. The rates for northern pintail and green-winged teal are 0.06% (3 of 5,341 recoveries) and 0.04% (5 of 12,274 recoveries), respectively. If immigration rates are similar to emigration rates, and if those rates are indicative of the proportion of eastern North American birds that are immigrants from Europe, the chance of sampling even one hunter-killed immigrant is very low. Some common eiders (Keith McAloney, Canadian Wildlife Service, personal communication) and North Atlantic Population Canada geese (Fox et al. 1996) move back and forth between northeastern North America and Greenland, where they could interact with birds from mainland Europe. Likewise, the high-arctic Atlantic brant that breed in North America and winter in Ireland also come into contact with European birds. Thus, these are additional species that should be considered when implementing surveillance for highly pathogenic H5N1 avian influenza virus in the Atlantic flyway.

Methodology

Alaska

The surveillance of live-captured birds strategy (Attachment 4) has provided the biological basis for identifying the primary species recommended for sampling in Alaska. Below is a list of these target species and sampling locations recommended for surveillance of hunter-killed birds.

Samples from fall hunters:

- Northern pintail: Mendenhall Refuge, Minto Flats, and Cook Inlet
- Lesser sandhill crane: Delta Junction
- Black brant: Cold Bay
- Common eider, king eider, long-tailed duck: Kodiak National Wildlife Refuge, and perhaps cooperating hunting guides along the coast of Alaska.

Samples from summer hunters:

- Seward Peninsula: lesser sandhill crane, bar-tailed godwit, long-billed dowitcher
- Yukon-Kuskokwim Delta: black brant, emperor goose, common eider, king eider, northern pintail, long-tailed duck
- Barrow: common eider, king eider, black brant, long-tailed duck, glaucous gull
- St. Lawrence Island: emperor goose, black brant, common eider, king eider

North American Flyways

There are at least 25 states in which either the state wildlife agencies or USFWS routinely check hunter harvested birds: 9 in the Atlantic Flyway, 7 in the Mississippi Flyway, 4 in the Central Flyway, and 5 in the Pacific Flyway. Those 25 states and most other states have already expressed some willingness to collect samples from hunter-killed birds. States are encouraged to develop specific implementation plans in consultation with their respective flyway council using the guidance provided in this strategic plan.

Four primary target species/populations have been identified for highly pathogenic H5N1 avian influenza virus sampling in the lower 48 states: northern pintail, Pacific black brant, Wrangel Island snow geese, and lesser sandhill crane (mid-continent population). Also, several secondary species that mix with the primary species in Alaska (and thus have an increased risk of exposure to highly pathogenic H5N1 avian influenza virus) and later winter in the 4 flyways have been identified (see below). Given the ephemeral nature of birds at specific sites along their migration routes, sampling efforts for both primary and secondary species should be concentrated on the wintering grounds.

The target species/populations and general sampling locations presented in this document are based on band recovery and in some cases radio telemetry data for birds banded in Alaska. Specific sampling sites (i.e., check stations or other areas where hunter-harvested birds could be sampled) will be determined by the individual states and National Wildlife Refuges that elect to participate in the sampling.

Pacific Flyway:

- Northern pintail (primary species): Central Valley of California
- Wrangel Island snow geese (primary): Skagit-Fraser Rivers Delta, Washington and British Columbia
- Black brant (primary): Humboldt Bay, California and San Quintin Bay, Mexico if permits to collect samples and ship them to the U.S. can be obtained
- American wigeon combined (secondary): Central Valley of California
- American green-winged teal (secondary): Central Valley of California
- Northern shoveler (secondary): Central Valley of California
- Cackling goose (secondary): Northwest Oregon/Southwest Washington permit goose areas
- Pacific greater white-fronted geese (secondary): Central Valley of California
- Tundra Swan (secondary): Montana and Utah
- Mallard (secondary): western Washington

Central Flyway:

- Lesser sandhill crane (primary): New Mexico, west Texas, Nebraska
- Northern pintail (primary, but few from Alaska): Gulf Coast, Texas
- Lesser snow goose (secondary): North Dakota, Nebraska
- Mid-continent greater white-fronted geese (secondary): Texas
- American wigeon (secondary): Gulf Coast, Texas
- American green-winged teal (secondary): Gulf Coast, Texas
- Northern shoveler (secondary): Gulf Coast, Texas

- Tundra Swan (secondary): North Dakota, South Dakota
- Mallard (secondary): Oklahoma, Texas, Nebraska

Mississippi Flyway:

- Northern pintail (primary, but few from Alaska): Gulf Coast, Louisiana
- Mid-continent greater white-fronted geese (secondary): Louisiana
- American wigeon (secondary): Gulf Coast, Louisiana
- American green-winged teal (secondary): Gulf Coast, Louisiana
- Northern shoveler (secondary): Gulf Coast, Louisiana
- Mallard (secondary): Arkansas, Mississippi, Louisiana
- Lesser scaup (secondary): Gulf Coast of Louisiana, Mississippi, Alabama

Atlantic Flyway

- Tundra swan (secondary): North Carolina, Virginia
- Greater scaup (secondary): East Coast from Massachusetts to Virginia
- Lesser scaup (secondary): Florida, East Coast from Chesapeake Bay south
- Canvasback (secondary): Chesapeake Bay
- Long-tailed duck (secondary): East Coast from Massachusetts to Virginia

Duck breeding population estimates can provide some indication of the relative likelihood (among species) that a given hunter-killed bird came from Alaska. That is, samples from species that have a larger proportion of their breeding population in Alaska are more likely to contain birds from Alaska than samples from species with a lower proportion of their breeding population occurring in Alaska. Based on that premise, we ranked the secondary species of ducks listed above according to sampling priority. In 2005, 39% of the American wigeon breeding population occurred in Alaska, making that the highest priority species according to this criterion. The others are ranked as follows: 2. northern pintail (35% of the breeding population occurred in Alaska), 3. American green-winged teal (33%), 4. greater and lesser scaup combined (28%), 5. northern shoveler (18%), 6. canvasback (18%), and 7. mallard (10%).

As in Alaska, a sample size of 200 birds from each sample population is needed to detect highly pathogenic H5N1 avian influenza virus prevalence of 1.5% or greater with 95% power. Therefore, it is recommended that a minimum sample size goal of 200 birds per species per wintering area. Tracheal and/or cloacal samples should be collected in accordance with protocols identified in this document (Attachment 8).

Discussion

Sampling of hunter-killed birds would supplement targeted surveillance in live wild birds (Attachment 4) and other strategies identified in this strategic plan. The advantage to this approach is that it is cost-effective because for most of the species that are classified as game birds, existing infrastructure (e.g., check stations) is in place in most wintering areas and sufficient numbers of birds are expected to be encountered. The disadvantages to this approach are: 1) most of the sampling in the lower 48 states will be of secondary species, thus the likelihood of sampling birds that have come into contact with infected primary species birds is small, especially in the Atlantic, Mississippi, and Central Flyways; and 2)

numerous sampling sites throughout the U.S. will require sufficient training of sampling personnel to ensure samples are properly acquired, preserved, and shipped. There are advantages and disadvantages in terms of public perceptions of sampling hunter-harvested birds. Public perceptions could be positive if user-groups will appreciate that samples are being taken. Conversely, if hunters do not have accurate information about highly pathogenic H5N1 avian influenza virus, they could become unnecessarily alarmed about exposure (especially if agency samplers are wearing protective gear). For spring and summer subsistence users, providing access to birds that were historically taken illegally may make sampling difficult and basic information on highly pathogenic H5N1 avian influenza virus and the sampling program may be harder to deliver.

Recommendation

Sampling hunter-killed birds would supplement other approaches in a cost-effective manner and may allow us to determine if certain species of birds (e.g., migratory game birds) are currently infected with highly pathogenic H5N1 avian influenza subtype or other AI viruses. This expanded effort to identify highly pathogenic H5N1 avian influenza virus should be considered a supplemental part of any surveillance system. Specific implementation plans and budgets should be developed in concert with affected state agencies and the flyway councils.

Unlike other surveillance approaches, the use of hunter-killed birds has high public profile. Therefore, the implementation of this action should be discussed with agencies and organizations that have experience regarding the public relations aspects of researching and assessing zoonoses (e.g., chronic wasting disease and West Nile virus) to ensure that we develop an appropriate and consistent message to hunters.

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ATTACHMENT 6

Sentinel Animal Methods

Overview

This methods section reviews two sentinel animal methods that have been used in avian disease surveillance programs and that may be used for the early detection of avian influenza (AI) virus infection along migratory flyways in the U.S.

Non-commercial Backyard Poultry Flocks

Backyard poultry are defined as domesticated fowl, including chickens, turkeys, waterfowl, and game birds (except doves and pigeons) maintained for hobby or noncommercial egg and meat production (NAHRS FAQ 2005). Backyard poultry are typically allowed to forage freely or may be confined in partially enclosed fenced areas. The evaluation of poultry flocks reared on backyard premises for diseases of interest to the commercial poultry industry has been used as a surveillance method to estimate seroprevalence of selected disease agents as part of health surveys in backyard flocks adjacent to commercial operations. (McBride; Hird; Carpenter; Snipes; Danaye-Elmi, and Utterback 1991;Johnson; Colby; Tablante; Hegngi; Salem; Gedamu, and Pope C. 2004)

In 2005, State animal health officials in Alaska sampled birds at fairs and exhibitions (concentration points). Most exhibitors were 4H or hobby farmers. Fairs and exhibitions are social events and are attended by large numbers of game bird fanciers from remote regions of Alaska. State animal health officials offered testing to exhibitors at three agricultural fairs with the goal of testing every entry to the fair (600 samples representing 100 flocks). This was a voluntary sampling program, but nearly 100% of owners of exhibition game agreed to test in 2005.

- 150 birds were sampled at the Fairbanks fair. Tanana Fair entries represent flocks from a 40 mile radius around the city of Fairbanks (Healy, Tanana, and the North Pole).
- 100 birds were sampled at the Kenai fair. Kenai Fair entries include flocks from Homer to Anchorage
- 300 samples were collected at the Alaskan State Fair in Palmer. Palmer Fair entries include Anchorage, Matanuska Valley (includes flocks as far north as Fairbanks)

In Alaska, poultry chicks are either purchased through mail order or from a few local breeders and may be reared in suburban areas or in remote villages throughout the State. Most backyard birds are reared for egg production and slaughtered for meat prior to the winter season, although there are a growing number of hobbyists that raise show birds. Birds

are often reared on open range or in outside enclosures and sometimes have an opportunity to intermingle with wild waterfowl. Limited resources prohibited widespread backyard bird surveillance testing over the large expanse of the state. Backyard flock surveillance is presently passive and owners request testing after morbidity or mortality events occur in their flock or after noting dead waterfowl or sick waterfowl on their premises. There is currently no census available to estimate the number of backyard flocks in Alaska.

Cloacal swab samples are placed in ethanol and evaluated at the University of Alaska, at Fairbanks by RT-PCR. If surveillance screening samples are positive by PCR, the premise is placed under quarantine and additional cloacal samples taken during the epidemiological investigation are placed in viral transport media and submitted for virus isolation to NVSL in Ames, Iowa.

In 2006, the Alaska Department of Environmental Conservation, Office of the State Veterinarian will sample backyard flocks, near summer water systems where wild and domestic waterfowl congregate and collect environmental samples (bird droppings, water samples) in six general areas:

- Southeast- 2 cities (Juneau, Ketchikan)
- Southcentral- 4 cities (Homer, Soldotna, Anchorage, Matanuska Valley
- Interior- 3 cities (Fairbanks, Healy, Talkeetna)
- Southwest- 2 cities (Bethel, King Salmon)
- Northwest- 2 cities (Nome, Kotezebue)
- Aleutians/Bering Sea- 4 cities (Kodiak, Dutch Harbor, Cold Bay, Pribilof Islands)

The areas listed in bold have the highest priority and cover a majority of the population where domestic poultry is kept. The other 3 areas have substantial populations of wild birds but few domestic backyard flocks. A sample size of 11 is needed to detect avian influenza at a prevalence rate of 25% at a 95% confidence interval in flocks ranging from 10 to 10,000 or more birds.

The Office of the State Veterinarian will sample poultry exhibited at the six agricultural fairs (concentration points):

• Deltana Fair	Date to be announced
• Haines Fair	7/26-30/2006
• Tanana Valley Fair	8/4-12/2006
• Kenney Lake Fair	Date to be announced (2nd Week August 2006)
• Kenai Peninsula Fair (Ninilchik)	8/18-20/2006
• Alaska State Fair (Palmer)	8/24 - 9/4/2006

The fair boards have expressed interest in promoting this sampling effort and it is expected that all entries will be tested. The state has just finished construction of a new diagnostic laboratory, Alaska Environmental Health Laboratory in Anchorage, and will develop the capacity to run the diagnostic tests using RT-PCR. If surveillance screening samples are positive by PCR, the premise will be placed under quarantine and additional cloacal samples

taken during the epidemiological investigation are placed in viral transport media and submitted for virus isolation to NVSL in Ames, Iowa.

This approach to sampling non-commercial poultry flocks may be adapted in other areas of the U.S. where there is widespread non-commercial poultry production.

Sentinel Duck Flocks

The second method described is the placement of sentinel duck flocks in wetland environments where they are potentially exposed to and infected with disease agents as they commingle with wild birds. The placement of sentinel flocks of domestic ducks has been used to recover AI and detect influenza epizootics in pelagic bird colonies, and yielded much higher isolation rates compared to isolations from wild birds (Turek; Gresikova, and Tumova 1984;Sinnecker; Sinnecker; Zilske, and Koehler 1982;Sinnecker; Sinnecker, and Zilske 1982). Sentinel ducks have been used to determine the presence of AI and timing of infection associated with the arrival of wild migratory waterfowl in wetland habitats adjacent to market turkey production flocks (Halvorson; Karunakaran; Senne; Kelleher; Bailey; Abraham; Hinshaw, and Newman 1983;Halvorson; Kelleher, and Senne 1985;Kelleher; Halvorson; Newman, and Senne 1985).

In North America, AI isolations from waterfowl have been reported from approximately 30 locations over the past 35 years (Hanson; Stallknecht; Swayne; Lewis, and Senne 2003). Many of these sites are located along each of the four migratory waterfowl flyways (Pacific, Central, Mississippi, and Atlantic) in the continental U.S. Bodies of water with large concentrations of migratory waterfowl and shorebirds might also serve as sentinel sites.

Ideally, surveillance activities should occur at sites at a time when migratory birds are actively nesting and at locations where they marshal and intermingle with other migratory birds transiting the area prior to winter migration. The onset of avian influenza infection in sentinel ducks has been shown to occur in late July and early August in summer breeding areas (infection of range reared turkey flocks was shown to occur about 6 to 8 weeks later) (Halvorson et al. 1985). Avian influenza virus prevalence estimates from published waterfowl surveys indicate that virus can first be detected in naïve juvenile birds in summer breeding areas in July or August (prevalence ranged from 11% to 61% in published surveys) as juveniles emerge from hiding and intermingle with other broods and a subsequent high rate of re-infection as birds marshal for winter migration in October (Hanson et al. 2003; Hinshaw et al. 1985). Avian influenza virus prevalence generally decreases during late fall and winter and may reach a level of 1% or less in over-wintering areas.(Stallknecht ;Webster; Bean; Gorman; Chambers, and Kawaoka 1992) However, virus was isolated from 11% of teals and from 15% of northern pintails in one recent survey of wintering ducks in Texas, suggesting that the avian influenza season may not be a fall season event (Hanson 2003). As a result of early migration, blue winged teal are thought to serve as an immunologically naïve host in wintering areas.

Most virus isolations have occurred in mallards and other species of dabbling ducks, but less commonly in wood ducks and similar species (Stallknecht). Mallards are commonly

associated with habitats located near man, livestock, and poultry and would be more likely to interact with backyard poultry flocks compared with other waterfowl species (Stallknecht and Shane 1988). Although H5, H7, and H9 subtypes have been poorly represented in most waterfowl surveys (H3, H4, or H6 subtypes have been isolated most frequently), pintails and mallards have been shown to be significant reservoirs in one recent survey where H5, H7, and H9 virus subtypes were isolated 21.5% of the time in Minnesota (Hanson and others 2003). The prevalence of AI isolated from blue winged teal on wintering grounds in February in Texas was found to be 22% in 2001 and 15% in 2002 (Hanson 2003). Migration of blue winged teal occurs in late summer and early fall (typically September), prior to the highest period of AI prevalence. Early migration of this species is thought to play a role in maintenance of AI infection on wintering grounds by providing a susceptible population with little or no prior exposure or immunity. However, blue winged teal are less likely to interact with man or livestock, so sites where blue winged teal congregate may not serve as the best sites for surveillance using backyard flocks of domestic waterfowl.

The role of shorebirds in avian influenza ecology should be considered separately from that of migratory waterfowl. The highest prevalence of avian influenza virus in shorebirds has been shown to occur in May and in September, which coincides with the times of peak shorebird migration in the northeastern U.S. (Kawaoka; Chambers; Sladen, and Webster 1988). Shorebirds migrating through the Delaware Bay have been shown to have the highest prevalence of AI virus compared with other shorebird populations surveyed at four other locations along the Atlantic flyway (Hanson 2003). Although most isolates reported from shorebirds in this survey were H10 and H12 (H9 and H13 in previous studies), H5 and H7 subtypes were isolated from a small percentage of shorebirds. During May, virus was isolated mostly from ruddy turnstones (9.1%).

The approach to the design of a targeted surveillance method for the detection of avian influenza using either of these two sentinel animal methods should incorporate what is presently known about the ecology and natural history of avian influenza infection in wild waterfowl reservoir species. Sentinel animals are most likely to become infected with AI if exposed to reservoirs in nature during periods of highest viral shedding. As described above, prevalence of infection as measured by virus isolations in published waterfowl surveys has been shown to vary temporally by location, age, season, and species. A targeted approach to sentinel animal surveillance should be designed to:

- Target specific locations where AI has been isolated from wild waterfowl historically;
- Target locations where known primary reservoir species (mallards, blue winged teal, ruddy turnstones) congregate for breeding (resulting in higher concentrations of juveniles susceptible to infection) or wintering (higher concentrations of species with little or no previous exposure) resulting in a higher prevalence of infection;
- Be timed to coincide with periods (seasons) of highest prevalence in the reservoir species, in particular migratory species that originate from an area having high incidence of AI (Southeast Asia).

Methods

Backyard Poultry Surveillance Method

Flock Selection

- Targeted flocks should consist of free range domestic waterfowl or poultry flocks located near marshlands or wetlands.
- Marshlands should contain high density populations of waterfowl or shorebirds.
- Flocks should have an opportunity to directly intermingle with waterfowl (especially mallards) at or near the common watershed via open range or open enclosure or by sharing a common source of water.
- Chose sites adjacent to wetlands where AI virus has been isolated historically.

Timing of Surveillance

- Surveillance should begin in late July and continue through October at sites near northern breeding areas.
- Although, prevalence rates in wild waterfowl were shown to decrease significantly in wintering areas in Louisiana (1%), prevalence in blue winged teal in wintering areas in Texas during February of >10% indicates that some wintering sites may be useful for sentinel surveillance.
- The seasonal peak of AI prevalence in shorebirds occurs in May rather than late summer, so surveillance of backyard flocks in the Delmarva (Delaware Bay) area should be planned for May to coincide with the time of highest prevalence.

Sample Size Estimates

- The average size of backyard poultry flocks in the U.S. is 35 birds (varies from 28 to 49 birds per flock by region). A prevalence estimate for avian influenza of 25% is assumed (NAHMS Poultry '04 Part I 2004).
- A sample size of 11 is needed to detect avian influenza at a prevalence rate of 25% at a 95% confidence interval in flocks ranging from 10 to 10,000 or more birds.(Cannon and Roe 1982)
- Cloacal and tracheal swab samples would be submitted to the appropriate diagnostic labs for RT-PCR testing and to a reference lab for virus isolation.

Sentinel Duck Method

Flock Preparation and Placement

- Construct pens or plan for open fenced enclosures that will hold 10 to 20 ducks and allow contact with released "messenger" ducks and wild ducks. Pens should allow exposure to water contaminated with wild duck feces.
- Deploy pens to selected wetlands (or construct fenced enclosures).
- Arrange to provide basic husbandry.
- Rear one day old ducks in isolation facilities for 6 to 7 weeks.
- Establish AI free status by cloacal swabbing and serologic testing.
- Release 10 to 20 isolation reared "messenger" pinioned mallard or white Peking ducks on selected body of water.
- Place 10 to 20 ducks in pens on selected body of water to intermingle with "messenger" ducks and wild ducks.
- Periodically bleed ducks to determine serologic status and replace H5 seropositive ducks with immunologically naïve ducks.

Timing of Surveillance

- Placement of sentinel duck flocks should coincide with backyard flock surveillance seasonally.

Sampling

- Retrieve fecal samples via cloacal swabs from 10 to 20 penned ducks to detect virus weekly and periodically trap messenger ducks for cloacal sampling.

Data Collection

For backyard flocks, a database similar to the one used for Exotic Newcastle Disease (END) surveillance would provide the ability to trace positive samples back to their flock of origin (Accession number; sending facility premises ID; submitter name, address, and contact information; location of animals including premises ID, latitude, and longitude; owner name; flock information including size, number affected, number dead; purpose of submission and relevant clinical information).

Data needed to create predictive geospatial models to evaluate spatial and temporal risk for sentinel duck flocks include: (1) lat/long (in unprojected decimal degrees with a WGS-84 or NAD-83 datum) of the sentinel cage's location; (2) front gate coordinates for the premises; (3) name, address, county, zip code, contact information for the land owner/manager, and occupations of all residents; (4) age, sex, and breed of birds; (5) number of sentinel birds and each bird must have a unique ID (e.g., numbered aluminum leg or wing bands work well); (6) environmental description of area where cage containing sentinel birds is placed; (7) AI

virus test status (birds are bled periodically to evaluate immune status and need for replacement); (8) presence and approximate distance to other birds and mammals; (9) exposure to wild birds and free ranging domestic birds; (10) estimated density of birds and mammals on premises and in the vicinity of the sentinels; (11) exposure of sentinels to human contact other than the avian phlebotomist; and (12) an environmental assessment of the vicinity (e.g., within 100 meters, within 500 meters, and within 1000 meters). These data should be captured on a site survey form. However, a separate form should be used to record: date and time blood samples were collected, the birds' ID number, and the vial number for the blood specimen. With this basic information, other data sources can be used to evaluate proximity to wetlands, bird roosts, position with in normal flyways, terrain features, and more. Access to extensive datasets (e.g. the National Wetlands Inventory and the National Landcover Dataset) and hydrologic models could be used to identify wetlands.

Discussion

Major advantages of the use of sentinel animals to detect AI:

- Backyard bird surveillance programs are already in existence in most states.
- State animal health officials are familiar with a targeted surveillance approach (i.e. surveillance of backyard flocks within a designated radius adjacent to commercial poultry operations).
- The placement of sentinel ducks has been used successfully to isolate AI from wild waterfowl in previous published surveys.
- Mortality in backyard poultry from H5N1 has occurred in other countries.
- Could be done in conjunction with other surveillance methods at the same location for comparison.

Major disadvantages of the use of sentinel animals:

- Locating suitable surveillance sites will require field surveillance or input from wildlife biologists.
- Expense of rearing AI free birds.
- Pen construction and husbandry costs.
- Sentinel flocks are subject to predation.

Recommendations

In order to implement an efficient active sentinel animal surveillance system, sentinel flock locations should be purposefully chosen. Appropriately allocating limited resources to achieve targeted sampling and reduce costs is an important objective of animal disease surveillance programs (McCluskey 2003). Knowledge of disease distribution allows us to focus surveillance activities. In this case, we can use our knowledge of the most likely entry points for H5N1 through migratory waterfowl to locate sentinel animal flocks. In order to target areas for sentinel surveillance with a higher probability of disease, flyway information

should be plotted over waterfowl management areas in order to select sites most likely to have migratory birds from areas where commingling with Eurasian species is most likely to occur. Specific locations in areas where migratory birds from possible northern exposure sites are most likely to be in highest concentration have been identified in other methods sections of this plan. National information on the health and management practices of backyard and small production flocks adjacent to commercial poultry operations in 18 states is available. All of this information should be combined with information on the geographic distribution of poultry producers including sizes and densities of operations in order to produce a risk map. Local animal health officials could then locate sentinel backyard flocks adjacent to waterfowl management areas in poultry dense regions where there is the highest probability of disease transmission. The health status of sentinel backyard flocks could be evaluated on a recurring basis (quarterly, or more often during seasons of the year that pose the highest probability of disease transmission due to higher prevalences) for an active disease surveillance program.

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ATTACHMENT 7

Environmental Sampling (Feces/Water)

Introduction

Avian Influenza viruses (AI) are released by waterfowl through the intestinal tract and viable virus can be detected in both feces and the water in which the birds swim, defecate and feed. This is the principle means of virus spread to new avian hosts and potentially to poultry and other susceptible livestock. Analysis of both water and fecal material from waterfowl habitat can provide evidence of AI virus circulating in wild bird populations, the specific AI subtypes, levels of pathogenicity, and possible risks to livestock.

Technical Aspects of Sampling Water

AI is relatively stable in water, especially at colder temperatures. The longevity of viable virus (weeks to months) allows for an integration of activity on a site basis. However, in the absence of established serial sampling, pinpointing the time at which a site becomes contaminated would be difficult. The advantages of including waterfowl sites as a point of sampling lie in the ease of collecting samples and the potential to sample the potential contaminating influence many birds at once. This method would provide a cost-effective, geographically explicit methodology. Moreover, given the ease of sampling, more sites could be sampled, providing for a higher resolution surveillance network.

Technical aspects to monitor water samples for AI involve collecting specified volumes of water (usually 50-500ml), transporting the samples on ice or frozen, concentrating the virus present either by filtration or precipitation/centrifugation, and inoculating the virus onto chicken eggs or cell culture for virus growth. The virus replicates in the cultures and is characterized by serological or molecular methods to determine specific subtypes. Alternative methods of analysis involve extracting viral genetic material from the sample with detection using molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and subsequent sequencing to determine subtype. Refinement of these methods still needs to be done. However, these techniques have the advantage of rapid results. All of the procedures for monitoring AI in water samples are generally established, and with proper expertise and equipment can be easily adapted to most laboratory settings.

Technical Aspects of Sampling Feces

Fecal sampling is used extensively in monitoring studies for AI in wild bird populations. The principal advantages of this method are that the costs and effort of capturing birds are avoided and large sample numbers can be quickly and easily obtained. It also is a

good method to determine the presence or absence of virus in bird populations present at a specific location. The disadvantage is that species identification is not always possible, determination of prevalence is complicated by the possibility of repeated sampling of individuals, and the sensitivity of the method is lower than for oral swabs. However, the increased sampling effort occurring because of reduced sampling costs are anticipated to offset any short comings owing to decreased sensitivity. Information from the field could be used to generate an environmental risk map related to specific areas (habitats) associated with potential AIV transmission.

Infectivity of the virus is maintained up to 4 days in wet feces at 25°C. Best analytical results come from fresh fecal samples that are either processed quickly or frozen until processing. Thus this method of sampling, while providing good information, is best applied while birds are present at a location such that the samples are as fresh as possible. By restricting fecal collection to fresh samples, it allows for population census data to be collected, and by inference estimates of the species sources of the contamination. Species and individual identification through genetic typing of feces would allow estimates of prevalence.

Accredited laboratories have the capacity and infrastructure to analyze a limited number of samples for AI. The anticipated sampling effort for this surveillance study will require an investment in equipment and staff to provide results in a timely fashion. Equipment needs include real-time PCR thermalcyclers, RNA extraction capabilities, DNA sequencing capabilities, tissue culture and egg culture facilities, ultracold freezers, centrifuges and vacuum pumps.

Methodology

Sampling for highly pathogenic avian influenza (HPAI), such as highly pathogenic H5N1 avian influenza, from environmental deposition of virus by waterfowl should be accomplished by collecting and analyzing feces (Attachment 10) and water from areas of known use by high risk species (e.g., transcontinental migrants). The general challenges faced include; 1) Determining locations used by high risk species, 2) Refinement of existing methods for detecting the virus in water and fecal samples and developing the analytical infrastructure and capacity, 3) Design of a sampling system using composite samples for analysis.

Table 7-1. Qualitative comparison of environmental sampling methods.

Fecal Sampling	
Pros	**Cons**
Technically easy sample acquisition. Sampling represents non technical approach and would not require extensive training or experience by field personnel.	Viable virus restricted to fresh samples (1-4 days)
Generate large sample numbers quickly.	Large sample numbers can swamp lab systems (applies to all methods)
Does not require handling or capturing animals	
Low cost, well established technique amenable for high through-put screening (modified APHIS RT-PCR method). Sample analysis is transferable across labs.	
Capable of identifying HPAI contaminated sites/locations/regions. Prevalence would be estimated on a site basis. Information from the field could be used to generate an environmental risk map related to specific areas (habitats) associated with potential AIV transmission.	Identity of species and individuals unlikely, estimates of prevalence not possible. Species identification possible through molecular fingerprinting, but at additional cost.
BSL-2 laboratory conditions sufficient for initial diagnostic screening.	Requires Biosafety level 3 capabilities for virus isolation
Summary: An approach based on fecal sampling could be immediately implemented and may represent the only reasonable approach in areas where bird capture is not practical.	
Water Sampling	
Pros	**Cons**
Low cost	Biosafety level 3 capabilities for virus isolation
Effectively sample all or most birds present on the body of water	Analyses potentially complicated if multiple strains of AI present in water samples.
Samples easily, quickly obtained	Large volumes of water needed to concentrate virus for analysis, transportation and logistical issue
Virus stable, especially at moderate pH and low temperature	Longevity complicates interpretation on initial timing of contamination.
Does not require handling or capturing animals	Identity of species and individuals not possible/difficult. Prevalence calculation restricted to a site basis system.
Generate large sample numbers	Large sample numbers can swamp lab systems- need analysis infrastructure
Can provide large scale spatial risk assessment of HPAI contamination.	May need to validate technique

Sampling strategies to detect highly pathogenic H5N1 avian influenza virus in waterfowl populations will change depending upon the risk assessment and management goals and prevailing status of the pathogen in North America. For first detection of highly pathogenic H5N1 avian influenza virus in migratory birds efforts should focus on likely cross-over routes of birds from Asia to North America (e.g., Alaska and North Slope). Efforts should focus on areas of high aggregations of waterfowl intersecting with logistical sampling support, e.g., National Wildlife Refuge (NWR) system and state waterfowl management areas. While highly pathogenic H5N1 avian influenza virus may cross from Asia to North America at any point the surveillance network needs to be

tactically practical without compromising its ability for detection. Once highly pathogenic H5N1 avian influenza virus gains a foothold in North America the surveillance network should be placed along known waterfowl movement paths from the point of origin (i.e., point of detection). These paths can be inferred from known migration routes based on waterfowl telemetry data. However, practically, and given the patterns emerging in Eurasia, once highly pathogenic H5N1 avian influenza virus gains a foothold in North America the likelihood of rapid and diffusive spread across the continent is high. At this point local waterfowl and environmental sampling should target areas of strategic value, e.g., human population centers and areas of high density of poultry production. In the former case, such areas would be represented by urban zoo-parks and lakes. These areas would represent the highest level of risk of human contact with contaminated water and/or waterfowl. In the latter case, ponds, lakes and waterfowl management areas around high density poultry production areas would provide the best ability to assess risk of transmission to humans and poultry. Surveillance efforts patterned on these areas are best amenable to local and state efforts for first detection and subsequent risk assessment once the highly pathogenic H5N1 avian influenza virus achieves enzootic status in North America.

There is an inherent conflict between the need for high resolution surveillance, the number of samples generated, the time to analyze those samples, and the cost of analysis. If the goal is first detection, methods that integrate across many individuals and species at a particular site without loss of sensitivity should be preferred. Currently analysis of fresh fecal samples is the best method to achieve these goals. For logistically practical and economic reasons sample analysis should focus on composite samples on a per site basis; this bulk sample minimizes effort in both data collection and analysis, while greatly increasing the probability of detection. Given the expected rarity of highly pathogenic H5N1 avian influenza virus in current migratory bird populations, this approach will allow for a substantially reduced number of samples to be analyzed. Table 7-2 provides a hypothetical, but plausible, example of the expected number of tests per composite fecal sample necessary to detect Highly pathogenic H5N1 avian influenza virus. When prevalence is very low (e.g., 10^{-7}) almost all composites will test negative and on average only a single test will be needed to determine the absence of highly pathogenic H5N1 avian influenza virus in that composite sample.

The approximate sample sizes necessary for assuring a high probability of detecting highly pathogenic H5N1 avian influenza virus depends on its prevalence in the population, which is currently unknown. However, a preliminary estimator is:

$$p^* = 1- (1-r/m)^{1/n} \qquad \text{(eq. 1)}$$

where p* is the proportion of infected individual samples across all composite samples, r is the number of composite samples that test positive for the presence of highly pathogenic H5N1 avian influenza virus, m is the total number of composite samples tested, and n is the number of individual samples in each composite sample (e.g., fecal count or volume). Rearranging eq. 1 provides an estimate of the number of individual

fecal samples needed to detect highly pathogenic H5N1 avian influenza virus, for a given population level prevalence;

$$n = \ln(1-r/m) / \ln(1-p^{*}) \qquad \text{(eq. 2)}$$

Table 7-2. Expected number of tests needed for a single positive reaction for each composite sample containing 100 individual fecal samples, *n*, as a function of expected prevalence of HPAI, *p*. Calculation is based on the binomial probability model describing the average number of tests needed as $(n+1) - n(1-p)^n$

Prevalence in Waterfowl (*p*)	Individual fecal samples/composite (*n*)	Mean # composite samples to test
10^{-3}	100	10.5
10^{-4}	100	2.0
10^{-5}	100	1.1
10^{-6}	100	1.0
10^{-7}	100	1.0

The results for various hypothetical values of r, m, n, and p^{*} are shown in Table 7-2. Thus, if highly pathogenic H5N1 avian influenza virus prevalence is 10^{-6} and 10,000 independent fecal samples are collected, analysis of 100 composite samples would result in detecting the presence of highly pathogenic H5N1 avian influenza virus in one composite. These two equations allow us to initially estimate the number of fecal samples to be collected and to estimate prevalence of highly pathogenic H5N1 avian influenza virus in the population.

Table 7-3. Number of individual fecal samples *n*, for a fixed prevalence p^{*}, needed to detect the presence of HPAI in 1 out of 100 composite samples. Calculation is based on the probability model given by eq. 2.

Prevalence in Waterfowl (p^*)	Number of positive composites (r)	Number of composites (m)	Number of individual samples (n)
10^{-3}	1	100	10
10^{-4}	1	100	100
10^{-5}	1	100	1005
10^{-6}	1	100	10050
10^{-7}	1	100	10050

Safety

Given the concern of introduction of highly pathogenic H5N1 avian influenza virus into North America, and the potential for human infection, significant precautions should be taken by workers conducting the environmental sampling and those handling the samples in the laboratory. In the lab, standard BSL-3 precautions are required for virus isolation, and BSL-2 precautions for molecular diagnostics. In the field, workers should wear disposable gloves and garments. Gloves should be decontaminated with 70% ethanol frequently, or changed often as necessary. Mucous membranes (eyes, nose, throat) should be protected from splashes and aerosols. This may require covering with protective equipment such as goggles and hepafiltered masks in some cases. Field workers should avoid direct contact with animals after handling environmental samples until decontamination procedures are completed (e.g. changing garments and gloves). Untrained workers (such as the general public) should be discouraged from collecting and submitting environmental samples for testing.

Summary

Monitoring of water and/or fecal samples gathered from waterfowl habitat is a reasonably cost effective, technologically achievable means to assess risks to poultry in the western hemisphere to new, potentially highly pathogenic subtypes of AI. A surveillance system based on water sampling is not ready to implement. However, the validation of this method could come on-line in a short period of time and would represent considerable cost savings without loss of sensitivity. Fecal sampling is an established technique and is ready for use in surveillance with the establishment of sampling guidelines. Both approaches yield advantages where individual bird sampling is too costly or logistically impractical. Either approach could yield a spatial and habitat risk assessment for site contamination with highly pathogenic H5N1 avian influenza virus. The main considerations are where and when to get the samples, ensuring proper storage and

transport, and the capacities and capabilities of the laboratories doing the analyses. Real-time reporting and the infrastructure to support such reporting is a serious constraint on any surveillance system. The ability to integrate, analyze, and responsibly disseminate these data is critical and needs to be addressed.

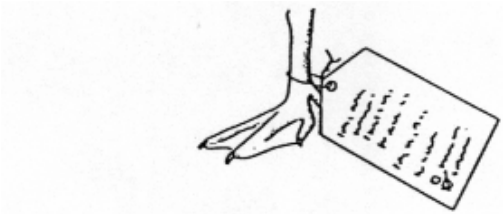
ATTACHMENT 8

Instructions for Collection and Shipment of Avian Carcasses for Diagnostic Evaluation

The following are general guidelines for collecting and shipping wildlife carcasses to veterinary diagnostic labs to insure adequate and well preserved specimens. Field biologists should contact the specific laboratory that they will be working with well in advance of any specimen collection and shipping to receive specific instruction for specimen submissions to that lab. Labs should always be notified ahead of time when a shipment is being made to their facility. Once you have determined what equipment and supplies will be needed for specimen shipping, keep adequate numbers of shipping containers, frozen ice packs, shipping labels and packing materials available at all times. If you plan to collect animals while in the field, take along a cooler with ice packs to chill the carcasses.

1. More than one disease may be affecting the population simultaneously. Different species may have varying susceptibility to disease agents. Therefore, collect and ship specimens representative of all species and geographic areas affected.

   Obtain good specimens for necropsy. Carcasses that are decomposed or scavenged are unacceptable. If the carcass has an odor, is soft and mushy, has skin discoloration, feathers or skin that easily rubs off, or has maggots present, it is too decomposed for testing.



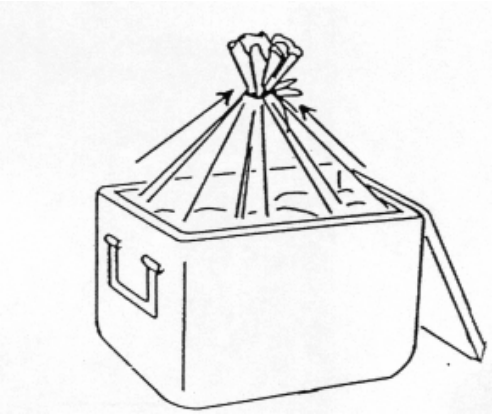
2. Collect animals under the assumption that an infectious disease or toxic substance is involved and other animals or humans may be at risk. Remember to protect yourself as some of these diseases and toxins are hazardous to humans. Guidelines for personal protection against disease exposure for individuals working with sick or dead wild animals can be obtained from the USGS National Wildlife Health Center, the Centers for Disease Control and Prevention, and OSHA websites.

   Always wear latex or nitrile gloves when picking up sick or dead animals. If you are dealing with a significant number of dead animals, or you suspect the presence of a zoonotic disease agent, additional protective equipment including coveralls, eye protection and N95 respiratory protection should be used.

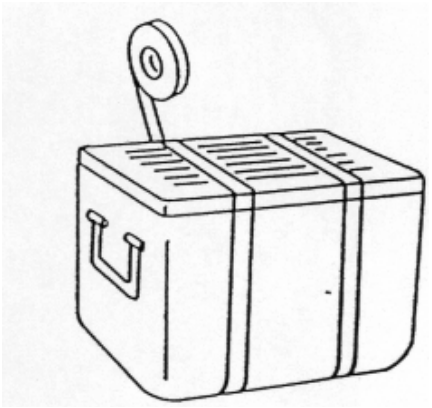
   Attach a leg tag to each animal with the following information in pencil/waterproof ink:
   - species
   - date collected
   - location (state, county, location name, and latitude/longitude if available)
   - found dead or euthanized
   - collector (name/address/phone)
   - additional history or comments on back of tag

Place each animal in a plastic bag, tie shut, then place inside a second bag and tie shut. This system of double bagging prevents cross-contamination of individual specimens and leaking shipping containers that can contaminate vehicle surfaces and handlers during transportation. Contact the diagnostic lab for guidance in assistance with collecting samples from animals that are too large to ship.

3. Ship animals in a sturdy hard sided plastic cooler. These coolers can be disinfected and returned to you if a pre-paid shipping label or commercial shipping company account number is provided to the diagnostic lab. Be sure to provide a street address for return of the cooler.

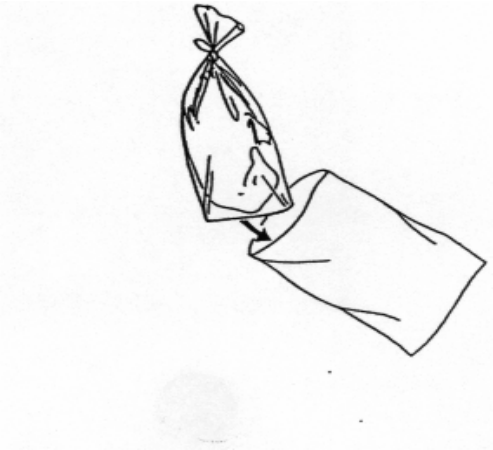


Line the shipping cooler with a large plastic bag and pack the individually bagged animal(s) in the cooler with enough blue ice to keep carcasses cold. Disperse blue ice packs among the carcasses so that all carcasses are kept chilled. If you are shipping blood tubes, culture tubes, or other specimen containers along with the carcasses, these specimens should be placed within a sturdy cardboard or plastic box or screw cap container with padding material to prevent breakage. That container should be place next to blue ice packs within the large cooler. Do not use bagged wet ice for shipments in order to avoid fluid leakage during shipment. Do not use dry ice unless instructed to do so by the diagnostic lab. Place crumpled newspaper or similar absorbent material in the cooler with the bagged carcasses to fill unused space, hold the ice in contact with carcasses, provide insulation, and absorb any liquids. Tape the cooler shut with sturdy strapping tape.



Place a detailed history of the animal and circumstances associated with the mortality event in a paper envelope or a plastic sleeve and tape it to the outside of the cooler. A copy of this history should be faxed or e-mailed to the diagnostic lab at the time of shipment. A standard wildlife specimen history form can be found on the last page of these instructions.

4. Prior to shipping contact the diagnostic lab to inform them of the type and number of specimens being shipped. Ship specimens for next day delivery (overnight service) from Monday through Wednesday to guarantee arrival at the diagnostic lab before the weekend. If specimens are fresh and need to be shipped on Thursday or Friday contact the diagnostic lab to make special arrangements for receipt of specimens.



Freezing and thawing can make isolation of some pathogens difficult and damage tissues needed for microscopic examination. Diagnostic labs prefer unfrozen specimens if

they can be sent within 24 – 48 hours of collection or death. The diagnostic lab can provide guidance on when or if to freeze samples on a case-by-case basis. If you are in the field and cannot call or ship within 24-48 hours, freeze the animal(s).

5. Prior to shipping contact the commercial shipping company to obtain guidelines for shipping diagnostic or biological specimens. Label coolers with clear, legible labels including the diagnostic lab name, street address, and telephone number. In addition to the mailing address, attach a label reading "**DIAGNOSTIC SPECIMENS –WILDLIFE"** to the side of the cooler. If dry ice was used in the shipment a standard dry ice warning label will be required. These can be obtained from the shipping company. Please make note of the tracking number in case packages are delayed.

Wildlife Specimen History Form
Always contact the diagnostic lab before shipping specimens!

Submitter's name: Affiliation:

Address: Telephone: E-mail:

Date collected: Collector's Name:

Method of collection: [found dead, euthanized (describe method) etc.]

Method of storage: [chilled, frozen, fixed, etc.]

Species Submitted:

Specific die-off location:
State: County: Latitude/longitude:

Environmental factors: (Record conditions such as storms, precipitation, temperature changes, or other changes that may contribute to stress.)

Disease onset: (The best estimate of when the outbreak started.)

Species affected: (The diversity of species affected may provide clues to the disease involved.)

Age/sex: (Any selective mortality related to age and sex.)

Morbidity/mortality: (Ratio of sick animals to dead animals.)

Known dead: (Actual carcass count)

Estimated dead: (Consider removal by scavengers or other means.)

Clinical signs: (Any unusual behavior and physical appearance.)

Population at risk: (Number of animals in the area that could be exposed to the disease.)

Population movement: (Recent changes in the number of animals on the area and their source or destination, if known.)

Problem area description: (Land use, habitat types, and other distinctive features.)

Comments: (Additional information/observations that may be of value such as past occurrences of disease in area.)

PLEASE USE ADDITIONAL SHEETS IF NECESSARY.

ATTACHMENT 9

Protocol for the Collection of Tracheal/Cloacal Swab Samples

1. Contact Laboratory to determine specific protocol to use. Laboratories may request samples be placed in tubes containing Viral Transport Medium (VTM) or brain-heart infusion broth (BHI).
2. Thaw appropriate number of pre-labeled tubes of Viral Transport Medium (VTM) or brain-heart infusion broth (BHI) at refrigerator temperature (4 °C) overnight and keep chilled with wet/blue ice packs in a cooler during the day of collection.
3. Unwrap a Dacron swab from the stem-end of the packaging.
4. Remove swab and insert the entire head of the swab into the trachea or cloaca. Use gentle pressure and in a circular motion, swab the inside circumference of the trachea/cloaca two or three times.
5. For Cloacal swabs, shake off large pieces of feces.
6. Inserting the swab into the tube containing VTM or BHI broth. With the swab in the media, swirl the stem end of the swab between fingers vigorously. Lift the swab approximately ¼" from the bottom of the vial and bend the stem over the edge of the vial to break off the stem so that the swab remains in the vial and the cap can be screwed tight.
   The entire swab end and a portion of the stem will be left in the tube. If the stems are unable to be broken (some small swabs will have metal stems) then they can be cut with scissors. Scissors should be wiped with 70% alcohol each time they are used to cut a stem.

7. Record sample tube number on banding sheet or the Sample History Sheet along with date, species, age, sex, and location data (GIS coordinates if possible)..
8. Replace tube into cooler for transport back to the base camp. Samples should be kept cold (<4 °C, frozen if possible) and out of direct sunlight.
9. At camp, transfer tubes into liquid nitrogen shippers or into a freezer as soon as possible. Note any exceptions to the collection or storage conditions in field sheets and note such information on the "Sample History and Packing List Form".
10. Place tubes into a hard plastic shipping container with enough frozen gel packs to keep samples cold for at least two days.
11. Notify laboratory that samples are being shipped, the method of shipment (FEDEX is preferred), and the expect date of arrival. Packages should only be shipped on Monday, Tuesday, or Wednesday.

Sample History and Packing List
H5N1 Avian Influenza Project

Submitter _____
Address _____
_____
Phone _____
E mail _____

Sample ID	Bird Species	Age	Sex	Band Number	Location	Comments or Sampling problems

ATTACHMENT 10

Fecal Sampling and Shipping Protocol

Fecal Sampling

Purpose

The purpose of this standard operating procedure is to describe the essential elements of proper handling and collection of field fecal samples for surveillance of avian influenza.

Procedures

- Before collecting samples, personnel should don the appropriate personal protective equipment (PPE). These include latex or nitrile gloves and face shields, and if it is determined to be necessary, PAPRs.

- Label plastic whirl pack bags with necessary information including date, location (GPS coordinate if possible), species (if possible), investigator and sample identification. Record all required information on data sheets.

- Feces must be less than 24 hours old. Feces should appear moist.

- For collection, turn a sterile Whirl-Pak inside out and pick up feces using the Whirl-Pak as a glove, then turn the bag right side in with the feces inside the closed bag. Release as much air from the inside of the bag.

- Label the Whirl-Pak using an indelible ink marker. The sample should be labeled with the sample number, date, time, collector's name, location, and Quality Assurance number (Protocol Number). This latter information describes in detail the context, purpose, and other procedural and review information of the samples collected.

- Place the Whirl-Paks with fecal samples into a large zip-lock bag, tape (e.g., duct tape or packaging tape) the opening and label the outer bulk bag with name, date, location, and Protocol number.

- Place the bulk collection bag in a cooler with wet or blue ice to keep the specimen cool. This precaution is for maximizing the chances for subsequent viral isolation. Pack samples with enough ice or frozen gel packs to keep samples cold for at least two days.

- Maintain the temperature of samples as constant as possible.

- Change gloves if soiled or contaminated. When finished collecting, wash hands with suitable antibacterial agent.

- Notify laboratory that samples are being shipped, the method of shipment (FEDEX is preferred), and the expect date of arrival. Packages should only be shipped on Monday, Tuesday, or Wednesday; this allows the laboratories time to process samples during a normal work-week, or allows for tracking if the shipment is delayed.

Shipping

Purpose

The purpose of this standard operating procedure is to ensure diagnostic specimens are shipped safely and in compliance with governing regulations and requirements. Shippers of diagnostic specimens where a relatively low probability exists that infectious substances are present (diagnostic specimens being transported to undergo routine screening tests or for the purpose of initial diagnosis may be considered to fall under this category) must comply with the International Air Transportation Association (IATA) Dangerous Goods Regulations. The shipper must also ensure that shipments are prepared in such a manner that they arrive at their destination in good condition and that they present no hazard to persons or animals during shipment.

Procedures:

- Federal Express is the preferred carrier for the USDA/NWRC. Use next day service.

- The inner packaging (appropriately labeled) must be comprised of a watertight primary receptacle, and must no exceed 500 ml total volume. Primary receptacles include those of glass, metal, or plastic (i.e., test tube, plastic jar, or taped zip-loc bag). Positive means of ensuring a leak-proof seal must be provided. Screw caps on primary receptacles must be reinforced with adhesive tape.

- A secondary packaging (also water tight) must be used, but must not exceed 4 L total volume.

- An absorbent material sufficient to absorb the entire contents of all primary receptacles must be placed between the primary receptacle and the secondary packaging.

- The outer packaging (i.e., cardboard box or cooler) must be of adequate strength for its capacity, weight, and intended use (capable to withstand being dropped at least 1.2 meters, without leakage of the primary receptacle or significant damage to the outer packaging).

- An itemized list of contents must be enclosed between the secondary packaging and the outer packaging. To protect against possible leakage, the list should be enclosed in a sealed plastic bag.

- A Shippers Declaration for Dangerous Goods is not required. However, both the air waybill and the outer box must show the text "***DIAGNOSTIC SPECIMEN PACKED IN COMPLIANCE WITH IATA PACKING INSTRUCTION 650"***.

ATTACHMENT 11

Veterinary Laboratories Currently Certified to Conduct Highly pathogenic H5N1 avian influenza Virus Diagnostics

State	Laboratory Director	Laboratory Name	Telephone	Shipping address 1	Shipping address 2	City	Zip code	Lab Director E-Mail	AI/END Contact
AL		Charles S. Roberts Veterinary Diagnostic Lab	334-844-4987	1001 Wire Road		Auburn	36830		Dr. Fred Hoerr
AR	Dr. Konnie Plumlee	Arkansas Livestock & Poultry Commission Lab	501-907-2400	One Natural Resources Dr.		Little Rock	72205	kpluml@arlpc.org	Dr. Paul Norris
AZ	Dr. Greg Bradley	Arizona Veterinary Diagnostic Laboratory	520-621-2356	2831 N. Freeway		Tucson	85705	gabrad@ag.arizona.edu	Dr. Greg Bradley
CA	Dr. Alex Ardans	California Animal Health & Food Safety Lab	530-752-8709	University of California, School of Vet Med	W. Health Science Drive	Davis	95616	aaardans@ucdavis.edu	Dr. Alex Ardans
CO	Dr. Barbra Powers	Colorado State University Veterinary Diag. Lab	970-297-1281	College of Vet. Med. & Biomedical Sciences	300 West Drake	Fort Collins	80523	bep@lamar.colostate.edu	Dr. Barbara Powers
CT	Dr. Herbert Van Kruiningen	Department of Pathobiology & Veterinary Science	860-486-0837	University of Connecticut, Unit 3089	61 N. Eagleville Rd.	Storrs	06269-3089	herbert.vandruiningen@uconn.edu	Dr. Sandra Bushmich
DE		University of Delaware Poultry Laboratory	302-856-1997	16684 County Seat Hi-Way		Georgetown	19947		Dr. Mariano Salem
FL	Dr. Betty Miguel	Kissimmee Diagnostic Laboratory	407-846-5200	Florida Department of Agriculture	2700 N. John Young Parkway	Kissimmee	34745	miguelb@doacs.state.fl.us	Dr. Betty Miguel
GA		Georgia Poultry Laboratory	770-535-5996	4457 Oakwood Road		Oakwood	30566		Dr. James Scroggs
GA	Dr. Doris Miller	Athens Veterinary	706-542-5568	University of Georgia	Building 1079	Athens	30602	miller@vet.uga	Dr. Doris Miller

State	Laboratory Director	Laboratory Name	Telephone	Shipping address 1	Shipping address 2	City	Zip code	Lab Director E-Mail	AI/END Contact
		Diagnostic Laboratory		College of Vet Med					
GA	Dr. Charles A. Baldwin	University of Georgia Veterinary Diag. Laboratory	229-386-3340	43 Brighton Road		Tifton	31793-3000	cbaldwin@uga.edu	Dr. Charles A. Baldwin
HI	Dr. David T. Horio	State Laboratories Division	808-453-5990	2725 Waimano Home Road		Pearl City	96782	david.horio@doh.hawaii.gov	Dr. David T. Horio
IA	Dr. Bruce Janke	Iowa State University	515-294-1950	Veterinary Diagnostic Laboratory	1600 S. 16th St.	Ames	50011	bhjanke@iastate.edu	Dr. Kyoung-Jin Yoon
IN	Dr. Leon Thacker	Purdue University Animal Disease Diagnostic Lab	765-494-7460	406 South Lafayette		West Lafayette	47907	thackerl@purdue.edu	Dr. Leon Thacker
LA	Dr. H.W. Taylor	Louisiana State University	225-578-9777	Veterinary Med Diag. Laboratory	1909 Skip Bertman Drive	Baton Rouge	70803	hwt@vetmed.lsu.edu	Dr. Alma Roy
MD	Dr. Daniel Bautista	Maryland Dept. of Ag & Animal Health Laboratory	410-543-6610	27722 Nanticoke Road		Salsbury	21801		Dr. Daniel Bautista
MI	Dr. Willie Reed	Diagnostic Center of Population and Animal Health	517-353-0635	Michigan State University	4125 Beaumont Rd, Ste 201H	Lansing	48910	reed@dcpah.msu.edu	Dr. Willie Reed
MN	Dr. James E. Collins	Minnesota Veterinary Diagnostic Laboratory	612-625-8787	University of Minnesota, Vet Diag Lab	1333 Gortner Ave, 244 Vet D L	St. Paul	55108	colli002@unm.edu	Dr. James E. Collins
MO	Dr. Alex Bermudez	University of Missouri	573-882-6811	Veterinary Medical Diagnostic Laboratory	1600 East Rollins	Columbia	65211	bermudeza@missouri.edu	Dr. Stanley Casteel
MS	Dr. Lanny Pace	Mississippi Vet Research & Diagnostic Laboratory	601-354-6089	2531 North West Street		Jackson	39216	pace@cvm.msstate.edu	Dr. Lanny Pace
NC	Dr. Gene Erickson	North Carolina Department of Agriculture	919-733-3986	Rollins Animal Disease Diagnostic	2101 Blue Ridge Rd.	Raleigh	27607	gene.erickson@ncmail.net	Dr. Gene Erickson

State	Laboratory Director	Laboratory Name	Telephone	Shipping address 1	Shipping address 2	City	Zip code	Lab Director E-Mail	AI/END Contact
				Lab					
NE	Dr. David Steffen	Veterinary Diagnostic Center	402-472-1434	University of Nebraska	137 VDC UNL	Lincoln	68583-0907	dsteffen1@unl.edu	Dr. David Steffen
NJ	Dr. Robert Eisner	New Jersey Dept of Ag, Division of Animal Health	609-984-2293	State Diagnostic Lab, H & A Building	Rm 201 John Fitch Plaza, P.O. Box 330	Trenton	08625	rjeisner1@comcast.net	Dr. Robert Eisner
NM	Dr. Flint Taylor	New Mexico Department of Agriculture	505-841-2576	Veterinary Diagnostic Services	700 Camino de Salud, NE	Albuquerque	87106	ftaylor@nmda.nmsu.edu	Dr. David Mills
NV	Dr. Anette Rink	Nevada Animal Disease Laboratory	775-668-1182	Nevada Department of Agriculture	350 Capitol Hill Ave.	Reno	89502-2923	arink@govmail.state.nv.us	Dr. Anette Rink
NY	Dr. Alfonso Torres	Animal Health Diagnostic Center	607-253-4136	Cornell University, College of Vet. Med.	S3 110 Schurman Hall, Upper Tower Rd.	Ithaca	14853	at97@cornell.edu	Dr. Sung Kim
OH	Dr. Beverly Byrum	Ohio Department of Agriculture	614-728-6220	Animal Disease Diagnostic Laboratory	8995 E. Main Street, Building 6	Reynoldsburg	43068	byrum@mail.agri.state.oh.us	Dr. Beverly Byrum
OK	Dr. Bill J. Johnson	Oklahoma Animal Disease Diagnostic Laboratory	405-744-6623	Oklahoma State Univ., College of Vet. Med.	Farm Road & Ridge Road	Stillwater	74078	billyjj@cvm.okstate.edu	Dr. W. C. Edwards
OR	Dr. Jerry Heidel	Oregon State Veterinary Diagnostic Lab	541-737-3261	Oregon State Univ., College of Vet. Med.	30th & Washington	Corvallis	97331	jerry.heidel@oregonstate.edu	Dr. Jerry Heidel
PA	Dr. Helen Acland	Pennsylvania State Vet Diagnostic Laboratory	717-787-8808	2305 N. Cameron Street		Harrisburg	17110	hacland@state.pa.us	Dr. Deepanker Tewari
PA		University of Pennsylvania	610-925-6210	Lab of Large Animal Pathology & Toxicology	New Bolton Center, 382 West Street Rd	Kennett Square	19348-1692		Dr. Sherrill Davison
SC	Dr. Pamela Parnell	Clemson Veterinary Diagnostic Center	803-788-2260	500 Clemson Road		Columbia	29229	pprnll@clemson.edu	Dr. Pamela Parnell
TX	Dr. Lelve Gayle	Texas Vet Medical Diagnostic Laboratory	979-845-9000	1 Sippel Road	Drawer 3040	College Station	77843	1-gayle@tvmdl.tamu.edu	Dr. Lelve Gayle

State	Laboratory Director	Laboratory Name	Telephone	Shipping address 1	Shipping address 2	City	Zip code	Lab Director E-Mail	AI/END Contact
UT	Dr. Tom Baldwin	Utah Veterinary Diagnostic Laboratory	435-797-1895	950 E. 1400 North		Logan	84322-5700	tjbald@cc.usu.edu	Dr. Tom Baldwin
VA	Dr. David W. Brown	Virginia Dept of Agriculture and Animal Health Lab	540-434-3897	116 Reservoir		Harrisonburg	22801		Dr. David Brown
WA	Dr. Terry McElwain	Washington Animal Disease Diagnostic Laboratory	509-335-9696	Bustad Hall	Room 155-N	Pullman	99164	tfm@vetmed.wsu.edu	Dr. Terry McElwain
WA		Avian Health and Food Safety Laboratory	253-445-4537	7613 Pioneer Way E.		Puyallup	98371-4919		Dr. A. S. Dhillon
WI	Dr. Leslie Dierauf	USGS National Wildlife Health Center	608-270-2400	6006 Schroeder Road		Madison	53711	ldierauf@usgs.gov	Dr. Leslie Dierauf
WI	Dr. Kathy Kurth and Dr. Pete Vanderloo	Wisconsin Veterinary Diagnostic Laboratory	608-262-5432	Wisconsin Department of Agriculture	6101 Mineral Point Road	Madison	53705	Kathy.Kurth@WVDL.wisc.ed	Dr. Kathy Kurth and Dr. Pete Vanderloo
WV		West Virginia Dept of Agriculture	304-558-2214	1900 Boulevard, East		Charleston	25305-0172		Dr. Jewell Plumley

[a] This list represents the National Animal Health Laboratory Network (NAHLN) labs certified as of 3/14/06 to conduct avian influenza screening. This list will be updated as new labs become certified. For the latest list of certified laboratories, please contact Thomas.J.Deliberto@aphis.usda.gov

Attachment 12

Real-Time Reverse Transcriptase-Polymerase Chain Reaction for the Detection of Type A Influenza and the Avian H5 and H7 Ha Subtypes In Tracheal and Cloacal Samples

Cepheid Smart Cycler Protocol

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Prepared by:
Erica Spackman, Ph.D.
Southeast Poultry Research Lab

U.S. Dept. of Agriculture, Agricultural Research Service
934 College Station Rd.
Athens, GA 30605
706-546-3617
espackman@seprl.usda.gov
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I. MATERIALS

Mention of trade names or commercial products in this procedure is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

General recommendations regarding reagents

This assay was optimized using the using the Qiagen RNeasy kit and Qiagen one-step RT-PCR kit, therefore these reagents are recommended for uniformity, if available. Additionally, it is highly recommended that the PCR primers and probe be highly purified (i.e. HPLC purified).

General

Reagent grade H2O (nuclease free)
Pipetors and tips for volumes between 1µl and 1ml
1.5 ml microcentrifuge tubes Microcentrifuge TE buffer pH 8.3 (Promega #V6231 or V6232) (optional)

RNA Extraction

Qiagen RNeasy Mini Kit (Qiagen #74104 or #74106)
100% Ethanol
70% Ethanol (in nuclease free water)
QiaVac 24 vacuum manifold (Optional)
2-Mercaptoethanol (BME) (Sigma #M-6250)

Trizol LS reagent (Invitrogen #10296028, 200ml)
Chloroform (Sigma #C-2432)
Isopropanol (Sigma #I-9516)
Glycogen 5mg/ml (Ambion #9510)

Real-time RT-PCR

Qiagen one step RT-PCR Kit (Qiagen #210210 or #210212)
Hydrolysis probes (IDT, Idaho Tech/Biochem or Qiagen-Operon) (Table 1)
Primers (IDT, Idaho Tech/Biochem or Qiagen-Operon) (Table 1)
25 mM $MgCl_2$ (Promega #A3511 or #A3513)
RNase Inhibitor (Promega #N2511 or # N2515)
Positive control RNA
Nuclease free H_2O
25µl Smart Cycler tubes (Cepheid #900-0022 or 900-0003)

Table 1. Influenza real-time RT-PCR probe and primer sequences. Protocols for H7 subtype Eurasian, H7 subtype South American strains, the H6 subtype and the H9 subtype strains are available as supplementary protocols.

Specificity		Sequence
Type A influenza-Matrix gene	M+25 5' Primer	5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'
	M+64 Probe	5'-FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1-3'
	M-124 3'Primer	5'-TGC AAA AAC ATC TTC AAG TCT CTG-3'
H7Subtype-North American strains	H7+ 1244 5' Primer	5'-ATT GGA CAC GAG ACG CAA TG-3'
	H7+1281 Probe	5'-FAM-TAA TGC TGA GCT GTT GGT GGC-BHQ1-3'
	H7-1342 3'Primer	5'-TTC TGA GTC CGC AAG ATC TAT TG-3'
H5 subtype-Any strain[a]	H5+1637 Probe	5'-FAM-TCA ACA GTG GCG AGT TCC CTA GCA-BHQ1-3'
	H5-1685 3'Primer	5'-AGA CCA GCT AYC ATG ATT gC-3'
H5 subytpe-North American strains	H5+1456 NA 5' Primer	5'-ACG TAT GAC TAT CCA CAA TAC TCA-3'
H5 subtype-Eurasian strains	H5+1456 EA 5' Primer	5'-ACG TAT GAC TAC CCG CAG TAT TCA-3'

a. H5 strain specificity is determined by the 5' primer. Use only one primer in the reaction.

Note on subtype determination: Due to the high level of sequence variation within each HA subtype, a negative RRT-PCR result for a specific subtype does not exclude the possibility that that subtype is present.

Primer and probe handling and dilution

Lyophilized primers and probes must be centrifuged briefly, to ensure that the DNA pellet is at the bottom of the tube, before they are opened and reconstituted. TE buffer should be used for the initial reconstitution of lyophilized primers and probes (Idaho Tech sends TE with probes and primers and it is commercially available). Concentrated stock solutions should be stored at -20°C. Primer stock solutions should be 200µM (200pmol/µl), probes should be 120µM (120pmol/µl). Quantitation information will be supplied for each oligo (primers and probes are DNA oligos) by the manufacturer.

An example of calculation for oligo reconstitution:

You have 17786 pmol of oligo (will be on oligo information sheet from manufacturer).

Need 200pmol/µl for stock concedntration.

Divide pmol of oligo by the pmol/µl needed or: $\frac{17786 \text{ pmol}}{200 \text{ pmol/µl}} = 88.9\text{µl}$

For 200pmol/µl resuspend the pellet in 89µl of TE or nuclease free H2O. The calculation for the probe is the same, except divide the number of probe pmol by 120pmol/µl. Mix gently by tapping the tube and allow the oligo to resuspend for about 10 minutes before use.

Working stocks of primers should be 20pmol/ul (20µM) and working stocks of probes should be 6µM. Dilute the primers 1:10 and dilute the probe 1:20 in nuclease free H_2O (do not use TE buffer) for the working stocks.
Working stocks should be stored at 4°C. The probes are stable at this concentration at 4° C for approximately 1 month. It may be useful to make up several aliquots (5-6) of working stocks of primers and probes which are a volume that can be used in about one month. Store the unused aliquots at -20°C.

Note: the probes are light sensitive; store them in amber tubes if available, and minimize their exposure to light.

Additional information on fluorescent probe handling and storage can found at: www.idahotech.com, www.operon.com and www.idtdna.com.

Suppliers

Biosearch Technologies, Inc.
81 Digital Drive
Novato, CA 94949-5750
1.800.436.6631
WWW.Biosearchtech.com

Promega
2800 Woods Hollow Rd
Madison, WI 53711-5399
1-800-356-9526
www.promega.com

Idaho Technology/Biochem
390 Wakara Way
Salt Lake City, UT 84108
1-800-735-6544
www.idahotech.com

IDT
1710 Commercial Park
Coralville, IA 52241
1-800-328-2661
www.idtdna.com

Qiagen Inc./Operon
28159 Avenue Stanford
Valencia, CA 91355
1-800-426-8157 www.qiagen.com
www.operon.com

II. METHODS

NOTE ON SAMPLE TYPES AND RNA EXTRACTION METHODS

The types of samples collected and the processing of those samples varies by species. The optimal sample types and processing methods for many species are given below.

Table 2. Sample types and optimal processing methods.

Species/ Type	Recommended Specimen	Processing Method	Notes
Gallinaceous Poultry (chickens, turkeys, quail)	Tracheal swab	RNeasy RNA extraction, then RRT-PCR	Virus primarily replicates in the respiratory tract (LPAI)
Waterfowl/ducks	Cloacal Swab	Trizol Reagent RNA extraction, then RRT-PCR	Virus primarily replicates in the intestinal tract. RNA extraction method must be modified for cloacal samples
Any species	Tissue samples	RNA extraction with Trizol Reagent, then RRT-PCR	For HPAI viruses high levels of virus may be in tissues.
Environmental samples	(Swab)	Virus isolation, RRT-PCR not recommended	RRT-PCR can detect inactivated virus

REVISED RNA EXTRACTION PROTOCOL FOR TRACHEAL SWABS (7/03)

RNA Extraction with Qiagen RNeasy Kit- Centrifuge Method

Notes:

- Adaptation of kit for fluid samples from manufacturer.
- All kit supplied buffers and reagents should be prepared in accordance with the kit instructions.
- Use only RNA grade reagents and supplies

1. Vortex the sample (cloacal or tracheal swabs in BHI or other media) for 3-5 seconds and withdraw 500µl and place in a 1.5 ml microcentrifuge tube.

2. Add 500 µl of RLT buffer. Close the tube and vortex the sample for 5 seconds.

3. Add 500 µl of RNA grade 70% ethanol to the tube and mix. Centrifuge the sample for 5 minutes at ~5KXg to pellet any debris.

4. Add 750µl of the supernatant from step 3 to the RNeasy column and centrifuge for 15 seconds at ~12 KXg, empty the flow through from the collection tube and repeat (all of the sample/RLT/70% ethanol mix should be applied to the column).

5. Add 700µl RW1 buffer to the RNeasy column and centrifuge for 15 seconds at ~12 KXg and place the column in a clean collection tube (the collection tube with RW1 flow through may be discarded and replaced with a fresh collection tube).

6. Add 500µl RPE buffer to the RNeasy column and centrifuge for 15 seconds at ~12 KXg, empty the flow through from the collection tube.

7. Repeat step 6 for a total of 2 washes with RPE buffer.

8. Centrifuge the empty RNeasy column an extra 2 minutes at ~14 KXg and discard the collection tube.

9. Place the RNeasy column in an elution tube (or 1.5ml microfuge tube) and add 50 µl nuclease free H_2O to the column. Incubate at room temperature for 1 minute. Elute RNA by centrifuging for 1 minute at ~14KXg. Discard RNeasy column.

RNA Extraction with Qiagen RNeasy Kit- QiaVac 24 Vacuum Manifold Method

Notes:

- Adaptation of the RNeasy kit for fluid samples from manufacturer.
- All kit supplied buffers and reagents should be prepared in accordance with the kit instructions.
- RNeasy column lids should be open whenever vacuum is being applied.
- Use only RNA grade reagents and supplies

1. Vortex the sample (cloacal or tracheal swabs in BHI or other media) for 3-5 seconds and withdraw 500 µl and place in a 1.5 ml microcentrifuge tube.

2. Add 500 µl of RLT buffer. Close the tube and vortex the sample for 5 seconds.

3. Add 500 µl of RNA grade 70% ethanol to the tube and mix. Centrifuge the sample for 5 minutes at ~5KXg to pellet any debris.

4. Place the appropriate number of RNeasy columns in the luer locks of the vacuum manifold, cover any empty positions with the luer caps supplied with the vacuum manifold.

5. Apply vacuum and add the entire sample/RLT/ethanol mixture to an RNeasy column for each sample.

6. Wash by applying 700µl RW1 buffer to each column.

7. Wash again by applying 500µl RPE buffer to the column and repeat for a total of 2 washes with buffer RPE.

8. Shut off the vacuum and place each RNeasy column in a 2ml collection tube. Centrifuge the column for 2 minutes at ~14 KXg and discard the collection tube.

9. Place each column in an elution tube (or 1.5ml microfuge tube) and add 50 µl nuclease free H_2O and incubate at room temperature 1 minute. Elute RNA by centrifuging for 1 minute at ~14KXg.

10. Use 8µl per PCR reaction. Store at -70°C for long term storage.

RNA EXTRACTION FROM CLOACAL SWABS OR TISSUE WITH TRIZOL REAGENT

1. Sample Prparation:
   a. Cloacal Swabs: Vortex vigorously for 7-10 seconds. Centrifuge for 5 min. at

12,000Xg.Extract RNA from the supernatant.

b. Tissues: Make a 10% homogenate of tissue in PBS. Centrifuge for 10 min. at 12,000Xg. Extract RNA from the supernatant.

2. Add 250µl of the supernatant from the sample prepared as described in step 1, to 750µl of Trizol LS reagent. Vortex. Pulse spin to remove liquid from the tube lid.

3. Add 200µl 100% chloroform to the sample/Trizol homogenate. Vortex for 15 sec. Incubate at room temperature for 7 min.

4. Centrifuge at 12,000 x g for 15 min at room temperature.

5. Transfer 400-450µl of the upper aqueous layer to a separate microcentrifuge tube marked with sample number. **Caution: The transfer of organic phase material with the aqueous layer will inhibit the PCR reaction.** Add 500µl of 100% isopropanol. Add carrier to the isopropanol to aid precipitation i.e. glycogen: 1µl of 5mg/ml stock (may be added prior to addition of the aqueous phase from the trizol). Invert tube several times to mix. Incubate at room temperature for 10 min.

6. Centrifuge at 10,000 x g for 10 min at 4 C.

7. Decant liquid. Care should be taken to assure that the RNA pellet is not disturbed. Add 1.0 ml of 70% or 80% ethanol. Mix gently.

8. Centrifuge at 10,000 X g for 5 min at 4 C.

9. Decant ethanol. Invert tube on a clean tissue wipe and allow to air dry for 10 min. or until all visible signs of moisture are gone. It is important not to let the RNA pellet over-dry, as this will decrease its solubility.

10. Hydrate pellet in 100µl of RNase free water and allow to sit at 4 C for 1 hr to overnight.

RNA Handling and Storage

The RNA sample may be stored at 4°C for < 1 week, storage for longer than one week should be at –70°C. Always wear gloves when handling RNA and use only RNase or nuclease free materials and reagents with RNA. Additional RNA handling and storage information can be found in: Sambrook, J. and Russell, D. *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.

Procedure for Real-time RT-PCR for type A Influenza (MA gene), the H5, H6, H7 and H9 HA subtypes

This procedure was designed for the Cephied Smart-Cycler (Cepheid, Sunnyvale, CA). A protocol for the Roche Light Cycler and Idaho Tech R.A.P.I.D. is available. The original reference and validation study for this assay is: Spackman, et. al.; Journal of Clinical Microbiology 40:3256-3260, 2002.

Information on setting-up and programming the Smart Cycler can be found in the Smart Cycler user's manual. The conditions for the influenza primers and probes on the Smart Cycler are shown in tables 2 and 3. The RT step is the same for all primer and probe sets, these conditions are specific for the Qiagen OneStep RT-PCR kit. Cycle times for the PCR phase may vary among different real-time PCR instruments.

Table 3. RT step thermocycling for Qiagen one-step RT-PCR Kit.

RT Step	1 cycle	30 min.	50° C
		15 min.	95° C

Table 4. Thermocycling conditions for gene specific probe and primer sets.

Probe/Primer set		Step	Time	Temp
Type A influenza	45 cycles	Denaturation	1 sec.	94° C
		Annealing[a]	20 sec.	60° C
H7 Subtype North American	40 cycles	Denaturation	1 sec.	94° C
		Annealing[a]	20 sec.	58° C
H5 subtype North American or Eurasian[b]	40 cycles	Denaturation	10 sec.	94° C
		Annealing[a]	20 sec.	57° C
		Extension	10 sec.	72° C

a. Fluorescence is acquired at the annealing step.
b. Use only one H5 subtype 5' primer for the H5 test.

The real-time RT-PCR reactions for type A influenza (M gene) and the H5 and H7 HA subtypes should be setup with the following components and volumes using the appropriate primer and probe set and cycling conditions. Set-up the reactions with the tubes in the cooling block and use aerosol resistant pipet-tips.

1. Prepare the reaction mix (everything but the template) by pipetting: H2O, kit supplied 5X reaction buffer, kit supplied dNTP's and 25mM $MgCl_2$ into a nuclease free microcentrifuge tube using the volumes per reaction for each reagent given in table 4. Next add the RNase inhibitor and enzyme. Add the probe last. Mix by vortexing for 3-5 seconds and centrifuge briefly. Once the probe has been added minimize exposure of the reaction mix to light.

2. Add the reaction mix (17µl) to the Smart Cycler tubes (add the mix to the bottom of the cup at the top of the reaction tube).

Table 5. Real-time RT-PCR reaction mix volumes and conditions for type A influenza (M gene), H5 and H7 HA subtypes.

	Volume Per Reaction	Final Concentration
H_2O	6.95µl	
5X	5	1X
25mM $MgCl_2$	1.25	3.75 mM
Enzyme Mix	1	
Forward Primer	0.5	10 pmol
Reverse Primer	0.5	10 pmol
dNTP's	0.8	320 µM ea. dNTP
Probe	0.5	0.12 µM
Rnase Inhibitor	0.5	13 units
MM per rxn	17	
Template	8	
Total	25µl	

3. Add the template to the smart cycler tubes (8µl per reaction). Note: The template for the positive controls is *in vitro* transcribed RNA from the appropriate gene and the template for the negative controls is H2O.

4. Centrifuge the reaction tubes briefly in the Smart Cycler centrifuge and run the real-time RT-PCR with the conditions described in tables 2 and 3 depending on the probe and primer set used. Note the RT step is the same for all probe and primer sets (the RT step is specific for the Qiagen one-step RT-PCR kit).

III. ANALYSIS OF RESULTS

Positive results on the Smart Cycler may be determined by the Smart Cycler software (shown on the results table in the Smart Cycler software) and are generally reliable; however results should be manually confirmed by examination of the fluorogram.

On the Smart Cycler the default minimum increase in fluorescence for a sample to be classified as positive by the software is 30 units. Because this is an arbitrary threshold, any samples which have an increase in fluorescence between 20 and 40 should be considered suspect and should be re-tested with the type A influenza (M gne) assay and/or subtype specific assays. In general, any questionable samples should be retested. If results of the second test are unsatisfactory additional sampling from the flock or premises should be considered if possible.

Recommendations for evaluating fluorograms

Evaluation of the fluorogram with the following conditions may be helpful in determining results manually:

- ③ All reactions with default settings.
- ③ Remove all reactions with greater than 100 units increase in fluoresce from the graph (this changes the scale, making it easier to identify weak negatives). (Figures 1a and 1b).
- ③ If there are samples which have a "V" shaped fluorescence trace incrementally lower the "background maximum cycles" (analysis settings table) to approximately 2 cycles below the cycle number where the base of the "V" is (Figures 2a and 2b).

Figure 1a. Example of a fluorogram from samples run on the Smart Cycler. All samples shown. Background subtraction is on. All analysis criteria are set to the default values. Note that scale is from 0 to 1000 fluorescence units (Y axis), making it difficult to evaluate weak positive samples.

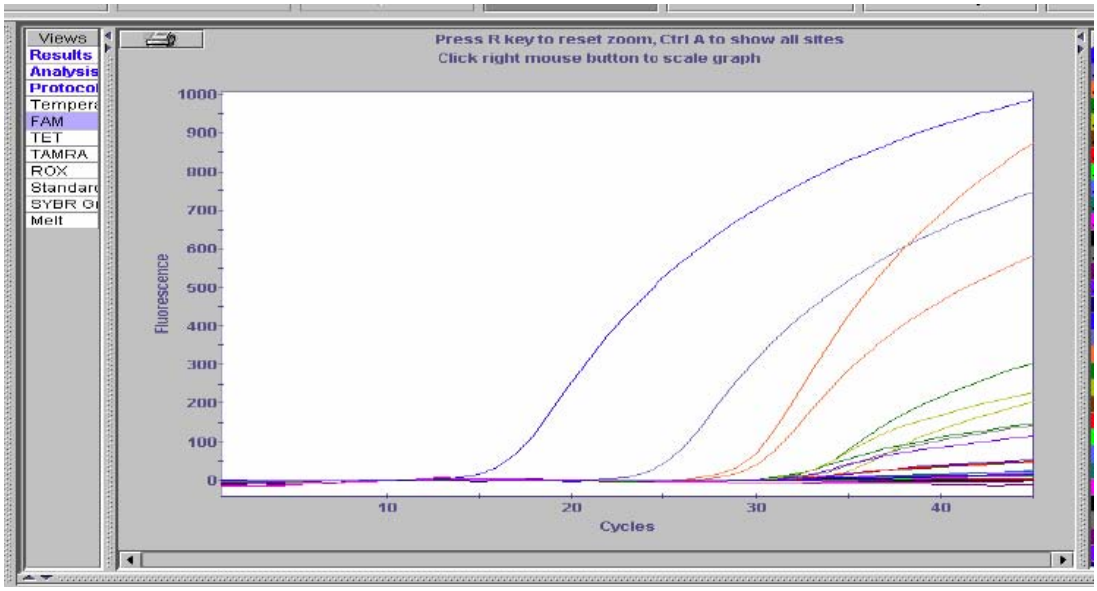


Figure 1b. Same fluorogram as figure 1a, however all samples which increased greater than 100units in fluorescence were removed from the graph. Note that the scale is from 0 to 120 fluorescence units (Y axis) making it easier to recognize weak positives.

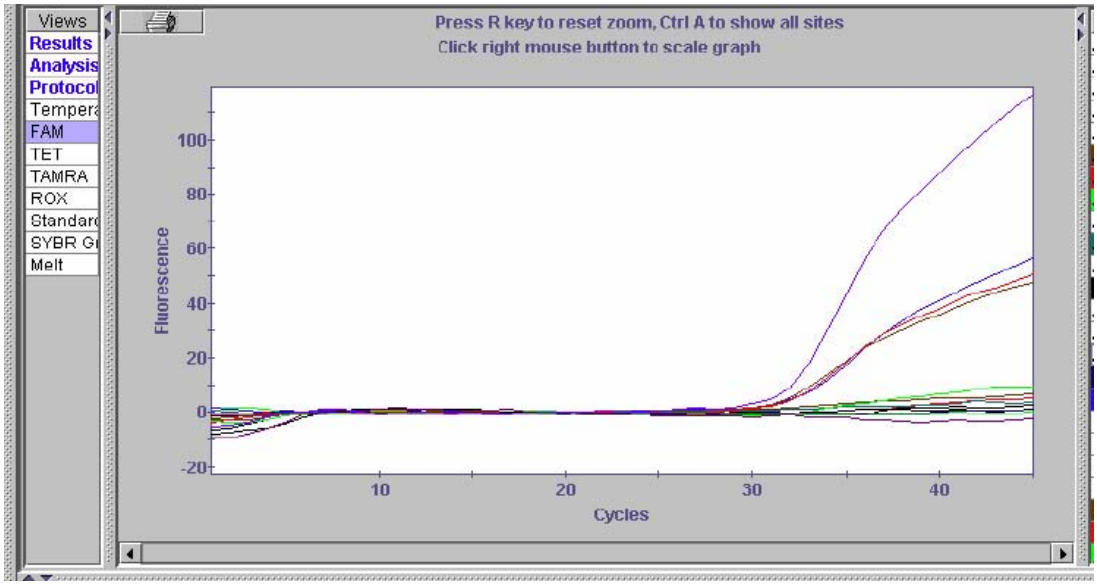


Figure 2a. Example of a "V" shaped fluorescence trace. The background maximum cycle is set to the default of 40 (red circle). All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero, light blue).

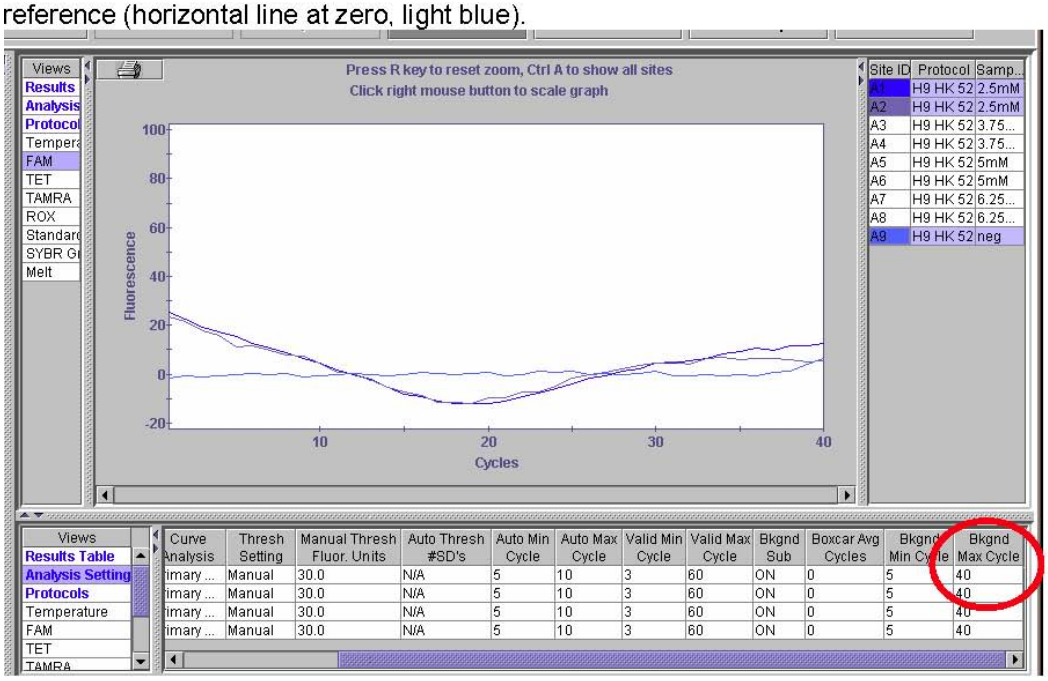
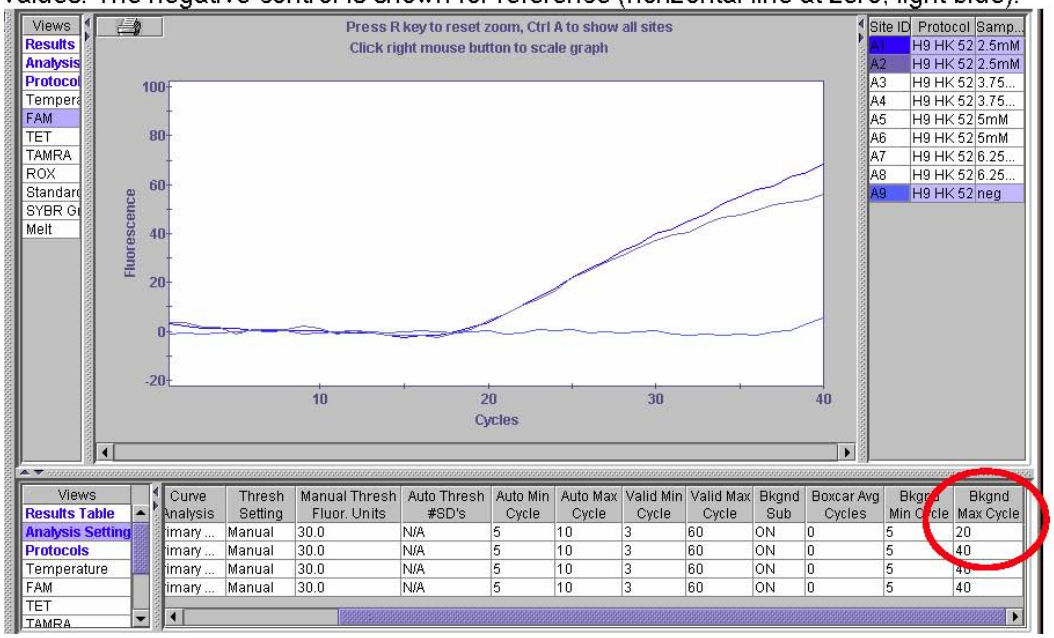


Figure 2b. Same fluorogram as figure 2a, however the background maximum cycles have been reduced to 20 (red circle) to align the background fluorescence at 0 units. All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero, light blue).



IV. APPENDIX

Troubleshooting

- Positive controls are negative:
  - Control template has degraded.
  - Probe may be old and the fluorescence may be dead.
  - The enzymes may be inactivated.
  - Incorrect thermal-cycling program or fluorescence acquisition (wrong setting or wrong channel being viewed).

- Negative controls are positive:
  - There may be cross contamination among the samples.
  - There may be non-specific probe degradation, use fresh probe and primers.

- Background level too high or too low (should be approximately 100-200 units):
  - The probe concentration may be wrong.
  - The probe may be degraded or too old if the background level is too low.

- Warning message in sample status on results screen.
  - Probe concentration too high (may cause ‘railing’; a sharp decrease in fluorescence after a steady increase).

Cross-contamination prevention

Due to the high sensitivity of RT-PCR based assays cross-contamination is an important issue. The following guidelines will help to prevent contamination of PCR samples in the lab:

- ③ Use aerosol resistant pipet tips
- ③ Centrifuge all reagents prior to use, especially freeze-dried materials
- ③ Preparation of samples in a biosafety cabinet
- ③ Use of separate areas (separate biosafety cabinets for RNA extraction and RT-PCR reaction preparation).
- ③ Minimizing sample handling
- ③ Change gloves often

Real-time PCR Basics

The general principle of real-time PCR is the same as standard PCR, however the reaction product can be monitored in real-time with a fluorogenic probe. There are several types of probes for real-time PCR: hydrolysis probes, hybridization probes and molecular beacons. This assay utilizes hydrolysis probes.

In the hydrolysis probe system, a DNA probe which binds the PCR product and which has a fluorogenic reporter dye on one end and a quencher dye on the other end, is added to the PCR reaction (figure 4). As the target PCR product increases the probe binds the amplicons and reporter dye is cleaved from the 5' end of the probe by *taq* polymerase (due to 5' exonuclease activity). As the reporter is cleaved from more and more probe molecules the fluorescence signal from the increases. The fluorescence signal is monitored every cycle, revealing increases in the PCR product as it occurs.

Additional information about Real-time PCR, primers and probes can be found at www.operon.com and www.idtdna.com.

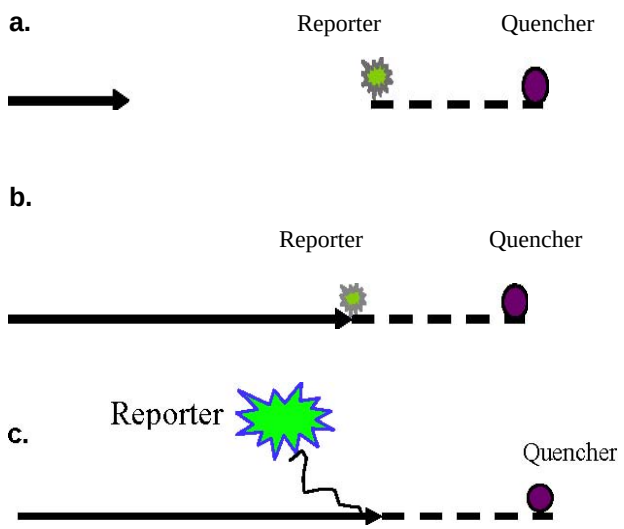


Figure 4. Hydrolysis probe mechanism. **a.** The probe (---) binds the PCR product (-) during amplification. **b.** The polymerase (➞) runs into the probe during synthesis of the PCR product. **c.** Taq polymerase cleaves the reporter dye from the probe, increasing the detectable fluorescence of the reporter dye.

ATTACHMENT 13

Proposed Highly pathogenic H5N1 avian influenza Data Integration, Data Management and Spatial Modeling

Data Standards

The Wildlife Disease Information Node (WDIN) system will accommodate a common set of data standards as developed by the Interagency HPAI Early Detection Working Group. It is expected that these standards will be compliant with those under consideration for use in the National Animal Health Laboratory Network (NAHLN). The needs for standards utilized in other systems (e.g. USGS Bird Banding Laboratory) will also be addressed. The WDIN will provide a data schema of required fields and elements that will be used through the system, as defined by the Working Group and collaborators in the data management field.

Data Security

Security for the system will be achieved through various checkpoints throughout the application. There will be several access roles each user can be assigned or revoked ("data entry", "data edit", "data verification", and "data administrator"). Users can be granted any number of the roles available. Without sufficient access, the user will be rejected from entering that portion of the system. Depending on access, a user's roles may include: (1) Data entry; (2) Data edit; (3) Data verification, and (4) Data administrator.

Data sharing will be achieved alongside the security measures. Each user/institution will have the ability to grant or revoke access to their data in agreed upon levels of access, and this access will be determined by the WDIN in collaboration with the Working Group. WDIN is envisioning low and high level access roles that can be granted to partner institutions.

Data System Environment

These are the existing components of the system proposed for HPAI data management:

- Java J2EE environment 1.4.2 (HTML, javascript, JSP) for the web application;
- Microsoft SQL (database) to house the entered data;
- Apache 2 (web server);
- Tomcat 5; (application server);
- ESRI ArcIMS 9 (web-enabled mapping);
- ESRI ArcSDE (spatial data engine component on top of MS SQL).

To keep the system up-to-date and fully functional, additional components, such as Rhapsody/Chameleon or another HL7 messaging software system will be needed to accommodate the transfer and receipt of HL7 messages.

Data Entry

Because of the multiple agencies and groups involved in sampling, there may be different procedures for field data capture and diversity of abilities and mechanisms for entering these data into an electronic system. The data management platform must accommodate these differences, and allow data entry to proceed in an efficient manner. WDIN is exploring the following options for data entry. Some or all of these may be implemented depending on user needs: (1) Direct web access; (2) File transfer; (3) Optical Mark-Read data forms; or (4) Handheld/PDA. WDIN will work with users whose preferences for data entry may change over time.

Data Access and Mapping

Based on the security protocols described above, and within the access guidelines determined by the Interagency Working Group, through the web portal, partners will be able to view all data that has been entered in a number of ways. Data can be browsed in entirety, or filtered by various parameters (e.g. species, sex, location). Standardized reports for individual partners, as well as grouped data will also be available. If permission has been obtained, subsets of raw data could also be downloaded.

Through the use of an interactive mapping tool (ArcIMS), maps will be available both on-line and printable. These maps are created on demand and can show whatever data fields the user desires, overlaid on a wide range of backgrounds, such as roads, political boundaries, species populations, topography, etc.

Spatial Analysis and Modeling

Once surveillance data has been collected and mapped, they can be used in spatial analysis both to assess the progress of the surveillance effort, and if HPAI is detected, observe the course of the disease and potentially model its spread, providing guidance for operational staff undertaking control and eradication measures. As the proposed WDIN Interagency Data Management System already contains a Geographic Information System (GIS) component, this process can be easily instituted. WDIN will work closely with the USDA APHIS Centers for Epidemiology and Animal Health (CEAH) to integrate GIS surveillance, mapping and modeling tools for application to HPAI analysis and response.