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Robert G. McLean
USDA, APHIS, Wildlife Services, National Wildlife Research Center

Jeffrey S. Hall
USDA, APHIS, Wildlife Services, National Wildlife Research Center

Alan B. Franklin
USDA, APHIS, Wildlife Services, National Wildlife Research Center, alan.b.franklin@aphis.usda.gov

Heather Sullivan
USDA/APHIS/WS National Wildlife Research Center, Heather.Sullivan@aphis.usda.gov

Kaci K. VanDalen
USDA/APHIS/WS National Wildlife Research Center, kaci.vandalen@aphis.usda.gov

See next page for additional authors

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ROBERT G. MCLEAN, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
JEFFERY S. HALL, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
ALAN B. FRANKLIN, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
HEATHER SULLIVAN, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
KACI VANDALEN, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
SUSAN SHRINER, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
MATTHEW FARNSWORTH, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
PAUL OESTERLE, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
GINGER YOUNG, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
JENNY CARLSON, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
KACY COBBLE, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
STACEY ELMORE, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
TED ANDERSON, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
SEAN HAUSER, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
KEVIN BENTLER, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
NICOLE MOOERS, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
KATHRYN P. HUYVAERT, USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, CO, USA
TOM DELIBERTO, USDA, APHIS, Wildlife Services, National Wildlife Disease Program, Fort Collins, CO, USA
SETH SWAFFORD, USDA, APHIS, Wildlife Services, National Wildlife Disease Program, Fort Collins, CO, USA

Abstract: All subtypes of influenza Type A viruses infect wild birds, especially waterfowl and shorebirds, but rarely cause disease or mortality in these aquatic species. Aquatic birds are the natural reservoirs for low pathogenic avian influenza viruses (LPAI) that are distributed globally. However, some AI subtypes can be virulent in other animals and humans and some highly

pathogenic AI viruses (HPAI) have caused major outbreaks in poultry and even pandemics in the human population. The emergence of a HPAI H5N1 subtype in southeast Asian poultry in 1997 subsequently involved migratory waterfowl in 2005 and has since spread westward throughout the Asian, European, and African continents. This rapid continental spread alarmed animal and human health agencies in North America and initiated the establishment of a National Strategy for Pandemic Influenza in the United States to increase and expand surveillance for the early detection of this virus, to improve and expand preventative measures, and to develop contingency responses to possible outbreaks. One of the methods of emergency surveillance developed and implemented was an interagency, early detection system for HPAI H5N1 avian influenza in wild migratory birds with the potential to bring in the virus from Asia or Europe and spread it throughout North America.

As part of this early detection system, the Wildlife Services National Wildlife Research Center developed testing methods, sampling protocols, guidelines, and analyzed 50,184 avian fecal samples collected by Wildlife Service biologists in 50 states and the U. S. territories. Samples were pooled in the laboratory (n = 10,541 pools) and analyzed using RT-PCR. AI viruses were detected in 4.0% of the 10,541 sample pools analyzed and H5/H7 subtypes were detected in 0.2% of the sample pools. Positive H5 and H7 subtypes were shipped to the National Veterinary Services Laboratory for further evaluation and confirmation. This monitoring effort was successful in detecting AI viruses in environmental samples and has proven to be a rapid and cost effective surveillance method.

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INTRODUCTION

Avian influenza viruses (*Influenzavirus*, *Orthomyxoviridae*) infect wild birds globally and the natural reservoirs are wild waterfowl, gulls and shorebirds (Webster 1998). Ducks, particularly dabbling ducks, are the primary species infected with low pathogenic avian influenza viruses that rarely cause disease or mortality in wild aquatic species. LPAI viruses have been isolated from more than 100 wild bird species using cloacal samples with an overall prevalence of 5.16% (Table 1) and nearly all of the AI virus subtypes have been detected in wild bird reservoirs or poultry (Olsen et al. 2006). Avian Influenza viruses are shed in the feces of infected waterfowl and AI virus can persist in feces for short periods and remain relatively stable and viable for days to months in water in which the birds swim, defecate, and feed (Stallknecht et al. 1990; Ito et al. 1995). Fecal/oral transmission is the primary method of virus spread to susceptible waterbirds and may be more efficient in shallow water where the virus is more concentrated and thus more likely to expose dabbling ducks that feed there.

Table 1. Groups of waterbirds infected with avian influenza viruses (adapted from Olsen et al. 2006).

Bird Group	No. Species	No. Tested	No. Positive	% Positive
Ducks	**36**	**34,503**	**3,275**	**9.5%**
Geese	**8**	**4,806**	**47**	**1.0%**
Swans	**3**	**5,009**	**94**	**1.9%**
Gulls	**9**	**14,505**	**199**	**1.4%**
Terns	**9**	**2,521**	**24**	**0.9%**
Waders	**10**	**2,637**	**21**	**0.8%**
Rails	**3**	**1,962**	**27**	**1.4%**
Petrels	**5**	**1,416**	**4**	**0.3%**
Cormorants	**1**	**4,500**	**18**	**0.4%**
Total	**84**	**71,859**	**3,709**	**5.16%**

AI virus strains can infect and have low virulence for domestic and wild birds. However, these viruses are unstable and evolution of AI viruses occur with unpredictable frequency through the constant mingling of multiple subtypes in wild waterfowl populations and the frequent exchange of genetic material (Webster et al. 1992). Therefore, some AI virus subtypes can become highly virulent and produce acute clinical disease and mortality in poultry. These highly pathogenic AI (HPAI) viruses are extremely infectious, and once established, can spread rapidly among poultry flocks and wild bird populations.

An outbreak of a new high pathogenic avian influenza (HPAI) H5N1 subtype virus occurred in Hong Kong poultry in 1997 and reemerged in 2002-03 (Webster et al. 2006). This H5N1 virus devastated the poultry industry in Southeast Asia since 2004 and caused an outbreak and mortality in migratory geese in Qinghai Lake, China during 2005 (Liu et al. 2005). HPAI H5N1 virus then spread from China south to Vietnam, Cambodia, Thailand, Laos, and Indonesia where numerous outbreaks occurred in poultry and numerous human cases were reported that were acquired from sick or dead poultry (WHO 2006). It has subsequently spread across Asia, Europe, Middle East, and into Africa in 2005-06 while causing mortality in poultry, swans, waterfowl species, and occasional other species. The outbreaks in poultry resulted in over 209 million birds dying or being culled around much of the world since January 2004 (Peiris et al. 2007).

The local and continental geographical spread of the HPAI H5N1 virus was a result of a combination of factors. Local spread was likely achieved by human movement of poultry and poultry products (Webster et al. 2006). Longer-distance spread within and across regions likely occurred as a result of commercial trade of poultry and poultry products and migratory birds. The role of migratory waterfowl and shorebirds has been implicated in the global spread of AI viruses, especially LPAI (Olsen et al. 2006). Millions of wild birds move within and between large continents along major routes or flyways where bird populations connect with each other and transmit viruses during the sharing of common wintering areas, staging areas, or breeding grounds. For example, birds migrating within the West Pacific and the East Asian-Australasian

flyways overlap with each other and with birds in Alaska where some of them share common breeding areas with North American birds (Webster et al. 2006).

Serious concerns have been raised about the potential impact of HPAI H5N1 virus on domestic poultry, wild bird populations, and humans in the event that it is introduced into the United States. One potential route of introduction of H5N1 into the United States could be through the migration of infected wild birds through Alaska and the Pacific flyway or through eastern Canada and the Atlantic flyway (Peterson 2006). In response to these concerns, the U.S. government developed a "National Strategy for Pandemic Influenza" (http://www.pandemicflu.gov/plan/tab1.html). One major component of this national strategy was an interagency strategic plan for an early detection system for highly pathogenic H5N1 avian influenza in wild migratory birds (USDA 2006). The plan outlined five major surveillance strategies for detecting H5N1: 1) investigation of morbidity/mortality events; 2) surveillance of live wild birds; 3) surveillance of hunter-killed birds; 4) sentinel species; and 5) environmental sampling. The National Wildlife Research Center (NWRC), Wildlife Services, Animal Plant Heath Inspection Service, United States Department of Agriculture, was designated to develop methods for the detection of AI viruses in environmental samples by direct PCR, organize field collection of samples, and test environmental samples collected nationwide from high-risk waterfowl habitats in the United States. The environmental sampling involved the analysis of both water and fecal material collected from waterfowl habitat to provide evidence of AI circulating in wild bird populations, the specific AI subtypes, levels of pathogenicity, and possible risks to humans and poultry. The Wildlife Disease Program of NWRC conducted the laboratory testing of fecal samples collected in all 50 states and United States territories by WS and associated field biologists. The methods developed and results of this sampling in 2006 are presented here.

METHODS

National surveillance utilizing environmental sampling was initially focused on Alaska, where H5N1 was likely to be introduced from Asia, and secondarily on the Atlantic coast, where HPAI could be introduced by migratory birds that cross over the Atlantic Ocean from Europe to Canada and south along the eastern coast of the United States (USDA 2006). Special attention was given to locations along major flyways, particularly the Pacific and Mississippi flyways, that migratory waterfowl use when moving south from Alaska and Canada during the fall and winter. These birds over winter in the southern United States and farther south into the Caribbean and Latin America. The goal was to select sites containing feeding waterfowl of priority species of dabbling ducks identified in the wild bird plan (USDA 2006) and to collect 20-30 fresh fecal samples from each location. The spacing of the collection was designed to represent, as much as possible, the distribution of all birds using the local body of water, and to collect 5 separate samples at multiple sites along the shore. A sample was collected from each fresh feces in the field with a swab that was immediately placed in a vial with transport media (BA-1) containing antibiotics. Samples were labeled with bar codes, transported from the field on ice packs, and shipped with ice packs to the NWRC in Colorado within 48 hours of collection.

Once sample shipments were received at the NWRC laboratory, bar codes on all samples and corresponding data sheets were scanned and entered into a laboratory

information management database system (limsExpress™, Dynamic Databases, Guthrie, OK), that was specifically designed to handle the large volume of samples that were received and tested for AI viruses. Samples were pooled with 1–5 samples per pool based on GPS coordinates and dates of sampling. Pooled samples were treated with inhibitex (Qiagen Inc., Valencia, CA) to reduce natural inhibitors and RNA was extracted by hand if there were ≤ 100 pooled samples, or with a robotic workstation (BioRobot MDx, Qiagen Inc., Valencia, CA), if there were >100 pooled samples to process at once. Extracted RNA was tested by PCR (7900 HT, Real-Time PCR System, Applied Biosystems, Foster City, CA) with a primer for the AI matrix gene. Any AI matrix positive pool was subsequently tested with H5 and H7 specific primers/probes following the procedures described in Spackman et al. (2002). The real-time PCR assay using the AI virus matrix gene was developed for the rapid detection of type A influenza virus and the H5 and H7 hemagglutinin subtype-specific probe sets were developed to detect potential HPAI viruses (Applied Biosystems, Foster City, CA). H5 and H7 positive pools were shipped overnight within 48 hours of our receiving the samples to the National Veterinary Service Laboratory (NVSL) in Ames, Iowa, where confirmation, virus isolation, subtyping, genetic analysis, and pathogenicity testing of H5N1 positives were conducted. The standard procedures for detection of influenza virus at NVSL were virus isolation in embryonated chicken eggs and hemagglutinin subtyping by hemagglutination inhibition (HI) assay (Swayne et al. 1998). The results were reported through established administrative channels.

An expert external committee was formed to develop a nationwide, statistically rigorous sampling design to provide scientific guidance to WS operations biologists in collecting samples from all 50 states for the detection of HPAI H5N1 virus. The committee will also provide guidance in implementing the design using an adaptive management approach and analysis of data resulting from implementation of the design.

RESULTS AND DISCUSSION

From May 1 to December 31, 2006, 50,184 fecal samples were collected from all 50 states, Guam, American Samoa, and the Marshall Islands with a mean of 1,004 samples (range = 114-1,505) collected per state or territory. These samples were tested in 10,541 pools. Of these pools, 419 pools (4.0%) were found PCR positive for AI viruses and 22 pools (0.2%) were PCR positive for H5/H7 subtypes (20 H5 and 2 H7), that were sent to NVSL for confirmation and identification. None of the AI positive sample pools were confirmed as H5N1. No AI viruses were detected in any sample pools from Hawaii, Maryland, Wyoming, or the Pacific Island territories. AI viruses were isolated in embryonated chicken eggs at NVSL from 14 of the 22 PCR positives (64%) and the AI subtypes isolated thus far included 8 H5 subtypes (6 H5N2 and 2 H5N8), 2 H3N2, and 1 each of H3N4, H4N6, H10N7, and H11N9 subtypes. No AI viruses were isolated from 5 of the PCR positives, but 3 low pathogenic avian paramyxovirus-1 viruses were isolated.

This sampling effort determined that fecal samples contain AI virus that can be detected directly by RT-PCR without BSL-3 containment. No H5N1 viruses were confirmed although other AI virus subtypes were identified. Environmental sampling was successful in detecting an overall prevalence of 4% AI viruses from a wide variety and quality of waterfowl habitats over an 8 month sampling period. This prevalence is similar to the overall AI prevalence of infection in individual wild

birds (5%) reported previously (Olsen et al. 2006). Environmental sampling of waterfowl fecal samples appears to be a viable method of monitoring AI viruses in waterfowl populations. Environmental sampling requires less effort than sampling live birds and has proven to be a rapid, cost effective surveillance method. Fecal sampling could complement regular bird sampling or substitute for capturing and sampling birds at sites where it may be the only reasonable approach.

Future activities will include the comparison of fecal sampling to cloacal/tracheal sampling of live birds for detecting AI infections in water birds. Water sampling for the detection of AI viruses by PCR was investigated in 2006 and will be fully developed and implemented as an additional surveillance method. The sampling design committee has reviewed band recovery data and the distribution of fecal samples collected from wild birds in 2006 to develop a targeted sampling strategy for fecal sampling for the 2007 national HPAI surveillance in wild migratory birds. This more targeted approach will focus sampling in areas with the highest risk of HPAI introduction.

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