Surveillance for Highly Pathogenic Avian Influenza Virus in Wild Birds during Outbreaks in Domestic Poultry, Minnesota, USA, 2015

Christopher S. Jennelle, Michelle Carstensen, Erik C. Hildebrand, Louis Cornicelli, Paul Wolf, Daniel A. Grear, Hon S. Ip, Kaci K. Vandalen, Larissa A. Minicucci

In 2015, a major outbreak of highly pathogenic avian influenza virus (HPAIV) infection devastated poultry facilities in Minnesota, USA. To understand the potential role of wild birds, we tested 3,139 waterfowl fecal samples and 104 sick and dead birds during March 9–June 4, 2015. HPAIV was isolated from a Cooper’s hawk but not from waterfowl fecal samples.

Wild birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls and shorebirds) are believed to be the predominant reservoir for avian influenza viruses (AIVs) (1), and most AIV subtypes are low pathogenicity (LPAIV) (2). Only subtypes H5 and H7 are commonly associated with highly pathogenic AIVs (HPAIVs), which sometimes arise from mutation after introduction of LPAIV in domestic poultry (3). The main transmission route of AIVs in birds is fecal-oral, with viral shedding in both feces and through the upper respiratory tract (4). Transmission involves direct or indirect contact between susceptible birds and infectious birds or fomites (5). A novel HPAIV (H5N2) strain discovered in North America in 2014, a reassortant with Eurasian (EA) and North American (AM) lineage genes (6), had been detected in domestic poultry and wild birds as far east as Kentucky, USA, through January 2016. Of 7,084 wild birds sampled by US federal and state agencies during December 2014–June 2015, a total of 98 (1.4%) tested positive for HPAIV (7).

We did not detect HPAIV, although 2 pooled samples containing LPAIV (detailed methods in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-2032-Techapp1.pdf).

In March 2015, we chose 5 counties with infected facilities (Kandiyohi, Lac Qui Parle, Meeker, Nobles, and Stearns) and 5 waterfowl production areas (online Technical Appendix 1) where facilities were uninfected (Figure 1) to test for a spatial difference in HPAIV shedding. Within these areas, we compiled a list of wetlands and lakes and scouted those areas for waterfowl activity and sampled feces. For each area, our goal was to collect 300 fecal samples. In counties with infected poultry, we choose sites within 16 km of infected facilities. We collected ≥20 samples from a given spatiotemporal point to obtain representation within a target area.

We solicited agency staff and the public to report any deceased wild birds or live birds exhibiting neurologic signs consistent with HPAIV infection, including raptors, wild turkeys, and groups of ≥5 dead birds from which we obtained samples. We refer to these as morbidity and mortality samples, and our collection efforts targeted birds that had died <24 h previously.

In Minnesota, USA, HPAIV subtype H5N2 was first confirmed in a poultry facility (hereafter termed facility) in Pope County on March 4, 2015. The scope of the outbreak in Minnesota was unprecedented, and by mid-June 2015, the virus had been found in 23 counties with confirmed cases at 104 sites (98 turkey facilities, 5 chicken facilities, 1 backyard flock). The outbreak resulted in the depopulation of 9 million birds (8) and an economic loss of at least $650 million (9). Given that wild waterfowl are reservoirs for AIVs and that their movement could contribute to HPAIV spread, we conducted surveillance to detect HPAIV in wild waterfowl feces, selected dead birds, and live birds displaying neurologic impairment.

The Study

On March 6, 2015, we conducted an aerial survey covering a 24-km radius around the Pope County facility and identified ≈100 resident mallards (Anas platyrhynchos) and 21 trumpeter swans (Cygnus buccinator). During March 9–12, 2015, we collected 148 representative waterfowl fecal samples, pooled in groups of up to 3, to determine whether wild birds were actively shedding HPAIV. We did not detect HPAIV, although 2 pooled samples contained LPAIV (detailed methods in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-2032-Techapp1.pdf).

In March 2015, we chose 5 counties with infected facilities (Kandiyohi, Lac Qui Parle, Meeker, Nobles, and Stearns) and 5 waterfowl production areas (online Technical Appendix 1) where facilities were uninfected (Figure 1) to test for a spatial difference in HPAIV shedding. Within these areas, we compiled a list of wetlands and lakes and scouted those areas for waterfowl activity and sampled feces. For each area, our goal was to collect 300 fecal samples. In counties with infected poultry, we choose sites within 16 km of infected facilities. We collected ≥20 samples from a given spatiotemporal point to obtain representation within a target area.

We solicited agency staff and the public to report any deceased wild birds or live birds exhibiting neurologic signs consistent with HPAIV infection, including raptors, wild turkeys, and groups of ≥5 dead birds from which we obtained samples. We refer to these as morbidity and mortality samples, and our collection efforts targeted birds that had died <24 h previously.

Author affiliations: Minnesota Department of Natural Resources, Forest Lake, Minnesota, USA (C.S. Jennelle, M. Carstensen, E.C. Hildebrand, L. Cornicelli); United States Department of Agriculture–Wildlife Services, St. Paul, Minnesota, USA (P. Wolf); US Geological Survey–National Wildlife Health Center, Madison, Wisconsin, USA (D.A. Grear, H.S. Ip); US Department of Agriculture Animal and Plant Health Inspection Service, Fort Collins, Colorado, USA (K.K. Vandalen); University of Minnesota College of Veterinary Medicine, St. Paul (L.A. Minicucci)

DOI: http://dx.doi.org/10.3201/eid2207.152032

In February 2015, a major outbreak of highly pathogenic avian influenza (HPAI) in domestic poultry was reported in Minnesota, USA (1). In March 2015, a new subtype of HPAI (H5N2) was isolated in the state (2). The first HPAI H5N2 outbreak in the United States was reported in Kentucky in June 2014 (3). In the United States, HPAI H5N2 has been detected in poultry and wild birds in Kentucky and Minnesota. The outbreak in Minnesota was unprecedented, and by mid-June 2015, the virus had been found in 23 counties with confirmed cases at 104 sites (98 turkey facilities, 5 chicken facilities, 1 backyard flock). The outbreak resulted in the depopulation of 9 million birds (4) and an economic loss of at least $650 million (5). Given that wild waterfowl are reservoirs for AIVs and that their movement could contribute to HPAI spread, we conducted surveillance to detect HPAI in wild waterfowl feces, selected dead birds, and live birds displaying neurologic impairment.

The Study

On March 6, 2015, we conducted an aerial survey covering a 24-km radius around the Pope County facility and identified ≈100 resident mallards (Anas platyrhynchos) and 21 trumpeter swans (Cygnus buccinator). During March 9–12, 2015, we collected 148 representative waterfowl fecal samples, pooled in groups of up to 3, to determine whether wild birds were actively shedding HPAI. We did not detect HPAI, although 2 pooled samples contained LPAI (detailed methods in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-2032-Techapp1.pdf).

In March 2015, we chose 5 counties with infected facilities (Kandiyohi, Lac Qui Parle, Meeker, Nobles, and Stearns) and 5 waterfowl production areas (online Technical Appendix 1) where facilities were uninfected (Figure 1) to test for a spatial difference in HPAI shedding. Within these areas, we compiled a list of wetlands and lakes and scouted those areas for waterfowl activity and sampled feces. For each area, our goal was to collect 300 fecal samples. In counties with infected poultry, we choose sites within 16 km of infected facilities. We collected ≥20 samples from a given spatiotemporal point to obtain representation within a target area.

We solicited agency staff and the public to report any deceased wild birds or live birds exhibiting neurologic signs consistent with HPAI infection, including raptors, wild turkeys, and groups of ≥5 dead birds from which we obtained samples. We refer to these as morbidity and mortality samples, and our collection efforts targeted birds that had died <24 h previously.

Author affiliations: Minnesota Department of Natural Resources, Forest Lake, Minnesota, USA (C.S. Jennelle, M. Carstensen, E.C. Hildebrand, L. Cornicelli); United States Department of Agriculture–Wildlife Services, St. Paul, Minnesota, USA (P. Wolf); US Geological Survey–National Wildlife Health Center, Madison, Wisconsin, USA (D.A. Grear, H.S. Ip); US Department of Agriculture Animal and Plant Health Inspection Service, Fort Collins, Colorado, USA (K.K. Vandalen); University of Minnesota College of Veterinary Medicine, St. Paul (L.A. Minicucci)

DOI: http://dx.doi.org/10.3201/eid2207.152032
In April 2015, which coincided with the peak rates of infection in Minnesota facilities (8), we collected 2,991 waterfowl fecal samples and pooled them into 1,027 brain-heart–infusion media vials; 1,591 samples (548 pooled) were obtained from counties with infected facilities, and 1,400 samples (479 pooled) were collected from waterfowl production areas without facilities (Figure 1). Although HPAIV was not detected in these samples, 30 pooled samples (representing 85 individual birds) tested positive for LPAIV. Apparent LPAIV fecal prevalence was 0.012 (95% CI 0.007–0.018) in counties with infected poultry, 0.008 (95% CI 0.004–0.014) in counties without infection, and 0.010 (95% CI 0.007–0.014) in the combined study area. Given that HPAIV was not detected and that we could not sample every individual bird in the waterfowl population, if HPAIV were present, there was a 95% probability that fecal prevalence was between 0 and 0.181% in areas with infection and 0 and 0.224% in areas without infection.

Through June 4, 2015 (last confirmed positive facility), we collected and tested 104 morbidity and mortality samples (Table) and detected a single HPAIV-positive bird, a Cooper’s hawk (Accipiter cooperii) from Yellow Medicine County (20 km from an infected facility); this infection was confirmed on April 29, 2015 (Figure 2). We suspect that this woodland predator and opportunistic scavenger was exposed to HPAIV through a food item. Although not discovered as part of Minnesota Department of Natural Resources surveillance, 3 black-capped chickadees (Poecile atricapillus) were found in an urban neighborhood exhibiting neurologic signs and submitted to the University of Minnesota Veterinary Diagnostic Laboratory by the Minnesota Wildlife Rehabilitation Center in June.
2015; in 1 bird there was weak detection of Eurasian H5 RNA, but no virus was recovered and no sequence could be obtained directly from the sample (7). All 3 birds demonstrated multifocal encephalitis, which was likely the cause for the neurologic signs (A. Armien, pers. comm.).

Conclusions

Morbidity and mortality samples yielded the only HPAIV detections within the Mississippi flyway in other states; 32% of HPAIV detections nationwide and 90% of HPAIV detections within the Mississippi flyway were derived from this source during December 2014–June 2015 (7). Evolving HPAIV strains can elicit clinical signs and death in young immunologically naive ducks (10), and targeted sampling of waterfowl postbreeding areas for dead or neurologically impaired hatch-year birds might prove useful for future HPAIV surveillance (11).

Careful thought has been given to the design of surveillance programs for avian influenza (12). The study objectives, coupled with the methodologic limitations of available approaches, drive the sampling tool ultimately applied. Although opportunistic sampling (e.g., morbidity and mortality surveillance) is accessible to most agencies, it is not suited for formal population-level inferences. For estimating AIV shedding prevalence, swab sampling of oropharyngeal and cloacal cavities in live birds or the trachea and cloaca in recently deceased birds is optimal because AIV replicates and sheds through the digestive tract (13) and the upper respiratory system (14). For investigating exposure history, sampling blood from live or recently dead birds for serologic testing would be more appropriate, although timing, location, and mechanism of exposure cannot be determined.

Most of our samples were obtained from waterfowl feces. The outbreak’s speed required a quickly deployable method to collect adequate sample sizes and implement spatial design elements that would allow a meaningful comparison between known areas with infection and areas of the state apparently without infection. Modeling has shown that AIV maintenance in wild bird populations is mediated by environmental transmission (15), and the detection of LPAIV in waterfowl fecal samples supports that conclusion. No HPAIV was detected in waterfowl feces, although there was 95% probability of apparent fecal prevalence throughout the study area of 0 to 0.1%. Thus, we conclude that during the 2015 HPAIV (H5N2) outbreak in Minnesota poultry, HPAIV contamination in wild waterfowl feces was not widespread.

### Table. Wild birds collected (n = 104) for highly pathogenic avian influenza virus screening as part of MNDNR morbidity and mortality sampling efforts, Minnesota, USA, March 9–June 4 2015

| Order* | Family     | Genus and species          | Common name               | Count |
|--------|------------|---------------------------|---------------------------|-------|
| Anseriformes | Anatidae   | Branta canadensis         | Canada goose              | 8     |
|         |           | Cygnus buccinator         | Trumpeter swan            | 3     |
|         |           | Aix sponsa                | Wood duck                 | 2     |
|         |           | Anas platyrhynchos        | Mallard                   | 2     |
| Galliformes | Phasianidae | Phasianus colchicus       | Ring-necked pheasant      | 8     |
|         |           | Meleagris gallopavo       | Wild turkey               | 17    |
| Pelicaniformes | Pelicanidae | Pelecanus erythrorhynchos| American white pelican    | 1     |
| Accipitriformes | Cathartidae | Cathartes aura           | Turkey vulture            | 1     |
|         |           | Haliaeetus leucocephalus  | Bald eagle                | 5     |
|         |           | Accipiter striatus        | Sharp-shinned hawk        | 8     |
|         |           | Accipiter cooperi†       | Cooper’s hawk             | 6     |
|         |           | Buteo platypterus        | Broad-winged hawk         | 1     |
|         |           | Buteo jamaicensis         | Red-tailed hawk           | 3     |
| Gruiformes | Rallidae   | Rallus limicola          | Virginia rail             | 1     |
|         |           | Porzana carolina         | Sora                      | 1     |
|         |           | Fulica americana         | American coot             | 9     |
|         |           | Grus canadensis          | Sandhill crane            | 1     |
| Charadriiformes | Laridae   | Larus delawarensis       | Ring-billed gull          | 1     |
|         |           | Larus argentatus         | Herring gull              | 1     |
| Columbiformes | Columbidae | Columba livia            | Rock pigeon               | 2     |
|         |           | Zenaida macroura         | Mourning dove             | 1     |
| Strigiformes | Strigidae  | Bubo virginianus         | Great horned owl          | 3     |
| Caprimulgiformes | Caprimulgidae | Chordeiles minor | Common nighthawk          | 1     |
| Passeriformes | Sturnidae  | Sturnus vulgaris         | European starling         | 10    |
|         |           | Setophaga striata        | Blackpoll warbler         | 1     |
|         |           | Setophaga palmarum      | Palm warbler              | 1     |
|         |           | Melospiza lincolnii      | Lincoln’s sparrow         | 1     |
|         |           | Euphagus carolinus       | Rusty blackbird           | 3     |
|         |           | Quiscalus quiscula      | Common grackle            | 1     |

*†1 HPAIV-positive Cooper’s hawk confirmed on April 29, 2015.
Acknowledgments

Many people were involved in the coordination of sampling, collection of samples, and logistical support for this study. We regret that we cannot name each person involved in these efforts, but we thank the participants from the Minnesota Department of Natural Resources; US Department of Agriculture–National Veterinary Services Laboratory; US Department of Agriculture–Wildlife Services; US Fish and Wildlife Service, US Geological Survey – National Wildlife Health Center; US Department of Agriculture–National Wildlife Research Center; University of Minnesota College of Veterinary Medicine, Public Health and Preventive Medicine Residents; University of Minnesota Veterinary Diagnostic Laboratory; and citizens of Minnesota who reported morbidity and mortality samples. We also thank Robert Dusek, Susan Shriner, and an anonymous reviewer for helpful comments on earlier drafts of the manuscript.

Dr. Jennelle is a research scientist in the Wildlife Health Program of the Minnesota Department of Natural Resources, Forest Lake, Minnesota, USA. He is interested in the ecology of wildlife diseases, quantitative methods for understanding wildlife disease dynamics, and conservation and management of wildlife populations.

References

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56:152–79.
2. Swayne DE, Suarez DL. Highly pathogenic avian influenza. Rev Sci Tech. 2000;19:463–82.
3. Kawaoka Y, Nestorowicz A, Alexander DJ, Webster RG. Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. Virology. 1987;158:218–27. http://dx.doi.org/10.1016/0042-6822(87)90256-X

Figure 2. Wild bird morbidity and mortality samples (n = 104) screened for highly pathogenic avian influenza virus (HPAIV) in Minnesota through June 4, 2015. A Cooper’s hawk was confirmed to be HPAIV positive in Yellow Medicine County on April 29, 2015, whereas weak titers of Eurasian H5 RNA were detected in a sampled black-capped chickadee from Ramsey County collected in June 2015.
4. Franҫa MS, Brown JD. Influenza pathobiology and pathogenesis in avian species. In: Compans RW, Oldstone MBA, editors. Influenza pathogenesis and control, vol. I. New York: Springer International Publishing; 2014. p. 221–42.
5. Stallknecht DE, Brown JD. Tenacity of avian influenza viruses. Rev Sci Tech. 2009;28:59–67.
6. World Organisation for Animal Health. Summary of immediate notifications and follow-ups—2014. Highly pathogenic avian influenza [cited 2015 Oct 14]. http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Immsummary
7. US Department of Agriculture. December 2014–June 2015 wild bird highly pathogenic avian influenza cases in the United States. 2015 Dec [cited 2016 Feb 15]. https://www.aphis.usda.gov/wildlife_damage/downloads/
8. USDA Animal and Plant Health Inspection Service. Update on avian influenza findings: poultry findings confirmed by USDA’s National Veterinary Services Laboratory. 2015 [cited 2015 Oct 16]. https://www.aphis.usda.gov/wps/portal/aphis/ourfocus/animalhealth/sa_animal_disease_information/
9. University of Minnesota Extension. Economic impact of the avian flu, updated 7/10/2015. 2015 Jul [cited 2015 Oct 16]. http://www.extension.umn.edu/community/economic-impact-analysis/reports/docs/Aavian-flu-update-fact-sheet.pdf
10. Pantin-Jackwood MJ, Swayne DE. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian Dis. 2007;51(Suppl):250–9. http://dx.doi.org/10.1637/7710-090606R.1
11. Hénaux V, Parmley J, Soos C, Samuel MD. Estimating transmission of avian influenza in wild birds from incomplete epizootic data: implications for surveillance and disease spread. J Appl Ecol. 2013;50:223–31. http://dx.doi.org/10.1111/1365-2664.12031
12. Hoyle BJ, Munster VJ, Nishiura H, Klaseen M, Fouchier RAM. Surveillance of wild birds for avian influenza virus. Emerg Infect Dis. 2010;16:1827–34. http://dx.doi.org/10.3201/eid1612.100589
13. Webster RG, Yakhmo M, Hinshaw VS, Bean WJ, Copal Murti K. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84:268–78. http://dx.doi.org/10.1016/0042-6822(78)90247-7
14. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, et al. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol. 2005;79:11269–79. http://dx.doi.org/10.1128/JVI.79.17.11269-11279.2005
15. Breban R, Drake JM, Stallknecht DE, Rohani P. The role of environmental transmission in recurrent avian influenza epidemics. PLOS Comput Biol. 2009;5:e1000346. http://dx.doi.org/10.1371/journal.pcbi.1000346

Address for correspondence: Christopher S. Jennelle, Minnesota Department of Natural Resources, 5463-C W Broadway Ave, Forest Lake, MN 55025, USA; email: christopher.jennelle@state.mn.us

Batrachochytrium salamandrivorans
[be-tray-koh-kit-ri-um sa-la-man-dri-vo'rans]

Batrachochytrium salamandrivorans is a recently discovered fungus that kills amphibians. It is related to B. dendrobatidis, which also kills amphibians (from the Greek dendron, “tree,” and Bates, “one who climbs,” referring to a genus of poison dart frogs). Batrachochytrium is derived from the Greek words batrachos, “frog,” and chytra, “earthen pot” (describing the structure that contains unreleased zoospores); salamandrivorans is from the Greek salamandra, “salamander,” and Latin vorans, “eating,” which refers to extensive skin destruction and rapid death in infected salamanders.

Sources
1. Longcore JE, Pessier AP, Nichols DK. Batrachochytrium dendrobatidis gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia. 1999;91:219–27. http://dx.doi.org/10.2307/3761366
2. Martel A, Spitzen-van der Sluijs A, Bloo M, Bert W, Ducatelle R, Fisher MC, et al. Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A. 2013;110:15325–9. http://dx.doi.org/10.1073/pnas.1307356110

Address for correspondence: Elizabeth Kurylo, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30329-4027, USA; email: kwq1@cdc.gov

DOI: http://dx.doi.org/10.3201/eid2207.ET2207
Surveillance for Highly Pathogenic Avian Influenza Virus in Wild Birds during Outbreaks in Domestic Poultry, Minnesota, 2015

Technical Appendix

Methods

Fecal Sampling

There is a precedent established for avian influenza virus (AIV) surveillance in wild birds by sampling waterfowl feces, with comparable AIV prevalence estimates for fecal and oropharyngeal/cloacal swabbing (1–5). Given the rapid emergence of infected poultry facilities in Minnesota during April 2015, we focused on collecting waterfowl feces for the following reasons: 1) it afforded us the most control over sampling design elements and permitted hypothesis-driven surveillance (6), 2) a large sample size could be collected relatively quickly, and 3) the timing of the outbreak occurred when birds are not available for efficient live capture.

From March 9–March 12, 2015, we used polyester-tipped swabs to collect 148 representative waterfowl fecal samples, pooled in groups of up to 3, to determine whether ≈100 resident mallards (*Anas platyrhynchos*) and 21 trumpeter swans (*Cygnus buccinator*) were actively shedding high pathogenicity AIV (HPAIV) in the surveillance zone (≈1,830 km²) around the Pope County index poultry facility. All fecal samples were submitted to the USDA National Wildlife Research Center (USDA-NWRC) in Fort Collins, Colorado, USA, for diagnostic testing.

For our designed sampling approach in areas of Minnesota with and without HPAIV-infected poultry, the waterfowl production areas we chose as control sites consisted of 5 wildlife management areas (WMA)/national wildlife refuges (NWR) without infected facilities (Carlos Avery WMA, Minnesota Valley NWR, Swan Lake WMA, Thief Lake WMA, and Whitewater...
These areas are managed by state or federal agencies to sustain and enhance wildlife habitat (especially wild waterfowl game birds) for wildlife conservation. We used polyester-tipped swabs to collect fecal samples deposited in 1 of 17 location types (Technical Appendix Table) during April 8–April 30, 2015. We sampled what we perceived to be fresh waterfowl feces (<24 h) that were at least 2 m apart. We assumed that each feces pile represented a unique individual bird and pooled up to 3 samples per vial, which was filled with brain-heart infusion medium, and refrigerated. Samples were submitted to the USDA-NWRC in Fort Collins, Colorado, for diagnostic testing.

**Morbidity and Mortality Sampling**

We made no fixed goals for this sample type because of the opportunistic nature of discovery and reporting, and targeted birds that had been dead for <24 h. We used these data as an auxiliary source of information in our surveillance efforts and obtained samples statewide. Depending on the resources available for staff, we either collected whole carcasses (double-bagged and frozen) or used polyester-tipped swabs to separately obtain tracheal and cloacal specimens from sampled birds. Both swab samples from a bird were pooled in blood-heart infusion media and refrigerated. Whole carcasses were submitted to the USGS National Wildlife Health Center (USGS-NWHC) or the Minnesota Veterinary Diagnostic Laboratory in St. Paul MN, and swab samples were submitted to USDA-NWRC for diagnostic testing.

**Sample Diagnostic Testing**

At the USDA-NWRC, nucleic acid was extracted from 50 μL of pooled fecal swab samples using the MagMax-96 AI/ND Viral RNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). Five microliters of nucleic acid extracts were analyzed by real-time reverse transcription PCR (rRT-PCR) with primers and probes specifically designed to detect the influenza virus type A matrix gene (7) and the iTaq Universal Probes One-Step Kit (BioRad Laboratories, Hercules, CA, USA). The rRT-PCR conditions were the following: 50°C for 10 min, 95°C for 30 s, and 40 cycles at 95°C for 15 s and 60°C for 30 s. Samples with Ct values ≤38 were forwarded to the USDA National Veterinary Services Laboratory (USDA-NVSL) in Ames, Iowa, USA, for confirmation and further H5 and H7 testing and isolation.

Swabs from carcasses of diseased or dead birds were submitted to USGS-NWHC where diagnostic necropsies were performed. Tracheal and cloacal swab specimens were collected from
all carcasses and used to screen for HPAIV, when a necropsy was not performed. Tissue samples for AIV testing were homogenized in viral transport media and centrifuged at 1,000 × g for 30 min at 4°C. RNA from 50 μL of the supernatant of the tissue homogenate or swab material were recovered and tested for AIV by the current National Animal Health Laboratories Network protocols (7,8). Aliquots of samples for subtypes H5 and H7 were sent to USDA-NVSL for confirmation on the day of detection and were further characterized by additional tests, including rRT-PCR for virulence, sequence analysis, and virus isolation.

**Data Analysis**

For estimating apparent prevalence of low pathogenicity AIV) in fecal specimens, we used rRT-PCR matrix test results determined by the USDA-NVSL and applied a Bayesian approach (9), accounting for variable-sized pooled samples and imperfect test sensitivity and specificity. We used a binomial distribution to model the response variable (proportion of rRT-PCR positive test results) and used an uninformative prior distribution for low pathogenicity AIV prevalence. Because no published diagnostics are currently available for sensitivity and specificity of rRT-PCR matrix results from waterfowl fecal samples when specifically testing for the HPAIV(H5N2) Eurasian-American strain, we assumed unity (SE = Sp = 1). For calculating the detection threshold for HPAIV shedding in fecal samples given zero positive tests, we assumed independence among samples and used a Bayesian approach (10), again assuming an uninformative prior distribution beta (α = β = 1) on HPAIV shedding prevalence.

**References**

1. VanDalen KK, Franklin AB, Mooers NL, Sullivan HJ, Shriner SA. Shedding light on avian influenza H4N6 infection in mallards: modes of transmission and implications for surveillance. PLoS ONE. 2010;5:e12851. PubMed [http://dx.doi.org/10.1371/journal.pone.0012851](http://dx.doi.org/10.1371/journal.pone.0012851)

2. Chen H, Smith GJD, Li KS, Wang J, Fan XH, Rayner JM, et al. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci U S A. 2006;103:2845–50. PubMed [http://dx.doi.org/10.1073/pnas.0511120103](http://dx.doi.org/10.1073/pnas.0511120103)

3. Stallknecht DE, Luttrell MP, Poulson R, Goekjian V, Niles L, Dey A, et al. Detection of avian influenza viruses from shorebirds: evaluation of surveillance and testing approaches. J Wildl Dis. 2012;48:382–93. PubMed [http://dx.doi.org/10.7589/0090-3558-48.2.382](http://dx.doi.org/10.7589/0090-3558-48.2.382)
4. Ofula VO, Franklin AB, Root JJ, Sullivan HJ, Gichuki P, Makio A, et al. Detection of avian influenza viruses in wild waterbirds in the Rift valley of Kenya using fecal sampling. Vector Borne Zoonotic Dis. 2013;13:394–400. PubMed http://dx.doi.org/10.1089/vbz.2011.0926

5. Grillo VL, Arzey K, Hansbro P, Hurt A, Warner S, Bergfeld J, et al. Avian influenza in Australia: a summary of 5 years of wild bird surveillance. Aust Vet J. 2015;93:387–93. PubMed http://dx.doi.org/10.1111/avj.12379

6. Høy BJ, Munster VJ, Nishiura H, Klaassen M, Fouchier RAM. Surveillance of wild birds for avian influenza virus. Emerg Infect Dis. 2010;16:1827–34. PubMed http://dx.doi.org/10.3201/eid1612.100589

7. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002;40:3256–60. PubMed http://dx.doi.org/10.1128/JCM.40.9.3256-3260.2002

8. Pedersen J, Killian ML, Hines N, Senne D, Panigrahy B, Ip HS, et al. Validation of a real-time reverse transcriptase-PCR assay for the detection of H7 avian influenza virus. Avian Dis. 2010;54(Suppl):639–43. PubMed http://dx.doi.org/10.1637/8911-043009-Reg.1

9. Williams CJ, Moffitt CM. Estimation of fish and wildlife disease prevalence from imperfect diagnostic tests on pooled samples with varying pool sizes. Ecol Inform. 2010;5:273–80. http://dx.doi.org/10.1016/j.ecoinf.2010.04.003

10. Heisey DM, Jennelle CS, Russell RE, Walsh DP. Using auxiliary information to improve wildlife disease surveillance when infected animals are not detected: a Bayesian approach. PLoS ONE. 2014;9:e89843. PubMed http://dx.doi.org/10.1371/journal.pone.0089843
Technical Appendix Table. Description of 17 location types in Minnesota searched for waterfowl feces, April 2015

| Location description                                                                 |
|-------------------------------------------------------------------------------------|
| Foam baiting stations in ditches, ponds, and marshes                                |
| Mowed grass and gravel around ponds or along dikes                                  |
| Top of gravel or grass dikes                                                        |
| Waste water ponds                                                                   |
| Sand bars in lakes                                                                  |
| Mud flats                                                                           |
| Vegetation mats on water                                                            |
| Golf courses                                                                        |
| Mowed ditches along roads                                                            |
| Gravel or grass shore along lakes                                                   |
| Upland hay meadows                                                                  |
| Sandy beaches on lakes                                                              |
| Loafering rocks in open water and logs along lakes or pond shoreline                 |
| Cleared areas along lakes or ponds                                                  |
| Softball fields                                                                     |
| Residence yards near ponds or lakes                                                 |
| Park grounds                                                                        |