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BRIEF REPORT

Increased detection of influenza A H16 in the United States

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Abstract As a result of an US interagency avian influenza surveillance effort in wild birds, four isolates of influenza A viruses were initially identified as H7 by hemagglutination inhibition (HI) but subsequently identified as H16 through genetic sequence analysis. We report the development of internal primers for amplification and cycle-sequencing of the full-length H16 gene, increased detection of H16 within the US, and possible steric inhibition or cross-reaction between H7 and H16 antigens during the conventional HI assay. The latter could have critical implications for poultry operations if H16 viruses are detected and mistakenly reported as H7 viruses.

Influenza A viruses have been isolated from a wide range of avian species throughout the world [1]. Wild birds, predominantly waterfowl and shorebirds, serve as the natural reservoir of influenza A viruses [2]. Until 2005, 15 hemagglutinin subtypes (H1–H15) had been isolated from wild birds. In 2005, the hemagglutinin subtype H16 was identified in black-headed gull (*Larus ridibundus*) samples from Sweden [3]. Subsequent sequencing of influenza isolates collected in the United States identified H16 in shorebird and gull samples collected in Delaware, New Jersey, and Alaska between 1975 and 1988 which had been previously identified as H1 or H13 subtypes [4].

While avian influenza viruses rarely affect their wild bird hosts, the potential transmission of highly pathogenic avian influenza (HPAI) viruses to hosts of agricultural and human health importance is of concern. In 2006, an interagency avian influenza surveillance effort was implemented throughout the United States and its territories to facilitate early detection of HPAI viruses in wild birds [5]. Since this effort began, USDA-APHIS-Wildlife Services National Wildlife Research Center (NWRC) tested approximately 75,000 fecal samples from wild birds across all 50 states, American Samoa, the Marshall Islands and Guam for the presence of avian influenza viruses [6]. Conventional subtyping and genetic sequencing have resulted in the identification of a wide range of low-pathogenic avian influenza (LPAI) viruses including 12 isolates of the H7 subtype and 18 isolates of the H5 subtype. However, 4 out of 12 samples originally identified as H7 by hemagglutination inhibition (HI) were subsequently identified as H16 following genetic sequencing at the NWRC and the USDA-APHIS National Veterinary Services Laboratory (NVSL). Here, we report the development of internal primers useful for amplification and cycle-sequencing of the full-length H16 gene, the increased detection of H16 within the US, and possible cross-reaction between H7 and H16 antigens or steric inhibition during the conventional HI assay.

Wild bird fecal samples collected as part of the US interagency strategic plan for early detection of HPAI were shipped to the NWRC, Fort Collins, CO, for analysis. Up to five samples were pooled for analysis, and viral RNA was

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extracted from these pools and analyzed by real-time reverse transcription-polymerase chain reaction (RRT-PCR) [7]. All RRT-PCR positive samples were inoculated into 11-day-old embryonated chicken eggs, and viral isolates were subtyped using HI and neuraminidase-inhibition (NI) tests at NVSL. After conventional subtyping, HA genes were amplified and sequenced. RT-PCR was performed using a suite of subtype-specific (H1–H15) primers developed by the Southeast Poultry Research Laboratory (SEPRL), Athens, GA, USA. Isolates that could not be amplified using the H1–H15-specific primers were re-amplified using the HA-end universal primers (Hgga+ and H-T7) obtained from SEPRL [8]. RT-PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA) and sequenced using ABI BigDye chemistry (Applied Biosystems, Foster City, CA, USA). The cycle-sequencing product was visualized on an ABI 3130×1 genetic analyzer. Sequences were aligned using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA) and compared to other published sequences in GenBank.

Fourteen different HA subtypes were identified from 186 influenza A viruses isolated from wild bird feces by the HI test. Genetic sequencing results agreed with HI subtyping results in all but a small number of isolates. Noteworthy were four viruses isolated from fecal samples collected in Alaska, New Hampshire, Rhode Island, and Utah. Originally, these isolates were identified as H7 by the HI assay but could not be sequenced using H7-specific primers. Subsequently, these isolates were amplified using HA-end universal primers (Hgga+ and H-T7) and compared to other published sequences in GenBank. These genetic sequence comparisons suggested the four isolates were actually H16 viruses. Primers specific for the H16 HA gene had not been previously published and were developed for confirmation and further characterization (Table 1).

To develop H16-specific primers, H16 gene sequences were assembled and checked for errors. Edited sequences were aligned with all similar H16 sequences available in GenBank: A/black-headed gull/Sweden/5/99(H16N3); A/black-legged kittywake/Alaska/295/1975(H16N3); A/herring gull/DE/712/1988(H16N3); A/shorebird/New Jersey/840/1986(H16N3); and A/shorebird/Delaware/168/06(H16N3). Priming sites were chosen in regions of high similarity across all available H16 sequences and evaluated for melting temperature and secondary structure using OligoAnalyzer 3.1. RT-PCR targeting the H16 coding region was performed using the Qiagen OneStep RT-PCR kit. Two microliters of RNA template was amplified in a 50-µl reaction containing 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 2.0 µl RT-PCR Enzyme Mix, 1.0 µM of a forward primer (H16+854 or H16+1063), and 1.0 µM of a reverse primer (H16−1097 or H16−1320) using the following PCR program: 30 min at 50°C; 15 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 52–56°C, 1 min at 72°C; and 10 min at 72°C.

Table 1 Internal primers developed for amplification and cycle-sequencing of the avian influenza H16 gene

Primer	Primer sequence (5′ → 3′)	T_m (°C)
H16+854	GAGAAATACGGTWCAGGACG	52.6
H16+1063	AGGTCTGTTTGGTGCGATTGC	58.4
H16−1097	TTCTATGAAGCCTGCAATWGC	53.3
H16−1320	CATCAACCCGATCAGCMAGC	57.7

The discovery of H16 viruses in wild birds during the interagency avian surveillance effort and our development of H16-specific primers are important for several reasons. First, the disagreement between the HI test results and the genetic sequencing results presents new research opportunities. The misclassification of the H7 and H16 subtypes may be due to steric inhibition as the H7 HI test was conducted with a reference reagent with the same NA antigen as the H16 viruses. Alternatively, the misclassifications could be due to some cross-reactivity between the H7 and H16 antigens [9]. Cross-neutralization studies and further sequence analyses using H16-specific primers are necessary to prevent false-positive reporting of H7 viruses. The false reporting of H7 has critical implications for agricultural poultry operations. Past outbreaks of highly pathogenic avian influenza in poultry have been mostly due to subtypes H5 or H7 [10], which have had severe economic impact through culling of potentially infected birds and trade embargoes [11].

Second, as a result of the surveillance, virus isolation, and sequencing effort, we have identified H16 in three states not previously reported: New Hampshire, Rhode Island, and Utah. As with the genetically related subtype H13, influenza A H16 viruses have only been isolated from shorebirds and gulls [1, 3, 4]. Because the H16 isolates in this study came from wild bird fecal samples, it is impossible to know the exact avian species that shed the virus. However, field collectors noted the 2–3 most prevalent avian species at sampling sites, which were gulls at the Alaska, New Hampshire, and Rhode Island sampling sites where H16 isolates were obtained. However, at the Utah site, the most prevalent species listed in collection notes were American green-winged teals and gadwalls. While gulls cannot be ruled out, this information may warrant further studies to characterize H16 viruses in the US and assess their ability to infect other species.

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