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Inability of Real-Time Reverse Transcriptase PCR Assay To Detect Subtype H7 Avian Influenza Viruses Isolated from Wild Birds▽

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We report a failure of the real-time reverse transcriptase PCR H7 subtyping protocol currently used in national avian influenza surveillance programs. Significant substitutions in primer and probe target sequences were identified, especially in wild bird viruses. The protocol, originally designed for detecting H7 influenza viruses in poultry, is not reliable for wild bird surveillance.

Avian influenza viruses (AIV) with H5 or H7 hemagglutinin (HA) subtypes can cause highly pathogenic avian influenza in susceptible poultry (16). Most subtype H5 and H7 isolates in poultry and wild birds are not highly pathogenic, but critical mutations can occur in these low-pathogenicity or nonpathogenic viruses that result in increased virulence (1, 8). Poultry are not considered to be natural hosts for AIV but are susceptible to infection with waterfowl-origin viruses (4, 5, 10). Because the reservoir hosts (wild birds) may not show symptoms of infection (15), the only way to monitor for the presence of H5 and H7 viruses is through active surveillance of wild birds.

Although waterfowl and shorebirds are the natural reservoir hosts of influenza A viruses, many species have not been surveyed in large numbers. The recent recognition that the highly pathogenic H5N1 AIV can infect wild birds and has the potential to be spread by these birds to new areas (3, 9) has resulted in a tremendous surge of interest in the surveillance of free-flying avian species for AIV. The result has been a rapid increase in the numbers of individual wild birds and the diversity of species tested for AIV and specifically for subtype H5 and H7 viruses. In 2005, the AIV test results from 983 individual birds were recorded in the HEDDS database, and by 2006, 163,451 birds nationwide were tested for AIV (11).

This report documents the failure of the real-time reverse transcriptase PCR (rRT-PCR) assay currently used in the highly pathogenic AIV early detection surveillance program (2) to identify three H7 viruses isolated from waterfowl in California. All three viruses were tested both in a university laboratory and in a National Animal Health Laboratory Network laboratory; none were detected as H7 viruses either from a pool of five samples or in individual allantoic fluids containing the isolated viruses.

Sample flow and virus isolation. The three samples from which the viruses reported here were collected were part of 1,517 swabs collected from hunter-killed waterfowl in California between October 2006 and March 2007. Cloacal swabs were collected, and placed into 3 ml of viral transport medium, immediately placed on dry ice, and sent overnight to the University of California, Davis. Upon receipt, the samples were stored at −80°C until they were split and an aliquot sent to the California Animal Health and Food Safety Laboratory, where five samples were pooled and screened for AIV by rRT-PCR per the procedures recommended by the USDA-APHIS Wildlife Services (2). Individual samples from positive pools were tested for H5 and H7 viruses by rRT-PCR. After screening, the stored aliquots of all the original samples were inoculated into embryonating chicken eggs by standard methods (17).

Matrix gene determination and H7 subtyping by rRT-PCR. AIV detection and subtyping were performed by rRT-PCR using assay protocols recommended by the avian influenza early detection program (2) in both the California Animal Health and Food Safety Laboratory (on the original sample and HA-positive allantoic fluid) and the university laboratory (on HA-positive allantoic fluid). Briefly, total RNA was recovered from 60 μl of cloacal swab fluid (50 μl was used at the university laboratory) using a commercial magnetic bead-based RNA extraction kit (MagMAX-96 viral RNA isolation kit; Ambion, Austin, TX). The extracted RNA was screened for the presence of A2 AIV by rRT-PCR using a previously published assay targeting the AIV matrix gene (13). Specimens testing positive for the AIV matrix gene were then evaluated by rRT-PCR for H5 and H7 subtypes (14). None of the samples or isolated viruses were either H5 or H7 positive (Table 1).

Subtyping by sequencing. The same RNA extraction and RT protocols were followed for generation of cDNA from isolated viruses. Universal HA and neuraminidase amplification primers were used to generate fragments (7) which were then fractionated in a 1.5% agarose gel, purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA), and partially sequenced

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TABLE 1. Summary of laboratory findings for California subtype H7 AIV

Virus	Sampling date (mo/day/yr)	Test result					
		Swab sample testing		Allantoic fluid testing			
		AIV RT-PCR[a]	H7 RT-PCR[a]	AIV RT-PCR[b]	H7 RT-PCR[a,b]	HA sequencing[b]	Virus serotyping[c]
A/cinnamon teal/CA/JN611/06 (H7N3)		−	ND[d]	+	−	H7	H7N3
A/cinnamon teal/CA/JN1310/07(H7N3)	01/20/07	+	−	+	−	H7	H7N3
A/Northern Shoveler/CA/JN1447/07 (H7N2)	02/03/07	−	ND	+	−	H7	H7N2

[a] Done at the California Animal Health and Food Safety Laboratory.
[b] Done in the Xing/Cardona laboratory, University of California, Davis.
[c] Done at the National Veterinary Services Laboratory, Ames, IA.
[d] ND, not done.

with the same primers. The HA subtypes of three isolates, JN611, JN1447, and JN1310, were identified as H7 through BLAST comparison with published sequences. After identification of the virus subtypes, primers were synthesized to amplify and sequence the complete HA genes.

Allantoic fluid containing AIV was subsequently sent to the National Veterinary Services laboratory for serological subtyping. The three isolates were identified as either H7N3 or H7N2 subtypes (Table 1).

Sequence analysis. The HA sequences were individually compared to sequences in the GenBank database using the BLASTN tool. The 24 published sequences with the most identity to each sequence were recorded and aligned (AlignX, VectorNTI Advance, version 10.1.1; Invitrogen, Carlsbad, CA) and then compared to the H7 primer and probe sequences (Fig. 1). The California isolates had 2 of 19 nucleotides in the forward primer and one (JN611 and JN1310) or two (JN1447) of 24 nucleotides in the reverse primer regions that were dif-

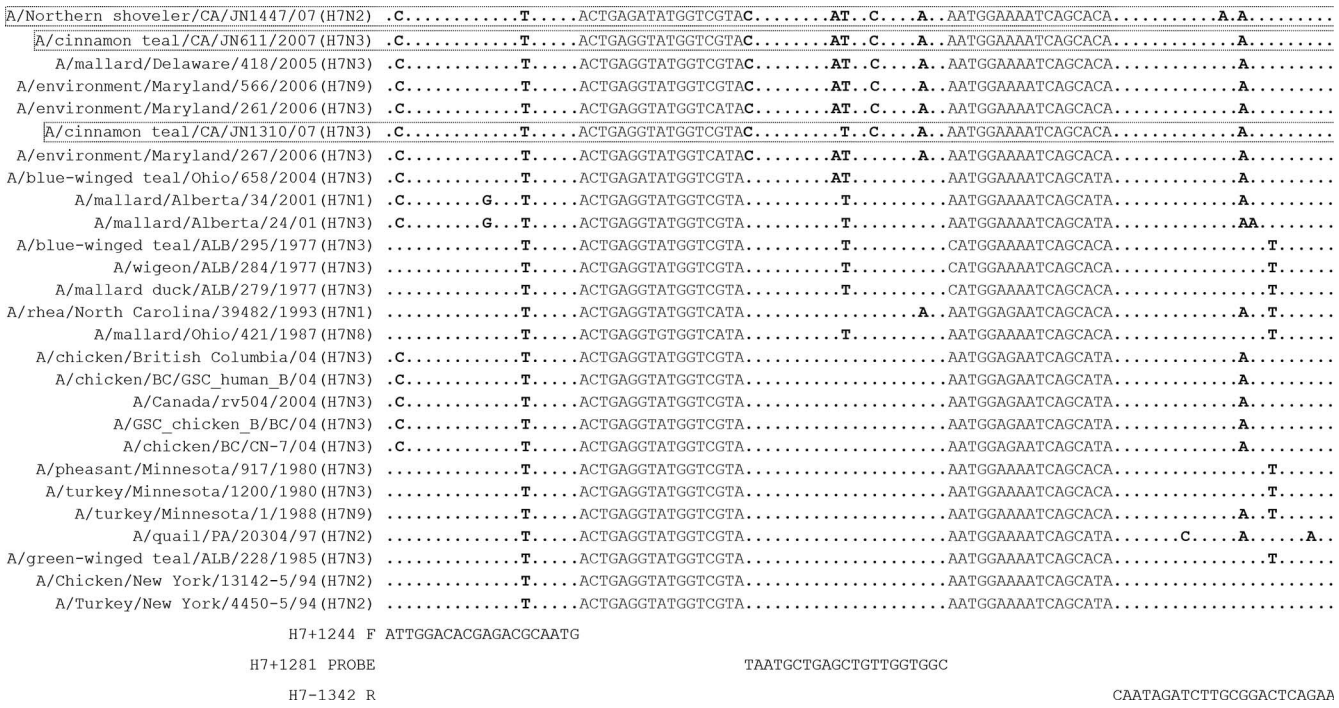

FIG. 1. Alignment of subtype H7 AIV. Subtype H7 AIV from California were aligned with the published sequences (GenBank) to which they were most closely related and compared to primer and probe sequences. Conserved nucleotides in the aligned sequences are represented by dots, while nonidentical bases are bold. The California viruses described in this paper are boxed. GenBank accession numbers for the other sequences are as follows: A/mallard/Delaware/418/2005(H7N3), CY021637.1; A/environment/Maryland/566/2006(H7N9), CY024818.1; A/environment/Maryland/261/2006(H7N3), CY022749.1; A/environment/Maryland/267/2006(H7N3), CY022757.1; A/blue-winged teal/Ohio/658/2004(H7N3), CY018901.1; A/mallard/Alberta/34/2001(H7N1), CY005983.1; A/mallard/Alberta/24/01(H7N3), DQ017504.1; A/blue-winged teal/ALB/295/1977(H7N3), CY005974; A/wigeon/ALB/284/1977(H7N3), CY005975.1; A/mallard duck/ALB/279/1977(H7N3), CY005976.1; A/rhea/North Carolina/39482/1993(H7N1), EF470586.1; A/mallard/Ohio/421/1987(H7N8), CY021621.1; A/chicken/British Columbia/04(H7N3), AY611524.1; A/chicken/BC/GSC_human_B/04(H7N3), AY646078.1; A/Canada/rv504/2004(H7N3), CY15006.1; A/GSC_chicken/BC/04(H7N3), AY650270.1; A/chicken/BC/CN-7/04(H7N3), EF470587.1; A/pheasant/Minnesota/917/1980(H7N3), CY014721.1; A/turkey/Minnesota/1200/1980(H7N3), CY014778.1; A/turkey/Minnesota/1/1988(H7N9), CY014786.1; A/quail/PA/20304/97(H7N2), AY240924; A/green-winged teal/ALB/228/1985(H7N3), CY005978; A/Chicken/New York/13142-5/94(H7N2), AF072384.2; A/Turkey/New York/4450-5/94(H7N2), AF072386.2.

ferent and, more importantly, 4 (JN1310) or 5 (JN1447 and JN611) of 21 nucleotides in the probe that were different in the HA genes (Fig. 1). These nucleic acid discrepancies are believed to have resulted in the false-negative identification of the California H7 viruses using the published primer and probe sequences.

Further analysis of the HA genes of all published North America H7 isolates showed that additional published wild bird sequences have similar mismatches with the H7 primers and probes, while sequences from domestic poultry are more identical to the primers and probes used in the protocol (Fig. 1). Prior to the isolation and identification of JN611, no subtype H7 viruses had been identified in the Pacific flyway since 2004 (6). Our findings suggest that H7 AIV may have been present, but not detected, in some samples. The development and validation of tests for wild birds should be a priority component of wild bird surveillance efforts.

The difficulties in utilizing rRT-PCR tests designed and validated for domestic poultry in free-flying wild birds have resulted in tests that demonstrate a lower diagnostic sensitivity for wild birds compared to domestic birds, based on the gold standard of virus isolation (Table 1) (A. Das, E. Spackman, M. J. Pantin Jackwood, D. E. Swayne, and D. L. Suarez, presented at the American Association of Veterinary Laboratory Diagnosticians Conference, Reno, NV, 2007). In the current study, two of the three H7 viruses detected by virus isolation were not detected by matrix gene rRT-PCR, resulting in false-negative tests, a potentially serious concern for AIV surveillance in wild birds. The decreased sensitivity of the AIV rRT-PCR has been attributed to the presence of inhibitors in samples from free-flying birds (12; Das et al., presented at the American Association of Veterinary Laboratory Diagnosticians Conference, Reno, NV, 2007). However, the failure of the rRT-PCR H7 test was not only with samples but also with RNA extracted from allantoic fluids, which contain large amounts of virus and few or no inhibitors. In fact, the failure of the rRT-PCR H7 assay is likely due to significant differences in the primer and probe sequences. Further, a review of published sequences indicates that the primer and probe mismatches identified in our isolates are not unique and may be common among the H7 AIV of waterfowl (Fig. 1).

This report demonstrates that the H7 subtyping rRT-PCR protocol used in the early detection surveillance program in the United States does not reliably detect H7 AIV from wild birds. Significant sequence differences exist between the primers and probe and isolates from wild birds. These findings elucidate the need for diagnostic testing methods that are validated with wildlife species.

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