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Short Communication

Wild Bird's-eye View of Influenza Virus A(H1N1) Phylogenetic Evolution

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Abstract: Wild bird fecal samples collected and characterized by the USDA as part of a national surveillance effort were sequenced to study the genetic relatedness of avian, swine, and human H1 and N1 subtypes. Our results find that the 2009 H1N1 human outbreak is closely related to swine virus, but falls into different clades in the H1 and N1 trees. Further, there is evidence of multiple viral genetic exchanges between birds and swine. Ongoing research across host species contributes to an understanding of the circulation of influenza viruses.

Keywords: Influenza virus A, H1N1, phylogeny, evolution, surveillance, zoonoses

Influenza A viruses ultimately originate from wild waterfowl, which are the primary reservoirs for these viruses (Webster et al., 1992). From wild waterfowl, influenza A viruses can subsequently spread to wild mammals, domestic poultry, swine, and humans. Often, these viruses evolve in animals and are subsequently transmitted to humans, where these have the potential to cause disease, sometimes at pandemic levels (Taubenberger et al., 2004; Trampuz et al., 2004). For example, the influenza A virus that caused the global pandemic in 1918 was an avian virus that adapted to humans (Taubenberger et al., 2004). Influenza A viruses are classified into different subtypes based on the antigenicity of the hemagglutinin (HA) and neuraminidase (NA) proteins (Spackman, 2008); there are 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) of influenza A virus currently identified (Fouchier et al., 2005) in wild waterfowl. Of these, the H1 and N1 subtypes are found in 5%–6% of wild ducks (Webster et al., 1992). Thus, wild waterfowl have the potential to contribute to both the evolution and potential transmission of H1N1 influenza A virus.

In April 2009, an outbreak of H1N1 occurred in humans—termed swine-origin influenza virus A(H1N1) [S-OIV]—which was closely related to a triple-reassortment of avian, swine, and human influenza virus A (Garten et al., 2009; Nava et al., 2009; Smith et al., 2009; Trifonov et al., 2009a, b). However, these recent studies did not distinguish between the contributions of wild avian (e.g., wild waterfowl species) and domestic avian (e.g., poultry) influenza virus, even though some wild bird sequences were included in some of their analyses (e.g., Fig. 2 in Garten et al., 2009; figures in Nava et al., 2009). Here, we specifically examined the relationship between wild waterfowl and the 2009 S-OIV H1N1 strain in humans, using H1 and N1 subtype influenza A viruses isolated and sequenced from fecal samples collected from wild birds during a U.S. interagency surveillance (years 2006–2007) effort to detect avian influenza virus in wild birds across the United

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States (U.S. Interagency Strategic Plan, 2006; McLean et al., 2007). Our goal was to infer whether influenza A viruses from wild birds specifically contributed to the 2009 S-OIV H1N1 outbreak.

A United States interagency avian influenza surveillance effort for the detection of highly pathogenic avian influenza (H5N1) was developed and implemented in 2006 (U.S. Interagency Strategic Plan, 2006). As part of this surveillance, Wildlife Services of the United States Department of Agriculture's Animal and Plant Health Inspection Service (USDA/APHIS) collected and tested 75,481 wild bird fecal samples for the presence of influenza A virus between April 2006 and March 2008 across all 50 states and the Pacific territories (McLean et al., 2007). Samples testing positive for influenza A were subsequently subtyped, and the HA and NA gene segments were sequenced from samples that were subtyped as H1 or N1. Wild bird fecal samples were tested using a molecular approach for positive influenza A viral infection (Spackman et al., 2002). Subsequently, all positive samples were inoculated into 11-day-old embryonated chicken eggs for virus isolation. All viral isolates were subtyped via hemagglutination inhibition (HI) assay at the National Veterinary Services Laboratories (Ames, IA). HA and NA genes were PCR-amplified using a suite of subtype-specific primers developed by the Southeast Poultry Research Laboratory ([SEPRL], Athens, GA). RT–PCR products were purified with the QIAquick gel extraction kit and sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Inc. [ABI]) on an ABI 3130xl DNA Analyzer. Our sequences (GenBank accession numbers GQ290657-GQ290692) and publicly available H1 and N1 nucleotide sequences including the recent S-OIV H1N1 outbreak in humans (NCBI Influenza Virus Resource, http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html; GISAID EpiFluDB, http://epiflu.vital-it.ch/GISAID) were aligned in MAFFT 6.523b (Katoh et al., 2005) for a total of 983 H1 sequences of 1705 nucleotides (nt) and 1451 N1 sequences of 1416 nt. The minimum length cut-off when mining public databases was 1200 nt, focusing more on complete sequences. The final alignments were inspected and edited manually where deemed necessary in Se-Al 2.0a11 (http://tree.bio.ed.ac.uk/software/seal; alignments are available from authors upon request). Phylogenetic analysis was accomplished in a maximum likelihood framework using the parallel Pthreads version of RAxML 7.1.0 (Stamatakis et al., 2005; Stamatakis, 2006) on an 8-way shared memory (32 GB RAM) server at the American Museum of Natural History (AMNH) implementing the GTR + Γ_4 substitution model (Lanave et al., 1984; Yang, 1994). Node support was evaluated with 100 rapid bootstrap pseudoreplicates (Stamatakis et al., 2008). Trees were visualized in FigTree 1.2.2 (http://tree.bio.ed.ac.uk/software/figtree).

We examined relatedness of H1 and N1 gene sequences from the wild bird influenza A viruses collected by the USDA surveillance effort (H1 = 12; N1 = 24) and publicly available sequences from birds (wild and domestic; H1 = 52; N1 = 308), swine (H1 = 229; N1 = 153), and humans (H1 = 643; N1 = 925) in Asia, Europe, and North America (including the 2009 H1N1 outbreak; H1 = 47; N1 = 39). The maximum likelihood phylogenetic analysis reveals similar topologies for both H1 and N1 gene trees (Fig. 1). Swine influenza virus (SwIV) H1 sequences are non-monophyletic with clade I arising out of a clade of predominantly human H1 sequences, clade II being closely related to but divergent from human H1 sequences, and clade III being more closely related to avian-derived influenza A virus sequences, including wild and domestic birds. A similar pattern is observed in N1; clade A is closely related, but divergent from human H1, while clade B arises out of an avian lineage. The polyphyletic nature of SwIV sequences suggests it arose on multiple occasions. The existence of branches and clades of SwIV (III and B) that are more closely related to avian—including wild bird—sequences suggests historical and recent exchange between wild birds and swine (Karasin et al., 2004). The USDA wild bird fecal viral sequences of H1 and N1 came from a broad geographic sampling area (Fig. 2) and they represent multiple H1 and N1 lineages circulating in North America, yet they group within other North American wild bird viral sequences. Some of the USDA N1 influenza A sequences from wild birds appear related to two samples from Alaska that group with two samples from Hokkaido, Japan, and these together are sister to California sequences, thus suggesting viral exchange between the U.S. and Japan (Duan et al., 2007). No clade in the H1 or N1 tree is monophyletic with respect to host species indicating ongoing genetic exchange of influenza between taxa (Fig. 1). For instance, within the H1 and N1 trees of the human-dominated clade, there are multiple SwIV sequences indicating human-to-swine H1N1 genetic exchange. In both gene trees, we observe a single monophyletic clade specific to the recent 2009 S-OIV H1N1 outbreak in humans arising from a SwIV clade. It is noteworthy that the SwIV clade that gave rise to the 2009 S-OIV outbreak has a different position in the H1 and N1 trees (II and B). In the H1 tree, the 2009

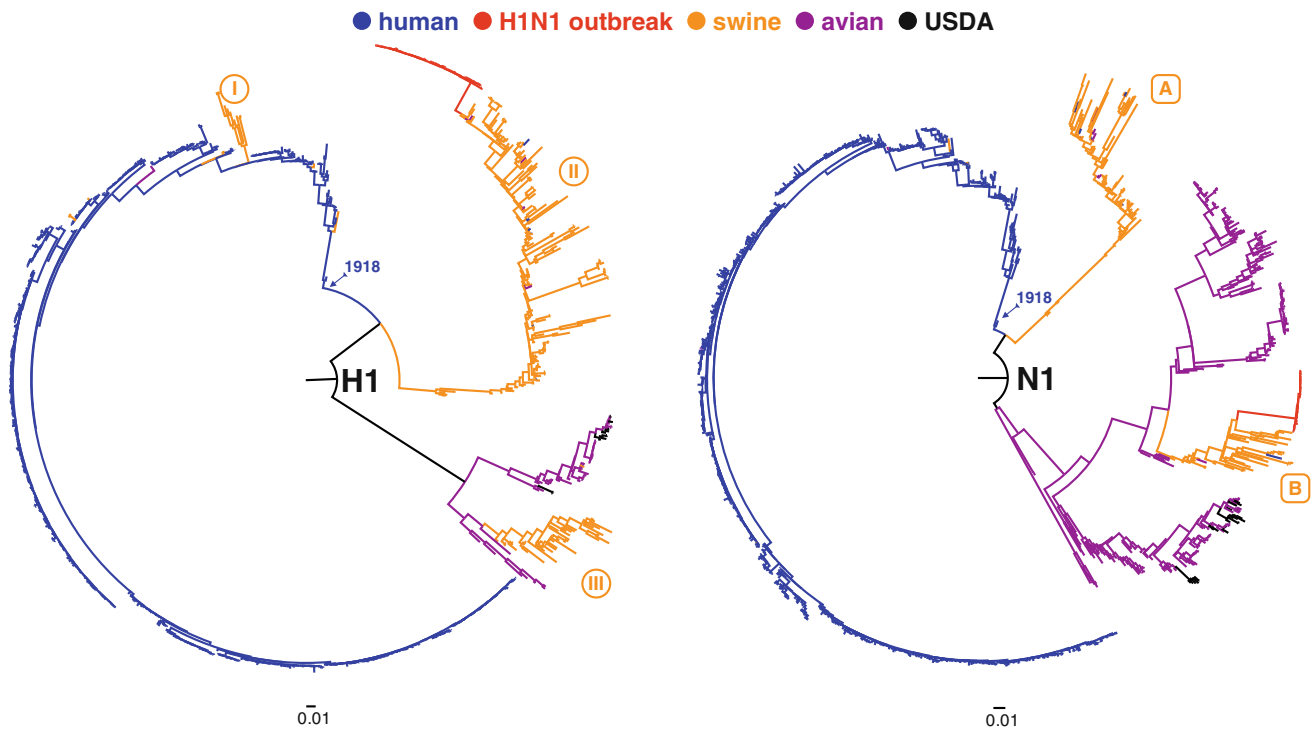

Figure 1. Maximum likelihood tree including H1 and N1 sequences from influenza A viruses from USDA wild bird surveillance samples and publicly available (NCBI database) swine, avian, and human influenza sequences. The sequences produced in this study are shown in black (USDA). Sequences from the 2009 H1N1 outbreak in humans are shown in red. The 1918 human H1N1 sequence is labeled on each tree. The scale bar denotes 0.01 substitutions per site. All major clades received 100% rapid bootstrap support; I, II, III, A, and B refer to clades discussed in the article.

Figure 2. Map of sampling localities of the USDA H1 and N1 viral sequences for wild birds in the United States.

S-OIV clade arises out of the SwIV clade II, which is closely related to the clade dominated by human sequences, while in the N1 tree, the 2009 S-OIV outbreak clade stems out of a mostly Eurasian avian-associated SwIV clade B (Fig. 1). The long branches leading to the human 2009 S-OIV outbreak in both trees are indicative of an accumulation of mutations over time and adaptation to its host, either directly or via a yet unknown host.

Here, we show that the 2009 S-OIV H1N1 human outbreak has a shared evolutionary history with swine

influenza but falls into different clades in the H1 and N1 trees, with evidence of distant, multiple, viral genetic exchanges between wild waterfowl and swine. The current human 2009 S-OIV H1N1 virus shares a common ancestor with H1N1 in swine and has little relationship to other human influenzas, including the H1N1 responsible for the 1918 influenza pandemic (Fig. 1).

We caution about conclusions made about the immediate relatives of the 2009 S-OIV H1N1 in humans in light of the apparent lack of Mexican, Central or South American H1 or N1 sequences from nonhuman taxa. Therefore, the conclusions of a recent report (Trifonov et al., 2009a, b) that the current H1N1 outbreak sequences are most closely related to SwIV from Europe/Asia (NA and M segments) and from North America (HA) may be sensitive to the addition of more sequence data. We find the 2009 S-OIV outbreak sequences to belong to different lineages in the H1 and N1 trees (clades II and B), which confirms reassortment as suggested by other authors (Garten et al., 2009; Nava et al., 2009; Smith et al., 2009; Trifonov et al., 2009a, b).

We conclude that the recent 2009 S-OIV H1N1 in humans is not a direct descendant of influenza A viruses from wild birds. However, in one of the gene segments (NA), the clade that contains swine N1 and the current human H1N1 outbreak is more closely related to N1 sequences from birds, including wild birds, than to other swine or human N1. This suggests that avian and swine N1 NA subtypes have a shared evolutionary history. However, no recently circulating influenza A virus in wild birds sampled in the U.S. is closely related to the current human H1N1 outbreak. Additional genetic information across potential host species, including wild birds and mammals, and broader geographic areas would help clarify these analyses and contribute to a deeper understanding of circulation of influenza viruses among hosts. Thus, understanding evolutionary relationships among influenza A viruses in wild and domestic birds, swine, and human hosts are critical for understanding the role of wild birds in the transmission of influenza A, which was the ultimate source of at least one global influenza pandemic (Taubenberger et al., 2004). However, one critical unanswered question that remains is: ''How are influenza A viruses transmitted between wild birds and domestic swine?'' Such transmission dynamics are critical to understanding and, hence, mitigating the influenza A viruses causing disease in humans, especially ones that reach pandemic levels as was the case of the 2009 H1N1 virus.

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