H7N9 influenza A virus in turkeys in Minnesota

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Introductions of H7 influenza A virus (IAV) from wild birds into poultry have been documented worldwide, resulting in varying degrees of morbidity and mortality. H7 IAV infection in domestic poultry has served as a source of human infection and disease. We report the detection of H7N9 subtype IAVs in Minnesota (MN) turkey farms during 2009 and 2011. The full genome was sequenced from eight isolates as well as the haemagglutinin (HA) and neuraminidase (NA) gene segments of H7 and N9 virus subtypes for 108 isolates from North American wild birds between 1986 and 2012. Through maximum-likelihood and coalescent phylogenetic analyses, we identified the recent H7 and N9 IAV ancestors of the turkey-origin H7N9 IAVs, estimated the time and geographical origin of the ancestral viruses, and determined the relatedness between the 2009 and 2011 turkey-origin H7N9 IAVs. Analyses supported that the 2009 and 2011 viruses were distantly related genetically, suggesting that the two outbreaks arose from independent introduction events from wild birds. Our findings further supported that the 2011 MN turkey-origin H7N9 virus was closely related to H7N9 IAVs isolated in poultry in Nebraska during the same year. Although the precise origin of the wild-bird donor of the turkey-origin H7N9 IAVs could not be determined, our findings suggested that, for both the NA and HA gene segments, the MN turkey-origin H7N9 viruses were related to viruses circulating in wild birds between 2006 and 2011 in the Mississippi Flyway.

INTRODUCTION

A high diversity of low-pathogenic (LP) influenza A viruses (IAVs) are maintained in wild aquatic birds (Olsen et al., 2006). LP IAVs can be introduced into domestic poultry, where some strains may adapt and become established. IAVs of the H5 and H7 subtypes have the added potential of becoming highly pathogenic (HP), which can result in significant mortality and economic losses in domestic
poultry. Additionally, the recent zoonosis of H7N9 IAV in China has raised concerns about the maintenance of H7 LP IAVs in domestic birds and the risks for spill-over into mammalian hosts, including humans (Gao et al., 2013; Jonges et al., 2013; Lam et al., 2013; Lebarbenchon et al., 2013). In North America, there have been multiple detections of H7 IAVs in domestic poultry, including H7N2 LP IAVs that circulated in live-bird markets in the north-eastern US for ~13 years (Senne et al., 2003; Spackman et al., 2003; Lebarbenchon & Stallknecht, 2011). Currently, there are many gaps in our understanding of the transmission of IAVs between wild birds and domestic poultry. In investigations of spill-over of IAVs from wild birds to domestic poultry, important information such as the wild avian source, precursor viruses or gene segments, risk factors and/or mechanisms of spill-over are rarely identified. This information is critical for improving biosecurity measures and effectively preventing spill-over events. When combined with epidemiological data and serological-based surveillance, virus isolation, genetic sequencing and subsequent analyses can provide important insights into IAV introduction events, viral evolution and adaptation processes in poultry.

During the winter and spring of 2009, antibodies to H7N9 IAV were identified by the National Veterinary Services Laboratory (NVSL), Animal and Plant Health Inspection Service (Ames, IA, USA), in birds at 11 commercial poultry farms in four Midwestern US states: Illinois (IL), Kentucky (KY), Tennessee (TN) and Minnesota (MN). Three of the affected farms were meat-type chicken breeder facilities and the remaining eight raised meat-type turkeys. None of the facilities were free-range operations; the turkeys were confined to the houses and did not have access to the outdoors. Four of the seropositive farms reported no overt disease or losses, whilst the remaining seven reported mild clinical disease (respiratory signs or slightly elevated mortality) and/or reduced egg production. IAV isolates were obtained from seropositive farms in MN which were later characterized as a LP H7N9 IAV at the NVSL. The spatiotemporal patterns of affected poultry farms suggested a spread of the virus over the spring, with seropositive farms detected first in KY (1 April), followed by IL (21 April) and TN (2 May), and finally in two geographically distinct areas in MN (mid April to May and mid June). After the final detection in MN there were no additional reports of H7N9 IAV detection (antibodies or virus) in US commercial poultry through the remainder of 2009 and 2010. During the spring of 2011, however, antibodies to H7N9 IAV were identified in two turkey farms in MN and viruses were isolated from one of the seropositive flocks. Turkeys in these facilities were also raised under confinement with no access to the outdoors. The 2011 H7N9 IAV was later characterized as LP at NVSL (Killian, 2010; Pedersen, 2012).

In this study, we investigated the origin of the 2009 and 2011 MN turkey-origin H7N9 IAVs. Based on sequencing of the haemagglutinin (HA) and neuraminidase (NA) genes, we attempted to identify the most recent H7 and N9 IAV ancestors in wild birds. The recent evolutionary history of these virus subtypes in North America was assessed in order to estimate the time and geographical origin of the MN turkey-origin H7N9 IAVs as well as the relatedness between the 2009 and 2011 viruses.

**RESULTS**

The maximum-likelihood phylogenetic analysis revealed genetic structuring of the North American H7 IAV HA gene within two main lineages (Fig. S1, available in the online Supplementary Material). The first lineage included only viruses that circulated in live-bird markets in the north-eastern US between 1994 and 2006; the second lineage included viruses isolated from wild and domestic birds from the 1970s onward. The 2009 and 2011 MN H7N9 isolates clustered with H7 IAVs recently isolated in wild birds in the US and Canada, as well as with H7N3 viruses isolated from chickens and humans in Mexico.

The coalescent analysis performed for this recent H7 HA genetic lineage provided more detailed information on the possible origins of the 2009 and 2011 MN turkey-origin H7N9 IAVs (Fig. 1). The phylogenetic structure of the resulting tree revealed that MN poultry-origin viruses recovered within the same year were closely related; however, clades of turkey-origin viruses recovered in 2009 and 2011 were more closely related to sequences for HA genes of wild-bird-origin IAVs than to each another, suggesting independent introduction events. The time of the most recent common ancestor (TMRCA) of the 2009 MN turkey-origin H7N9 IAVs was estimated to be late 2008 [TMRCA; ± 95% highest posterior density (HPD): 2008.7; 2008.2–2008.9]. The TMRCA between these viruses and the closest wild-bird ancestral H7 IAVs was estimated to be late 2007 (2007.9; 2006.7–2008.6), although the identification of the donor of the HA of the 2009 MN turkey-origin H7N9 IAVs could not be determined. Viruses isolated from wild ducks sampled in MN and other Mississippi Flyway states were distributed throughout the phylogeny (Figs 1 and S1), but none of them was clearly evidenced as the source of the introduction of the

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**Fig. 1.** (a) Maximum clade credibility tree of the HA gene for North American H7 IAVs (82 sequences; red branches in b). Posterior probability values >0.8 are reported. Blue bars indicate the 95% HPD for TMRCA. Branches for MN turkey-origin H7N9 IAVs are coloured red. GenBank accession numbers are reported for sequences generated in this study. (b) Maximum-likelihood consensus phylogram including 481 H7 HA nucleotide sequences from viruses isolated in North America. The root of the recent H7 genetic lineage is highlighted with a red star. The detailed phylogenetic tree is available in Fig. S1.
H7N9 IAVs into the MN turkey farms. For the 2011 MN turkey-origin H7N9 IAVs, the TMRCA of the two turkey-origin isolates was estimated to be late 2010 (2010.9; 2010.7–2011.0), with support for close genetic relationship to the H7N9 IAVs isolated in Nebraska (NE) in poultry in 2011. The TMRCA between the MN and the NE poultry isolates was estimated to be 2010 (2010.4; 2010.0–2010.8). Similar to the 2009 MN turkey-origin H7N9 IAVs, the identification of the donor of the HA could not be determined for these viruses although they were closely related to H7N3, H7N7 and H7N9 viruses isolated from wild ducks in Missouri and Mississippi (Figs 1 and S1).

The maximum-likelihood phylogenetic analysis revealed structuring of North American N9 IAVs NA genes from wild and domestic bird isolates within four major genetic lineages (Fig. S2). As for HA, the 2009 and 2011 turkey-origin H7N9 IAV NA sequences clustered in separate clades. The coalescent analysis (Fig. 2) supported that the NA gene of the 2009 MN turkey-origin H7N9 IAVs shared a common origin that was dated to 2008 (2008.7; 2008.1–2009.0); the TMRCA of the 2011 MN turkey-origin H7N9 IAVs was dated to late 2010 (2010.9; 2010.6–2011.0). The donor of the NA of the 2009 MN turkey-origin H7N9 IAVs was not identified although most genetically related viruses were isolated from wild ducks in MN, Texas and Wisconsin. The TMRCA between these wild duck viruses and 2009 MN turkey-origin H7N9 IAVs was dated to 2006 (2006.2; 2005.4–2006.8). Similar to the HA, the NA of the 2011 MN turkey-origin H7N9 IAVs was closely related to those from the NE poultry isolates, with a TMRCA dated to 2010 (2010.2; 2009.5–2010.7).

Partial nucleotide sequence comparison between viruses revealed >99% similarity among all gene segments of the 2009 MN turkey-origin viruses, as well as between the two 2011 MN turkey-origin viruses (Fig. 3). This finding further supports an independent introduction of H7N9 IAVs in MN poultry farms rather than viral maintenance between 2009 and 2011, following a single introduction.

Similarly, analysis of the NA gene suggests that viruses from wild ducks sampled within the Mississippi Flyway during 2008 and 2009 may be closely related to the clade comprised turkey-origin H7N9 sequences; however, the most closely related clades included isolates from wild ducks sampled within the Mississippi Flyway. Similarly, analysis of the NA gene suggests that viruses (H2N9, H3N9 and H11N9) circulating in wild ducks in the Mississippi Flyway during 2008 and 2009 may be closely related to the NA donor strain of the 2009 MN turkey-origin H7N9 IAVs. For both the HA and NA gene segments, TMRCA dates suggest that the wild-bird ancestral IAVs could have been isolated in wild ducks in MN during 2007 and 2008 branched at the root of the clad comprised turkey-origin H7N9 sequences; however, the most closely related clades included isolates from wild ducks sampled within the Mississippi Flyway. This possibility is supported by the reported isolation of both H7N3 and H11N9 viruses during 2007 and 2008 from wild ducks in MN (Wilcox et al., 2011).

Human cases of H7N9 and the associated high case fatality have raised concerns of human infection with influenza viruses and specifically those of the H7N9 subtype. Binding specificity of the HA for sialic acids on the host cell is discussed.
considered a major determinant of infection and transmission, with avian influenza HAs preferentially binding 2,3-α-linked sialic acids and human influenza HAs preferentially binding 2,6-α-linked sialic acids on host cells (Imai & Kawaoka, 2012). The zoonotic H7N9 viruses have been extensively studied since emerging, and receptor binding and X-ray crystallographic studies suggest aa 186 and 226 (H3 numbering) are determinants of 2,3-α-linked sialic acids on host cells (Imai & Kawaoka, 2012). The zoonotic H7N9 viruses have been extensively studied since emerging, and receptor binding and X-ray crystallographic studies suggest aa 186 and 226 (H3 numbering) are determinants of 2,3-α-linked and 2,6-α-linked sialic acids.
with the human H7N9 isolates having Val186 and Leu/Ile226, whilst avian H7N9 HAs have Gly186 and Gln226 (Dortmans et al., 2013; Xiong et al., 2013). Both the 2009 and 2011 MN turkey-origin viruses have Gly186 and Gln226 residues, suggesting these viruses are 2,3-α-sialic acid-restricted.

The position of the 2011 MN turkey-origin H7N9 IAV in phylogenetic trees for the HA and NA genes, as well as the analysis of the nucleotide similarity, provide support that the 2011 outbreak arose from an independent wild-bird introduction. The 2011 MN turkey-origin viruses were closely related to H7N9 IAVs isolated from a guinea fowl and a goose in NE during the same year, suggesting that the outbreak may have spread between poultry farms of neighbouring states. These viruses were closely related to IAVs circulating in waterfowl in the Mississippi Flyway in 2010 and 2011; however, the precise wild-bird origin donor of the MN and NE poultry-origin H7N9 IAVs could not be determined.

As with most introductions of IAVs into poultry, the mechanisms of spill-over were not identified in either the 2009 or 2011 outbreaks. All of the affected farms raised their turkeys in confinement without access to the outdoors; however, small wild birds (i.e. songbirds) could enter into the facilities. No obvious sources of introduction or spread were identified during routine disease investigations.

Active surveillance and evolutionary analyses are needed to understand the source of IAVs that circulate in poultry (Lam et al., 2013). Such a combined approach can not only provide information on the epidemiology and genetics of circulating viruses, it can also provide critical insights into viral evolution and adaptation processes in domestic birds. These analyses, however, are dependent on availability of sequence data and, consequently, are not possible unless IAV is isolated from infected flocks and reported in a timely manner. For example, in this study, no IAVs were isolated from H7N9-seropositive poultry flocks in TN, KY or IL. Consequently, whilst the 2009 H7N9-seropositive detections in poultry were spatially, temporally and antigenically linked, it cannot be ruled out that multiple unrelated H7N9 IAV introductions occurred. Furthermore, although active surveillance for IAV in wild birds has been conducted with increased intensity during the past decade, including areas within the Mississippi Flyway, most sampling efforts have been focused in autumn when viral prevalence has previously been reported to be highest; however, this sampling bias may have contributed to incomplete reference data for the genetic diversity of H7 subtype viruses circulating in wild birds in North America prior to the 2009 and 2011 outbreaks in domestic turkeys. Recent evidence suggests that viruses of the H7 subtype may be relatively more abundant at locations within the Mississippi Flyway during spring (Ramey et al., 2014), a season during which active surveillance for IAV in wild birds has been limited. Thus, increased surveillance efforts during this season may be useful for assessing spill-over of IAVs of the H7 HA subtype between wild birds and poultry.

The emergence of the zoonotic H7N9 avian influenza subtype in eastern China has demonstrated that the H7 subtype IAVs remain relevant to public and domestic animal health. For the outbreak we report in MN turkey farms as well as the emergence of the HP H7N3 in poultry in Mexico, the identification of the wild-bird species responsible for the introduction of the North American H7 viruses is complicated because of incomplete information on local circulation of LP IAVs in wild birds. Surveillance efforts for detection of LP H7 IAVs in wild birds, in particular in the vicinity of poultry farms, needs to be developed to identify spatial and temporal patterns of viral emergence in order to limit virus introduction into poultry production systems and prevent transmission to human populations.

**METHODS**

**Virus sequencing.** Sequencing of six 2009 and two 2011 turkey-origin H7N9 IAVs was performed at NVSL. Fourteen IAVs of
subtypes H7 or N9 isolated in MN wild ducks in 2007 and 2008 were sequenced at the University of Minnesota (St Paul, MN, USA). In addition, the HA and NA genes of H7 and N9 IAVs also were sequenced for 94 viruses isolated in wild birds in North America between 1986 and 2012. Sequencing was performed at the University of Georgia (Athens, GA, USA), Texas A&M University (College Station, TX, USA) and the J. Craig Venter Institute (Rockville, MD, USA). Details related to sequencing methodology are available upon request. GenBank accession numbers for the sequences of the HA and NA gene segments are indicated in Figs 1, 2, S1 and S2.

**Genetic analyses.** Full-length nucleotide sequences of the HA and NA gene segments of all available North American IAV H7 HA and N9 NA gene segments were downloaded from the Influenza Sequence Database (Bao et al., 2008). Sequences with unidentified hosts, duplicate sequences from the same strain and sequences previously identified as reflecting potential laboratory errors (Krasnitz et al., 2008; Lebarbenchon & Stallknecht, 2011) were excluded from the dataset. The final dataset included 481 and 346 sequences for the H7 HA and N9 NA segments, respectively. The coding region of nucleotide sequences was aligned with CLC 6.6.2 (CLC bio). A maximum-likelihood analysis was performed with PhyML 3.0 (Guindon et al., 2010), with the GTR + I + G evolutionary model. Nodal supports were assessed with 1000 bootstrap replicates.

Bayesian Markov Chain Monte Carlo coalescent analyses were performed for genetic lineages of H7 and N9 IAVs, including the 2009 and 2011 turkey-origin H7N9 IAVs (Figs S1 and S2). In total, 82 and 74 HA and NA sequences were included in the analyses, respectively. TMRCA dates as well as most probable location states were obtained with BEAST 1.7.4 (Drummond & Rambaut, 2007; Lemey et al., 2009). The uncorrelated exponential molecular clock was selected following Bayes factors comparison with estimates obtained with the strict clock and uncorrelated log-normal clocks. The SRD06 nucleotide substitution model (Shapiro et al., 2006) and a Bayesian skyline coalescent tree prior were used in all simulations (Drummond et al., 2005). Two independent analyses were performed with chain lengths of 120 million generations sampled every 1000 iterations; the first 10% trees were discarded as burn-in.

For the MN turkey viruses, similarity matrices were calculated using PHYLIP 3.68 (Felsenstein, 2005) based on the following sequence alignment sizes: PB2, 772 bp; PB1, 2157 bp; PA, 899 bp; HA, 1683 bp; NP, 1472 bp; NA, 1022 bp; M, 982 bp; NS, 838 bp. Shared nucleotide identity of >99% at all gene segments compared was inferred as evidence for epidemiological relatedness among viral strains (Reeves et al., 2011). Analyses were also performed to identify the most similar internal gene segments referenced in GenBank with BLASTN 2.2.29+ (Zhang et al., 2000). Finally, N-linked glycosylation sites in the HA protein sequences were located using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc). We also looked for deletion in the stem region of the NA protein sequences that may be involved in virus adaptation to terrestrial birds (Liu et al., 2013; Wu et al., 2013).

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