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Short communication

Complete genome sequence of a natural reassortant H9N2 avian influenza virus found in bean goose (*Anser fabalis*): Direct evidence for virus exchange between Korea and China via wild birds

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ABSTRACT

In 2011, we isolated a natural recombinant H9N2 avian influenza virus from fecal droppings of bean goose (*Anser fabalis*) in Korea. Phylogenetic analyses showed that the A/bean goose/Korea/220/2011(H9N2) isolate is a reassortant of Eurasian and North American lineages of avian influenza virus. In addition, the complete genome sequence, including all 8 gene segments, was associated with Chinese H9N2 viruses isolated from wild birds in the Hunan East Dongting Lake National Nature Reserve. These data provide direct evidence for the exchange of avian influenza viruses between Korea and China via wild birds.

1. Introduction

Avian influenza virus (AIV) belongs to the *Influenza A virus* genus of the Orthomyxoviridae family (Webster et al., 1992). The AIV genome consists of 8 single-stranded, negative-sense segments, including the polymerase basic (PB) 2, PB1, polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural (NS) genes. AIVs are classified into subtypes based on antigenic differences between their two surface glycoproteins, HA and NA. Sixteen HA subtypes (H1–H16) and nine NA subtypes (N1–N9) have been identified among influenza A viruses, and viruses of all subtypes and of the majority of all possible combinations have been isolated from wild birds. In addition to classification based on HA and NA subtypes, AIVs can also be genetically distinguished according to their geographical origin, North American or Eurasian (Olsen et al., 2006). The genetic features and/or severity of disease resulting from AIV infection in poultry determine whether the virus is classified as low-pathogenic avian influenza (LPAI) or highly pathogenic avian influenza (HPAI). HPAI has traditionally been either the H5 or H7 subtype (Alexander, 2007).

Wild waterfowl and shorebirds form the primary reservoir for AIV. Since wild waterfowl are potential long-distance AIV vectors, AIV can be spread through flyways during bird migration (Keawcharoen et al., 2008). A previous study suggested that global HPAI H5N1 outbreaks correspond with bird migration patterns at the flyway scale (Si et al., 2009). The first outbreak of HPAI H5N1 in wild birds was detected in May 2005 at Qinghai Lake, China. Since 2005, the wide spread of HPAI H5N1 has caused infection to wild waterfowl and domestic poultry in Central and South Asia, the Middle East, Europe, and Africa (Munster and Fouchier, 2009), prompting research on the role of wild birds in the geographical spread of AIV. Despite the extensive surveillance studies performed over the past decade in East Asia (Abao et al., 2013; Jahangir et al., 2008; Kang et al., 2010; Peng et al., 2013), in-depth understanding of the international movement of AIV with respect to the migration of waterfowl species remains limited.

In the present study, an H9N2 strain, A/bean goose/Korea/220/2011(H9N2) (designated H9N2/220), was isolated from fecal droppings of bean goose (*Anser fabalis*) in Korea in 2011. Subsequently, we sequenced the complete genome of this isolate to analyze its genetic features and trace the origin of this virus currently circulating in the Korean wild bird population. Phylogenetic analysis

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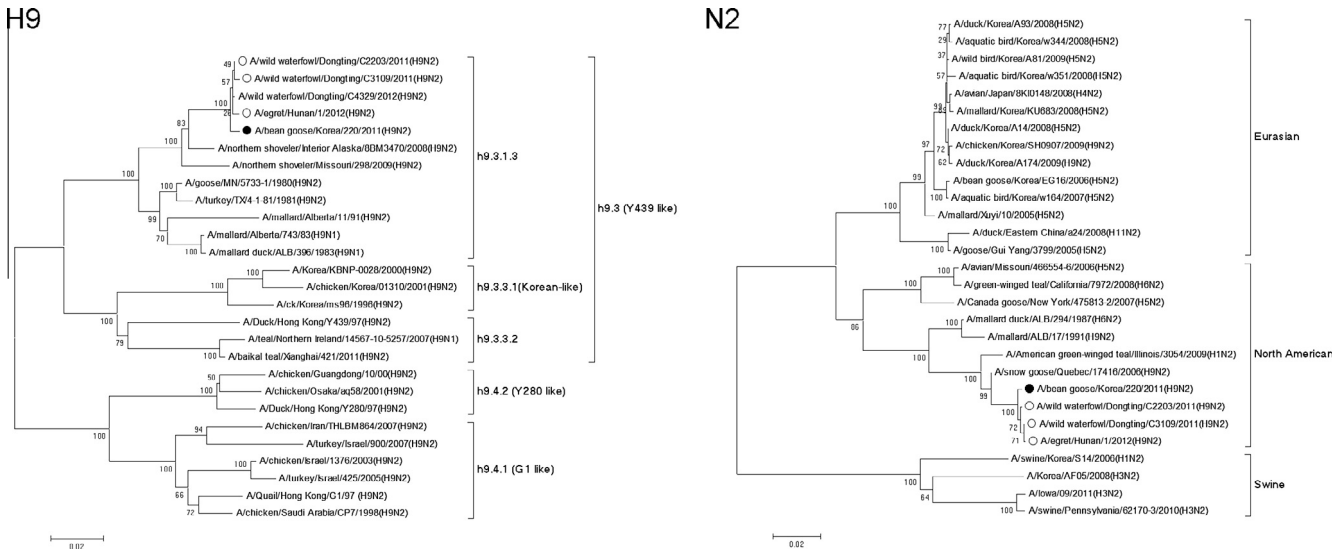

Fig. 1. Phylogenetic trees of the surface genes. Unrooted phylogenetic trees were generated by the distance-based neighbor-joining algorithm (nucleotide positions: HA, 31-1637; NA, 17-1397). The black circle (●) identifies the A/bean goose/Korea/220/2011(H9N2) virus and the open circle (○) identifies Chinese H9N2 isolates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

demonstrated that all 8 gene segments are associated with H9N2 viruses from wild birds in China.

2. Materials and methods

2.1. Virus

Fresh fecal samples (n = 229) were collected on 11 October 2011 from Cheon-su Bay (36°64′N, 126°42′E) in Korea. Fecal samples were dissolved in phosphate-based saline for AIV isolation. Virus isolation was performed in 10-day-old specific pathogen-free chicken embryonated eggs. After 72 h of incubation at 37 °C, the eggs were chilled, and allantoic fluids were harvested and tested for hemagglutinin activity. Of the 7 AIV isolates obtained from fecal samples, 6 H3 and 1 H9N2 LPAI viruses were identified. The H9N2 strain, named A/bean goose/Korea/220/2011(H9N2), was used in this study.

2.2. Nucleotide sequencing and molecular analysis

For molecular analysis, RNA was extracted using the RNeasy kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. RNA was tested for the presence of AIV by real-time reverse transcription-polymerase chain reaction (PCR) of the M gene (Spackman et al., 2003). The 8 genes of each virus were amplified using reverse-transcription PCR as previously described (Hoffmann et al., 2001). Purified products were ligated into a pGEM-T Vector (Promega; Madison, WI, USA). Nucleotide sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit, and the products were analyzed using the ABI PRISM 3730xl genetic analyzer (Applied Biosystems; Foster City, CA). A Basic Local Alignment Search Tool (BLAST) search was performed for all newly identified sequences against the AIV sequences published in GenBank. Phylogenetic analysis was performed using the MEGA 5 program, applying the neighbor-joining method with a maximum composite likelihood model (Tamura et al., 2011). Bootstrap analysis with 1000 replicates was performed to determine the statistical reliability of the phylogenetic trees generated. The genome sequences of H9N2/220 virus have been deposited in GenBank under accession numbers KJ013294 to KJ013301. For species identification of fecal samples, we analyzed the cytochrome oxidase I gene as previously described (Lee et al., 2010; Park et al., 2011).

3. Results and discussion

The genome lengths of each segment (PB2, PB1, PA, HA, NP, NA, M, and NS) were 2341, 2341, 2233, 1742, 1565, 1466, 1027, and 890 nucleotides, respectively. The eight genes encoded the following proteins, presented with their deduced amino acid lengths: PB2, 759; PB1, 757; PB1-F2, 90; PA, 716; PA-X, 252; HA, 560; NP, 498; NA, 469; M1, 252; M2, 97; NS1, 230; and NS2, 121.

Phylogenetic analysis revealed that the HA gene of H9N2/220 virus was classified into the Y439-like lineage. In particular, the HA gene clustered with the North American WI/1/66 or h9.3.1.3 sub-lineage, according to the H9 gene classification methods suggested by Dong et al. (2011) or Jiang et al. (2012), respectively (Fig. 1). The NA gene was also related to North American lineage isolates. Phylogenetic analysis of the internal genes revealed that PA and NS genes were related to North American lineage, but PB2, PB1, NP, and M genes were related to Eurasian lineages circulating in Asian migratory waterfowl (Fig. 2). Interestingly, as shown in Table 1, all 8 gene segments were associated with Chinese H9N2 viruses isolated from wild birds in the Hunan East Dongting Lake National Nature Reserve in 2011–2012 (Zhu et al., 2014).

The molecular characteristics of the H9N2/220 virus were identical to Chinese H9N2 viruses isolated from the Hunan East Dongting Lake National Nature Reserve in 2011–2012. The deduced amino acids at the cleavage site of the HA protein were PAASDR↓GLF, showing the characteristic of a low-pathogenic virus. The HA receptor-binding pocket included the avian-like motif, Q226 and G228 (H3 numbering). The stalk of NA does not have a 3-amino-acid deletion at positions 63–65. No substitutions associated with increased virulence and enhanced transmission in mammals were detected in the PB2 (Ala 199, Glu 158, Glu 627, Ala 661, Val 667, Asp 701, Lys 702), PB1-F2 (Asn 66), PA (Ser 409) or NS1 (Thr 92) proteins. Additionally, no amino acid mutations associated with amantadine and oseltamivir resistance were observed in the M2 ion channel protein and NA protein, respectively.

AIV in wild bird population forms transient "genome constellations," which are continually reshuffled by reassortment (Dugan