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Short communication

Novel reassortant highly pathogenic H5N6 avian influenza viruses in poultry in China

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ABSTRACT

We characterized two novel highly pathogenic H5N6 influenza viruses isolated from Chinese poultry in 2013. Genomic analysis showed that both isolates were reassortants, and derived their genes from H5 and H6 subtype viruses found in poultry in China. The virulence of the two isolates was examined in chickens and mice, and both isolates were found to be highly pathogenic in chickens and only moderately virulent for mice. Our results show that continued circulation of these viruses could endanger both avian species and humans.

Based on the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins, influenza A viruses are classified into 18 HA and 11 NA subtypes (Tong et al., 2013; Zhu et al., 2013). The highly pathogenic avian influenza (HPAI) strain H5N1 emerged in Asian countries in 1997, and has caused severe epidemics in poultry that have resulted in extensive damage to the poultry industry (Li et al., 2004, 2010). As of 24 January 2014, 650 cases of human infection with H5N1 viruses had been reported to the WHO, of those 386 (59.4%) were fatal (WHO, 2014a). On 6 May 2014, a novel H5N6 avian influenza virus (AIV) was reported in China, and linked to the death of a 49-year-old man, believed to be the world's first human infected with an H5N6 AIV (WHO, 2014b). The persistence of HPAI H5 viruses in the human population in many countries raises the possibility that this is an emerging human influenza pandemic virus. Because live poultry markets (LPMs) in China are a major site of dissemination for AIVs for potential influenza virus reassortment and interspecies transfers (Chen et al., 2013; Liu et al., 2003), LPMs should be actively surveyed for H5 AIVs as part of an early warning system for avian influenza outbreaks.

During active surveillance of LPMs for emerging poultry AIVs in Anji county, Huzhou city, Zhejiang Province, in eastern China, two H5N6 AIVs, A/chicken/Zhejiang/6C2/2013(H5N6) (6C2) and A/duck/Zhejiang/6D2/2013 (H5N6) (6D2), were isolated on 14 December 2013 from the same apparently healthy group of poultry from one LPM. To better understand the genetic relationship between AIVs from China and the isolated viruses, the gene segments of 6C2 and 6D2 were sequenced and compared with sequences available in GenBank. The virulence of the two isolates was examined in chickens and mice, and both isolates were found to be highly pathogenic in chickens and only moderately virulent for mice.

The virus was isolated from cloacal swabs and then inoculated into embryonated chicken eggs as described elsewhere (Hai-bo et al., 2012). All experiments with 6C2 and 6D2 were performed in a Biosafety Level 3 laboratory. RNA was extracted using the Viral RNA mini kit (Qiagen), according to the manufacturer's instructions. All segments were amplified with primers (Table S1), fragment sequencing and sequence analysis have been described

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elsewhere (Tamura et al., 2011). The sequence data obtained were submitted to GenBank under the accession Nos. KJ807773–KJ807788.

All eight segments, HA, NA, polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), nucleoprotein (NP), matrix protein (M), and nonstructural protein (NS), in 6C2 and 6D2 shared 99.9–100% sequence similarity (Fig. 1, Fig. S1 and Table S2). We chose the A/duck/Zhejiang/6D2/2013 (H5N6) (6D2) as strain representative of the H5N6 isolates. The HA genes of the two HPAI H5N6 viruses were very closely related to the H5 viruses circulating in China, from 2010 to 2012 that belong to clade 2.3.4.6. The phylogenies of eight segments indicated that 6D2 and the 1997 H5N1 influenza viruses, which possessed similar internal genes as the H6 influenza viruses had different ancestors for these segments (Chin et al., 2002; Zhao et al., 2011). Sequence analyses suggested that both H5N6 viruses were most closely related to isolates found in Chinese poultry since 2010. Our current results increase the evidence that there is active evolution of clade 2.3.4 H5 viruses, which have been the prevalent lineage in China since 2005 recently caused an outbreak of a new H5N8 strain in South Korea in 2014 (Lee et al., 2014; Smith et al., 2006).

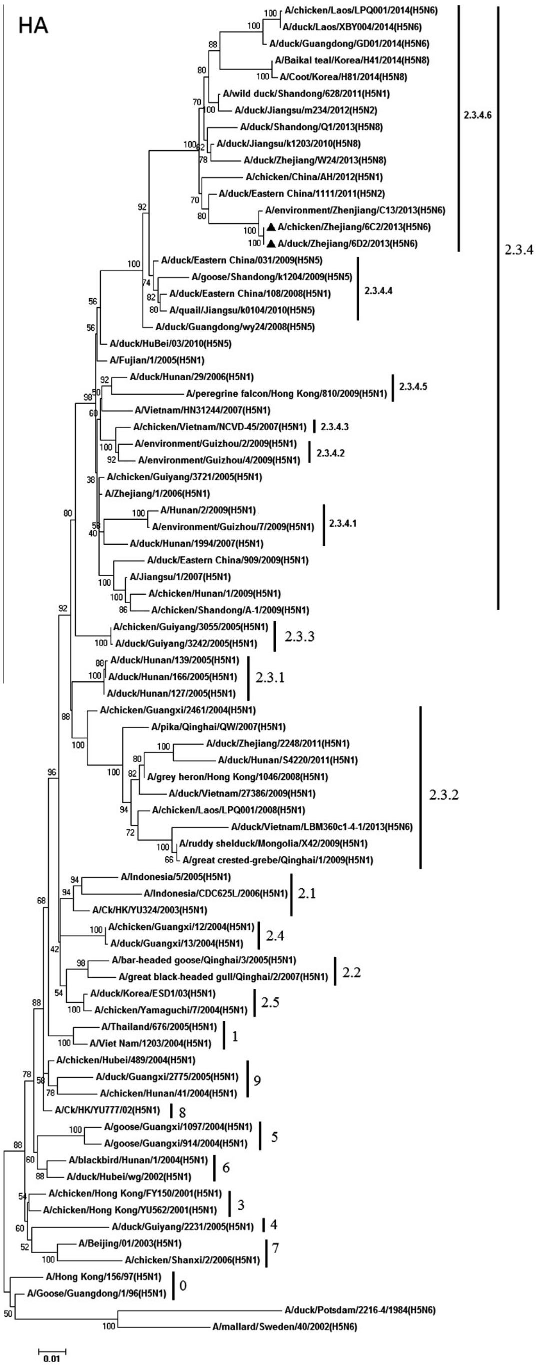

Fig. 1. Phylogenetic trees based on the HA (positions 1–1644) and NA (positions 40–1352) genes of the novel HPAI H5 viruses 6C2 and 6D2. The tree was created using maximum likelihood analysis (bootstrapped with 1000 replicates) with the Tamura-Nei model in the MEGA program (version 5.05). 6C2 and 6D2 are indicated by a triangle. The scale bar represents the distance unit between sequence pairs.

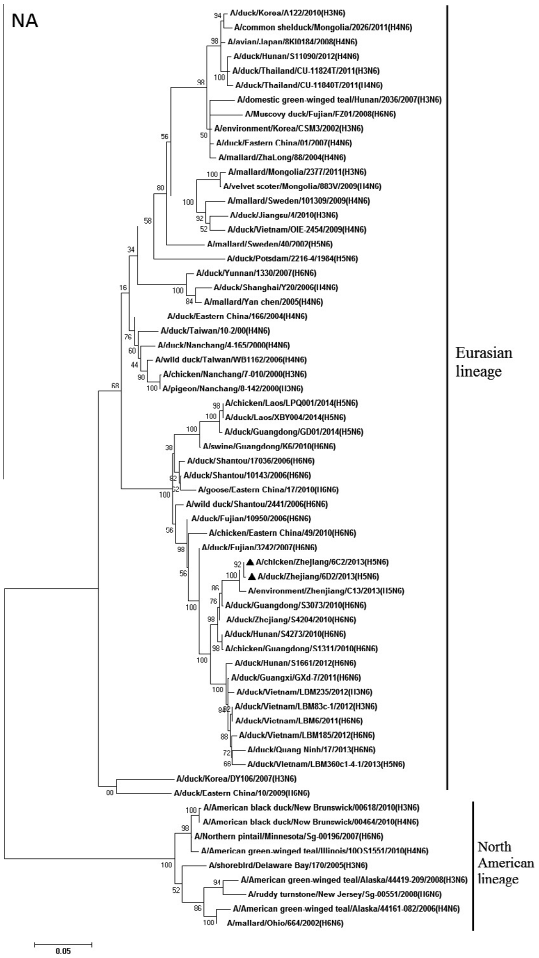

Fig. 1 (*continued*)

The PB2, PA, NP, M, and NS genes of 6D2 were most closely related to A/duck/Hunan/S4220/2011(H5N1). The PB1 and HA genes were most closely related to the H5 viruses, A/duck/Zhejiang/2248/2011(H5N1) and A/duck/Eastern China/1111/2011(H5N2), respectively. The NA gene of 6D2 was most closely related to A/duck/Guangdong/S3073/2010 (H6N6) (Fig. 2 and Table S3). H6 viruses are known to circulate widely in Chinese ducks, and to undergo frequent reassortment with multiple virus subtypes from the natural gene pool (Huang et al., 2012; Zhao et al., 2011). Our results suggest that 6D2 was a reassortant virus that derived its genes from H5 and H6 subtype viruses found in poultry in China.

The deduced amino acid sequence of the HA gene of 6C2 and 6D2 contains the HA cleavage site pattern, PLREKRRKR, indicating that these viruses were likely HPAI viruses. The amino acid sequences of 6C2 and 6D2 was NGQR GR at position 236–241 and GVSAA at positions 146–150. The receptor binding sites of