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Avian influenza viruses (AIVs) H9N2 are in the course of reassorting into novel AIVs*#

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In 2013, two episodes of influenza emerged in China and caused worldwide concern. A new H7N9 avian influenza virus (AIV) first appeared in China on February 19, 2013. By August 31, 2013, the virus had spread to ten provinces and two metropolitan cities. Of 134 patients with H7N9 influenza, 45 died (Chen et al., 2013; Li et al., 2014). From then on, epidemics emerged sporadically in China and resulted in several victims. On November 30, 2013, a 73-year-old woman presented with an influenza-like illness. She developed multiple organ failure and died 9 d after the onset of disease. A novel reassortant AIV, H10N8, was isolated from a tracheal aspirate specimen that was obtained from the patient 7 d after onset. This case was the first human case of influenza A subtype H10N8 (Chen et al., 2014). On 4 February, 2014, another death due to H10N8 avian influenza was reported in Jiangxi Province, China (Liu et al., 2015).

Since the time that human infection with AIV H7N9 was first reported by the Chinese Center for Disease Control and Prevention in March 2013, mainland China has experienced four epidemics of H7N9 influenza. Most infections in the first epidemic were identified during March to April 2013, whereas the majority of infections identified in the subsequent three epidemics occurred during November to April of 2013–2014, 2014–2015, and 2015–2016 (Tang et al., 2017). The previous four epidemics had resulted in 135, 320, 226, and 119 human infections, respectively, and most (83%) were reported in five eastern or southeastern coastal provinces. During the ongoing fifth epidemic (beginning October 1, 2016), the reported human infections represent a significant increase compared with the first four epidemics. As of May 16, 2017, a total of 686 laboratory-confirmed cases of human infection with AIV H7N9 have been reported to World Health Organization (WHO). The number of human infections and the geographical distribution of human cases in the fifth epidemic are greater than those in any earlier waves (Iuliano et al., 2017; WHO, 2017).

Previous studies had revealed that H7N9 and H10N8 were of avian-origin and had derived the six internal gene segments in common from AIVs H9N2 (Chen et al., 2013, 2014), and most of the homogenous sequences of the six internal gene segments were

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associated with Asian strains that were isolated during 2005–2013. In order to discuss and predict the risk of forming new subtype pathogens by reassortment of AIVs H9N2, we here comprehensively investigated their co-originating process and explored the donors' distribution. Detailed materials and methods are described in Data S1.

In this study, in total 5926 sequences of PB2, as well as 5526 of PB1, 5237 of PA, 5028 of NP, 5839 of MP, and 6027 of NS, were involved. After convergence, matrices used successively for further analyses contained 858 PB2, 906 PB1, 879 PA, 1026 NP, 1011 MP, and 930 NS sequences. AIVs containing one or more internal segments that were of high phylogenetic identity to H7N9 and H10N8 were distributed in China extensively, usually by the forms of AIVs H9N2 and mainly in eastern China. The municipality of Shanghai and provinces of Zhejiang, Jiangsu, Anhui, and Shandong were seriously affected by these AIVs H9N2.

The time to most recent common ancestor (tMRCA) analysis confirmed that the most recent common ancestor of the novel AIVs H10N8 and H7N9 emerged before March 2012, and many segments, such as PB2, might have been reassorted into H9N2 in early 2011 and subsequently into these two novel AIVs. Values of tMRCA (with their 95% higher posterior density (HPD) intervals) were 2011.31 (with 95% HPD interval being 2010.68 to 2011.87, similarly hereinafter) of PB2, 2011.81 (2011.39 to 2012.23) of PB1, 2011.59 (2011.06 to 2012.09) of PA, 2012.28 (2011.99 to 2012.54) of NP, 2012.09 (2011.62 to 2012.51) of MP, and 2011.74 (2011.16 to 2012.26) of NS (Fig. 1).

Although the most recent common ancestor of PB2 might have reached a status where they reassorted into the novel AIVs H10N8 and H7N9 in early 2011, the phylogenetic tree demonstrated that AIVs H9N2 possessing similar segments had emerged before 2009 in Shanghai (KC768062 duck; KC779062 swine), and by the end of 2013, such AIVs H9N2 were broadly distributed in Jiangsu, Zhejiang, Shandong, Hunan, Guangxi, and Hebei. Moreover, in 2013, this segment was sporadic reassorted into an AIV H5N2 in Jiangsu (KF150631 chicken). AIVs H9N2 circulating in eastern China had donated six internal segments for two novel subtypes H10N8 and H7N9. Their internal segments might have reached a status where they reassorted into the novel AIVs during 2011–2012. Having accomplished two spillovers from poultry to humans by means of H10N8 and H7N9 in such a short period, AIVs H9N2 demonstrated a high frequency and efficiency of genetic reassortment.

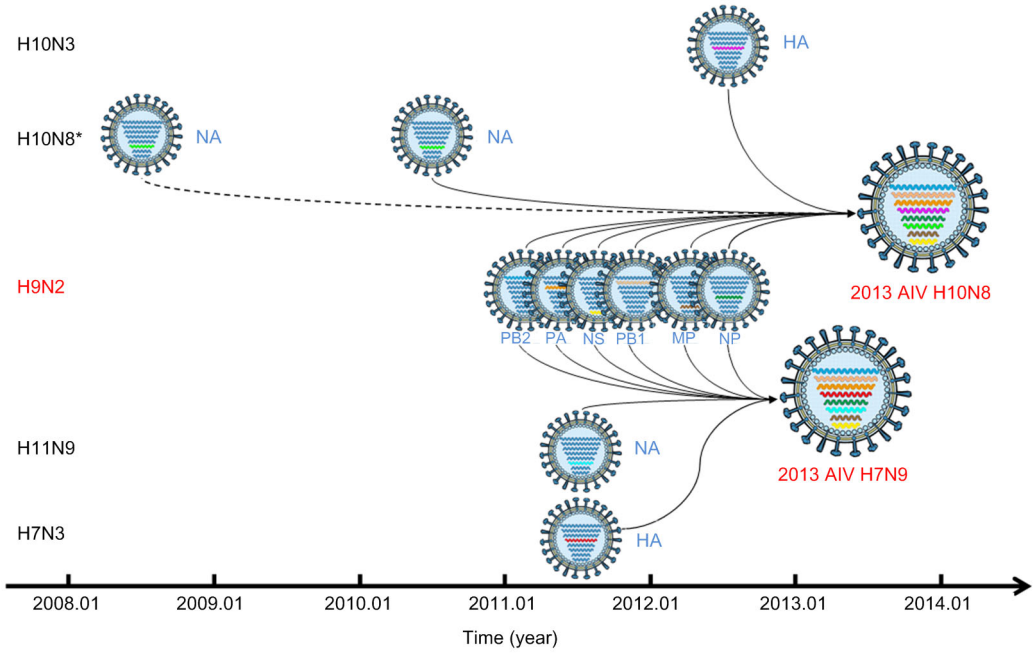

Fig. 1 Proceeding of co-originating of novel AIVs H7N9 and H10N8 in China, 2013

The novel AIVs H7N9 and H10N8 derived their six internal gene segments from H9N2. * References (Chen et al., 2013, 2014; Li et al., 2014) were consulted for the HA and NA gene segments. According to these references, the NA segments of H10N8 may have been derived from two H10N8 viruses that were isolated during different years

As the donor of the six internal gene segments for H10N8 and H7N9, AIVs H9N2 had circulated in eastern China for many years. They commonly act as donors and provide gene segments to form novel AIV isolates (Gu et al., 2010; Dong et al., 2011). Reassortment can only occur among viruses which replicate within the same cells. The prerequisite for reassortment is an individual host that is simultaneously infected with multiple divergent viral strains, which form a quasispecies pool that consists of closely related viruses (Padidam et al., 1999). The pathogenic characteristic of AIVs H9N2 endows it with abundant capabilities to serve as the genomic segment contributor. H9N2 is typically a low pathogenic avian influenza virus, and it can infect a number of types of poultry without phanerous sickness. In eastern China, the perennial rate of H9N2 antibody positivity fluctuates between 5.3% and 12.8%, and the rate of virus isolation had reached 9% in poultry; however, no epidemic with mass poultry deaths has been observed (Cheng et al., 2002). A high prevalence rate of AIVs H9N2 in poultry is always associated with a high risk of co-infections with other influenza viruses, and this increases the risk of virus reassortment.

In China, the people and the government suffer greatly from the descendants of AIVs H9N2, such as H7N9, H5N1, and H5N6. Since 2013, highly pathogenic AIVs H5N6 have emerged in poultry in Asia, especially Southeast Asia. These viruses have also caused sporadic infections in humans within the same geographic areas (Kang et al., 2017). Isolates of AIVs H5N6 established from both human and poultry were very heterogeneous; during 2014–2016, at least 34 distinct variants had been noted. Notably, genotype G1.2 virus, with internal genes from the chicken H9N2/H7N9/H10N8 gene pool, was responsible for at least five human H5N6 infections (Bi et al., 2016; Zhang et al., 2016; Chen et al., 2017). H9N2 subtype AIVs are extensively distributed worldwide, generally divided into two major lineages, a North-American lineage and a Eurasian lineage. The Eurasian lineage further blooms into various virus clusters (Gu et al., 2017), and four stem evolutionary clades of h9.1–h9.4 have been distinguished further (Jiang et al., 2012). The six internal genes may have been derived from at least two separate H9N2 lineages, which have been circulating within Chinese poultry populations for several years. Interestingly, the donor AIVs H9N2 for these outbreaks exhibits relatively diversified features according to previous studies (Cui et al., 2014; Liu et al., 2015; Bi et al., 2016; Jiao et al., 2016; Du et al., 2017). According to this study, AIVs H7N9/H10N8/H5N6 might have derived their PB2 segments from the subclade of h9.4.1, MP segments from h9.4.2.5, and all of the segments of PB1, PA, NP, and NEP from the subclade of h9.4.2.1 (Fig. 2). This might imply that there were frequent reassortment events within the subtype H9N2 itself. Such a high frequency of gene reassortment within AIVs H9N2 or between them and other subtypes of influenza viruses suggests that novel AIV reassortants from H9N2 viruses may appear and prevail occasionally in eastern China. PB2 is considered as a pivotal protein that determines the tropism of influenza viruses (Chen et al., 2013). AIVs H9N2 containing PB2 segments similar to those of H10N8/H7N9/H5N6 have been notified broadly in Jiangsu, Zhejiang, Shandong, Hunan, Guangxi, and Hebei Provinces. It could exacerbate the risk of forming novel flu viruses with the ability to cross the species barrier through reassortment and resulting in bird-to-human transmission.

H9N2 served not only as the donor of reassortment for assembling into novel AIVs, but also as a pathogen directly causing human spillover infection (WHO, 2017). Given that the virus continues to be detected in poultry populations, more human cases might be predicted. Briefly, AIVs H9N2 are typically mild virulence isolates, which often resulted in simultaneous infections with multiple divergent viral strains in poultry. Due to their constantly reassorting characteristics, high prevalence rates in poultry may inevitably lead to a high frequency of reassortment, which successively increases the risks of human epidemic caused by novel reassortants. In view of the fact of its long-lasting maintenance in poultries and wild birds, AIVs H9N2 should be supervised closely, especially the ones circulating in eastern and southern China. Sentinel surveillance for detecting virological and epidemiological changes associated with animal AIVs should be the conventional duty of the Centers for Animal Disease Control and Prevention (animal CDC). This is similar to the regular surveillance of human flu in sentinel hospitals and network laboratories. Furthermore, regular sequencing for the whole genome, including six internal gene segments, rather than HA and NA segments only for subtyping, is also

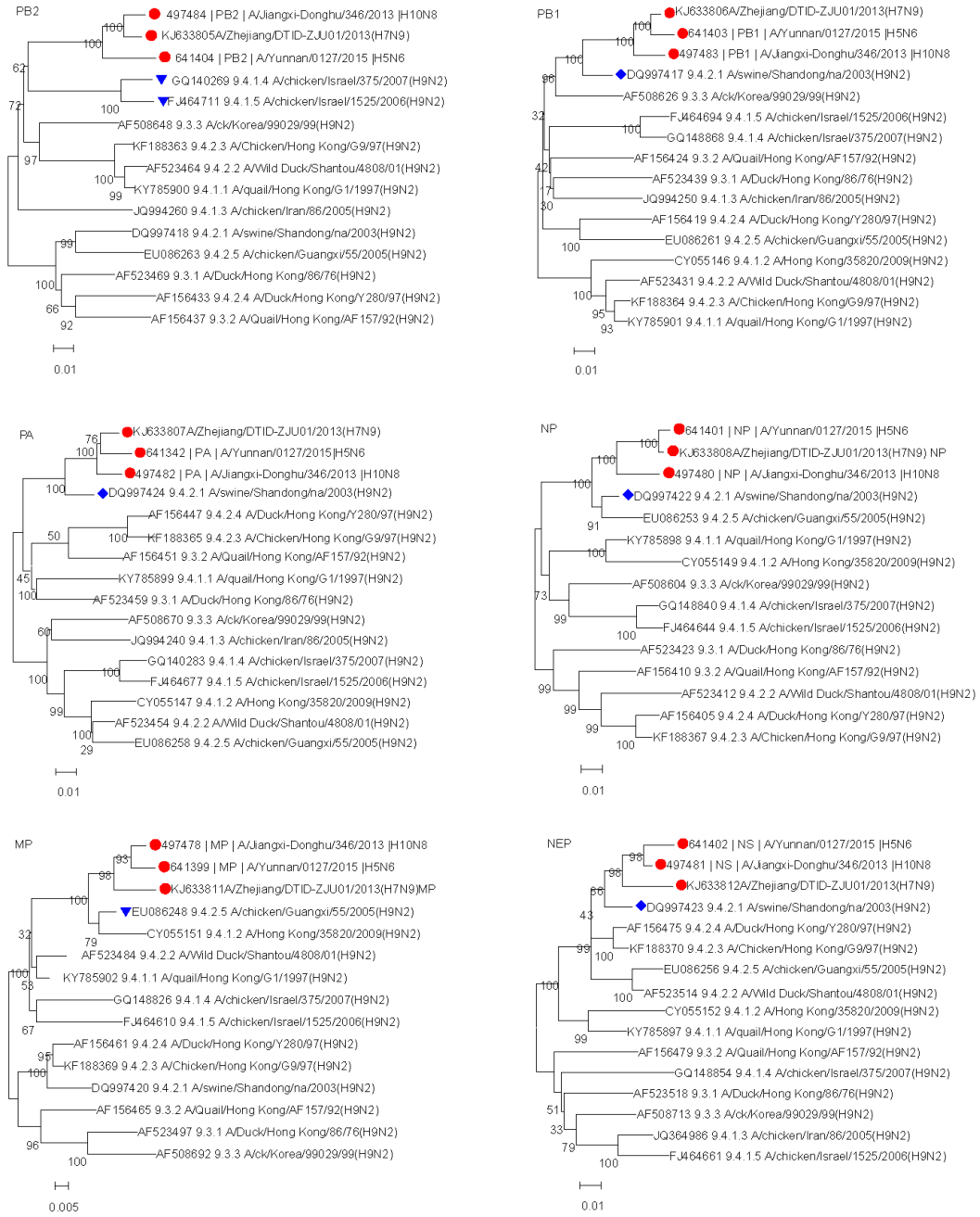

Fig. 2 Phylogenetic relationships of the internal gene segments amongst novel AIVs H7N9, H10N8, H5N6, and donor H9N2

It illustrated that AIVs H7N9/H10N8/H5N6 might have derived their PB2 segments from the subclade of h9.4.1, while MP segments from the subclade of h9.4.2.5, and all the segments of PB1, PA, NP, and NEP from the subclade of h9.4.2.1. 9.*x*.*x*.*x*.: Following the GenBank access No. of a sequence is a subclade of AIVs H9N2 according to references (Jiang et al., 2012; Gu et al., 2017). Sequences labelled by red circle are of AIVs H7N9/H10N8/H5N6, and sequences labelled by blue triangles or diamonds are of the representatives of AIVs H9N2 subclades

necessary; by this means, the dynamics of AIVs genetic evolution and variation will be known well and the risk of forming new subtype viruses will also be predicted well.

Compliance with ethics guidelines

Hui-ping CHANG, Li PENG, Liang CHEN, Lu-fang JIANG, Zhi-jie ZHANG, Cheng-long XIONG, Gen-ming ZHAO, Yue CHEN, and Qing-wu JIANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Extensive reading

Part of details about this research had been presented as a public preprint archive on bioRxiv, which was entitled Notorious Novel Avian Influenza Viruses H10N8 and H7N9 in China in 2013 Co-originated from H9N2. It can be extensively read at the website: http://www.biorxiv.org/content/early/2014/04/21/004390.article-metrics

List of electronic supplementary materials

Data S1 Materials and methods

中文概要

题　目：H9N2，行进在新亚型流感病毒的途中

目　的：分析 H9N2 禽流感病毒通过基因重配形成 H7N9 和 H10N8 人间禽流感病毒的进程，探讨作为供体的禽流感病毒 H9N2 在当前中国的主要分布及其内部 6 个基因节段的进化关系。

创新点：人间禽流感病毒 H7N9 和 H10N8 共起源于 H9N2 禽流感病毒早已成为共识，但共起源的时间节点、作为供体的禽流感病毒 H9N2 在当前中国的分布及其内部 6 个基因节段的进化关系鲜有论及。2014 年，H5N6 禽流感被多次报道造成人类感染。研究表明，H5N6 禽流感具有复杂的重配来源，H9N2 正是其一，加之 H7N9 第五波流行的严峻形势，亟需明确 H9N2 禽流感病毒通过基因重配形成新亚型的能力以及它在我国的当前主要分布。

方　法：从流感病毒公共数据库下载基因序列，评估查找适当的碱基替代模型，通过进化树查看与 H7N9、H10N8 及 H5N6 具有高度相似性的 H9N2 病毒的分布地区以及它们在内部 6 个基因节段上的进化关系，同时通过碱基替代速率的计算追溯最近共同祖先（tMRCA）及其分歧时间。

结　论：人间禽流感病毒 H7N9 与 H10N8 均在 2012 年之前形成，短期内通过碱基替代与基因重配形成了两种可感染人类的禽流感病毒，证实了 H9N2 通过重配形成新亚型的高效性。作为重配供体的 H9N2 至今仍广布于华东、华南及东南亚。其内部基因节段的重配复杂，发生在亚型内部的重配以及通过重配形成新的病毒亚型的风险都很高，需在禽畜中加强流感病毒的流行动态监测，特别是那些一向被忽视的编码内部蛋白的基因组节段。

关键词：禽流感病毒；H9N2；H10N8；H7N9；中国；共起源