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Title: High Genetic Diversity and Frequent Genetic Reassortment of Avian Influenza A(H9N2) Viruses along the East Asian-Australian Migratory Flyway

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ABSTRACT

To understand the molecular epidemiology and evolution of avian influenza viruses (AIV) along the East Asian-Australian migration flyway, we collected faecal samples (n=2859) between November 2014 and March 2015 from poultry, environmental sources and wild birds in Dongying, Shandong province and Yancheng, Jiangsu province in eastern China. The presence of AIV RNA was evaluated by real-time PCR and the positivity rate ranged from 0 to 29.3%. In both Dongying and Yancheng, samples collected from live poultry markets had the highest positivity rate for AIV RNA. AIV whole genomes were generated and phylogenetically analysed. Our results demonstrate that all of the viruses belonged to the H9N2 subtype, and could be classified into nine novel genotypes based on the phylogenetic analysis of the eight gene segments of the AIV genomes. This revealed a high genetic diversity of H9N2 in this region and suggested that they might have undergone frequent genetic reassortment. In addition, the internal genes (PB2, etc.) of two viruses from wild birds and several viruses from poultry belonged to the same gene constellation, suggesting a potential inter-host transmission of AIV between wild birds and poultry in live markets along routes of migratory flyways. Our results highlight the high genetic diversity of AIV along the East Asian-Australian migration flyway and the

need for more extensive AIV surveillance in eastern China.

Keywords: Avian influenza virus, H9N2, genetic diversity, reassortment, East Asian-Australian migration flyway

Influenza A virus reservoirs in animals have provided novel genetic elements leading to the emergence of global pandemics in humans. Most influenza A viruses circulate in waterfowl, but those that could infect mammalian hosts are thought to pose the greatest risk for zoonotic spread to humans and the generation of pandemic or panzootic viruses.

H9N2 subtype avian influenza viruses (AIVs) are distributed worldwide and in China, H9N2 is the most prevalent subtype currently circulating in the poultry population (Shen et al., 2015). Poultry with H9N2 monoinfections are predominantly asymptomatic or just suffer minor decreases in egg laying and respiratory distress. Sporadic H9N2 virus transmission from poultry to humans has also been detected (Gao et al., 2009). Previous studies have shown that human infections with the H7N9 or H10N8 viruses can have high morbidity and mortality and the internal genes of these two influenza subtypes are derived from the H9N2 AIVs (Cui et al., 2014; Chen et al., 2014; Shi et al., 2014; Qi et al., 2014). Therefore, although H9N2 is characterised as a low pathogenic

AIV (LPAIV), it can be directly transmitted to humans and in addition, poultry and wild birds carrying H9N2 could act as reservoirs for novel human avian influenza viruses (Zhang et al., 2009; Liu et al., 2014), which is a cause of significant concern due to the potential for the generation of new pandemic influenza strains with high transmissibility and high virulence.

To date, H9N2 subtype AIVs have been primarily detected from poultry, however, wild birds and waterfowl are the natural hosts of AIVs (Olsen et al., 2006). Since wild waterfowl are long-distance AIV vectors, AIV can be potentially disseminated along migratory routes during bird migration (Keawcharoen et al., 2008). There are eight major flyways of wild birds in the world, of which the East Asian-Australian route is the most important one, involving 21 provinces (autonomous regions and municipalities) in central and eastern China. It has been previously reported that Shandong and Jiangsu provinces in eastern China have had human cases of avian influenza, subtypes H5N1 (OIE et al., 2014) and H7N9 (Liu et al., 2013; Lam et al., 2013). As the two most important wintering sites and habitats of wildfowl along this migration route, Dongying (N36°55' to 38°10', E118°07' to 119°10') in Shandong Province and Yancheng (N32°34' to 34°28', E119°27' to 120°54') in Jiangsu Province were selected as the sampling sites for surveillance and genetic characterisation of AIVs.

In the present study, fresh fecal samples (n=2859) were collected in

November 2014 and March 2015 from poultry, environment and wild birds from Dongying and Yancheng (Fig. S1). Faecal samples were dissolved in phosphate-based saline (PBS) for viral isolation (Tang et al., 2014). Approximately 1 g of each sample was placed in a sterile 1.5 ml microcentrifuge tube and mixed with 1 ml PBS. Samples were subjected to centrifugation, and the clarified supernatant was collected and stored at -80°C .

Total RNA was extracted from allantoic fluids using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. cDNA was synthesised from RNA using the PrimeScriptTMRT Reagent Kit (TaKaRa, Dalian, China). The primer used for RT of the viral RNA genome was 5'-AGCAAAAGCAGG-3'. Real-time PCR reactions were performed with the following conditions on the ABI 7500 FAST platform: 95°C for 3 min, followed by 45 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 30 s. The primers were F-5'-AAGACCAATCCTGTCACCTCTGA-3' and R-5'-CAAAGCGTCTACGCTGCAGTCC-3'. Sterile DEPC-treated water was used as a negative control.

The presence of AIV RNA in faecal samples from farmed chickens and ducks, wild birds from sanctuaries and chickens from live poultry markets ranged from 0 to 29.3% and the positivity rate of live poultry markets was much higher than those of wild and farmed birds (Table 1). The

highest positivity rate was observed in live poultry markets in Dongying (29.3%) and Yancheng (22.6%), during sampling in March 2015 (Table 1). Overall, the detection of AIV RNA in wild birds was lower (0.3-5.0%). Of note, the AIV RNA positivity rates of live poultry markets in Dongying varied greatly between November 2014 (8.8%) and March 2015 (29.3%).

For influenza RNA positive samples with C_t values ≤ 24 , whole genome amplification was performed directly from the extracted RNA, however, in samples with $C_t > 24$, the supernatant was inoculated into the allantoic cavity of 9 or 10-day-old specific pathogen-free (SPF) chicken embryos (Jinan Sipafasi Poultry Co., Ltd.) for viral isolation. All of the embryos were harvested after 96 h and were blindly passaged for two generations using the allantoic fluid. The allantoic fluid of the second passage was harvested, and RNA was extracted and tested using the above real-time PCR methodology and whole genome sequencing was performed as described above.

Overall, complete genomes of 25 AIV strains were successfully sequenced (Table 2). GenBank accession numbers of the newly sequenced genomes have been provided as Table S1. Two of them were isolated from wild birds in the Yellow River Delta National Nature Reserve (Dongying) and were named A/wild bird/Dongying/DY084/2014 and A/wild bird/Dongying/DY049/2014, respectively. Three of them were isolated from environmental samples. The remaining 20 strains were

isolated from chickens from live poultry markets: three from Yancheng and 17 from Dongying. Reference sequences for the eight influenza gene segments were retrieved from the Influenza Virus Resource at GenBank using Blast and the new AIV sequences were employed as queries. The new AIV sequences and reference sequences were then combined, which produced eight datasets, corresponding to the eight gene segments of influenza A viruses which were then subsequently aligned using Muscle (Edgar, 2004). Phylogenetic trees were inferred using RAXML (Stamatakis, 2014) and one thousand bootstrap replicates were performed for each dataset.

Phylogenetic analyses showed that all of the 25 strains belonged to the H9N2 subtype (Fig. 1). In the HA tree, the novel 25 sequences fell within three distinct groups based on the tree topology and bootstrap values. The two viral HA gene segments from wild birds (A/wild bird/Dongying/DY084/2014 and A/wild bird/Dongying/DY049/2014) formed a distinct lineage (group 1) with chicken isolates from Jiangxi, Shanghai and Guangdong (Fig. 1A). Viral HA gene segments from chickens from Yancheng and Dongying from 2015 consisted of group 2. The majority (70%) of AIV HA gene segments segregating with group 3 were viruses isolated from Dongying from 2014. However, in the NA gene tree (Fig. 1B), the two viruses isolated from wild birds had high sequence homology with viruses (n=3) circulating contemporaneously in Dongying

(group 1). The remaining AIV NA gene segments (n=20) clustered together (group 2).

Following the same strategy, we classified the 25 viruses into different lineages for the internal genes (Table 2 and Figs. 2, S2-S7). For example, in the M gene tree (Fig. 2 and S2), the 25 viruses were classified into two distinct major groups and group 2 could be further classified into two sub-groups, 2.1 and 2.2 (Table 2 and Figs. 2 and S2). However, in contrast, the NS gene sequences from the viruses characterised in the present study were closely related and could not further classified into distinct groups.

Overall, the 25 viruses could be divided into nine distinct genotypes (Table 2), which have not been previously described. The 22 strains isolated from Dongying were distributed in each of the nine genotypes, suggesting frequent genetic reassortment events occurred in the local H9N2 subtype AIVs and an unexpectedly high genetic diversity in this region. The three sequences from Yancheng belonged to a single genotype, genotype 5, likely due to the lower sampling from Yancheng. Jiangsu Province, where Yancheng is located, has shown a high genetic diversity of H9N2 and has been regarded as one of the sources of human cases of H7N9 (Cui et al., 2014).

It is noteworthy that all of the eight gene segments of the three Yancheng strains were most similar to the counterparts of

A/Chicken/Dongying/150/2015 (Table 2), which also belonged to genotype 5. This revealed the widespread nature of genotype 5 and suggested that it might have gained a selective advantage of transmission potential. Also, although Yancheng and Dongying are located along the East Asian-Australian migration route and are natural habitats and stopovers for migratory birds, we consider that the spread of genotype 5 may be accomplished via poultry transportation based on the current evidence.

Interestingly, the two strains (genotype 1) isolated from wild birds possessed the same gene constellation with five viruses (genotype 3) isolated from chickens from Dongying, in 2014 and 2015 (Table 2). However, strikingly, they possessed different HA and NA gene segments. In addition, the internal genes of genotype 6 were also similar to those of genotype 1, with the single difference in the PB1 gene (Table 2). This evidence might suggest a potential inter-host transmission of AIV between wild birds and poultry, although the transmission direction could not be determined.

The cleavage site (amino acid residues 335-338) of the HA protein of the 25 strains was RSSR, indicating low pathogenic potential for land poultry (Guo et al., 2000). The receptor-binding pocket of HA1 retained the critical amino acid residues, Gly226 and Arg228, which preferentially bind to α 2,3 linked sialic acid residues. The AIV strains characterised in

the present study did not possess any amino acid deletions in the stalk of NA. All strains had an Arg at position 289 of NA, and were thus supposed to be sensitive to oseltamivir (Kiso et al., 2011). The PB2 gene segment of these viruses possessed Glu158, Glu627 and Asp701 (avian-signatures), and PA possessed Thr515, indicating low pathogenicity of this virus in mammals and poultry hosts (Hatta et al., 2001; Hulse-Post et al., 2007; Zhou et al., 2011). Furthermore, the Ser31Asn mutation, which is associated with adamantane resistance, was noted in the viral M2 protein (Chen et al., 2013).

In summary, the genetic diversity of avian influenza A (H9N2) viruses along the East Asian-Australian flyway was extremely high with evidence based on the diversifying viral genotypes. A number of viruses isolated from wild birds and poultry possessed the same internal genes, suggesting that there might be viral transmission between wild birds and poultry. Our findings are helpful for understanding the epidemiology and evolution of AIV along the East Asian-Australian migration route. Considering the fact that the novel human-infecting H7N9, H6N1 and H10N8 influenza viruses originated from this region, extensive surveillance should be performed to monitor AIV transmission via the wild birds-poultry and human interfaces in eastern China, where avian infection with H9N2 AIVs has become endemic.

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Figure legends

Fig. 1. Maximum likelihood phylogenetic trees of AIV H9 and N2 gene segments. The chicken isolates from Dongying are labeled in red, the

chicken isolates from Yancheng in green and the wild bird viruses are depicted in blue.

Fig. 2. Schematic phylogenetic trees of the internal genes of AIV H9N2 from Dongying and Yancheng.

Table 1

Sampling information and results of AIV RT-PCR testing							
Time	City	Location of sample collection	Source host	Sample type	No. of samples	No. of RT-PCR positive samples	RT-PCR positivity rate (%)
2014.11	Dongying	Live poultry markets	Chicken	Faeces/swabs	250	22	8.8
		Farms	Duck	Faeces	177	15	8.5
		Wild bird sanctuary	Wild bird	Faeces	601	30	5.0
2015.3	Dongying	Live poultry markets	Chicken	Faeces/swabs	659	193	29.3
		Wild bird sanctuary	Wild bird	Faeces	688	2	0.3
2015.3	Yancheng	Live poultry markets	Chicken	Faeces/swabs	340	77	22.6
		Wild bird sanctuary	Wild bird	Faeces	54	1	1.9
		Farms	Chicken	Faeces/swabs	90	0	0

Table 2

Phylogenetic classification of the avian influenza H9N2 viruses

NO.	AIV Strain Name	Collection date	Gene segment					
			HA	NA	M	NP	PA	PB1
1	A/wild bird/Dongying/084/2014	2014.11.27	1	1	2.1	1.2	2	1.3
2	A/wild bird/Dongying/049/2014	2014.11.28	1	1	2.1	1.2	2	1.3
3	A/chicken/Dongying/010/2014	2014.11.30	3	1	2.1	2	1.1	1.1
4	A/chicken/Dongying/015/2014	2014.11.30	3	1	2.1	2	1.1	1.1
5	A/chicken/Dongying/021/2014	2014.11.30	3	1	2.1	2	1.1	1.1
6	A/chicken/Dongying/201/2014	2014.11.29	3	2	2.1	1.2	2	1.3
7	A/chicken/Dongying/029/2014	2014.11.29	3	2	2.1	1.2	2	1.3
8	A/chicken/Dongying/006/2015	2015.3.12	3	2	2.1	1.2	2	1.3
9	A/chicken/Dongying/059/2015	2015.3.12	3	2	2.1	1.2	2	1.3
10	A/chicken/Dongying/079/2015	2015.3.12	3	2	2.1	1.2	2	1.3
11	A/chicken/Dongying/103/2015	2015.3.12	2	2	1	1.1	1.2	1.2
12	A/chicken/Dongying/164/2015	2015.3.13	2	2	1	1.1	1.2	1.2
13	A/chicken/Dongying/191/2015	2015.3.13	2	2	1	1.1	1.2	1.2
14	A/chicken/Dongying/258/2015	2015.3.13	2	2	1	1.1	1.2	1.2
15	A/environment/Dongying/002/2015	2015.3.12	2	2	1	1.1	1.2	1.2
16	A/environment/Dongying/014/2015	2015.3.12	2	2	1	1.1	1.2	1.2
17	A/environment/Dongying/016/2015	2015.3.12	2	2	1	1.1	1.2	1.2
18	A/chicken/Dongying/150/2015	2015.3.13	2	2	2.2	1.1	1.2	1.2
19	A/chicken/Yancheng/032/2015	2015.3.18	2	2	2.2	1.1	1.2	1.2
20	A/chicken/Yancheng/182/2015	2015.3.19	2	2	2.2	1.1	1.2	1.2
21	A/chicken/Yancheng/273/2015	2015.3.20	2	2	2.2	1.1	1.2	1.2
22	A/chicken/Dongying/012/2014	2014.11.29	3	2	2.1	1.2	2	2
23	A/chicken/Dongying/023/2014	2014.11.29	3	2	2.1	2	2	1.1
24	A/chicken/Dongying/037/2015	2015.3.12	2	2	1	1.2	1.2	1.2
25	A/chicken/Dongying/094/2015	2015.3.12	2	2	2.1	1.1	1.2	1.2

Figure 1

Figure 2

NS

M

NP

PA

PB1

PB2