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Genetics, Receptor Binding, and Virulence in Mice of H10N8 Influenza Viruses Isolated from Ducks and Chickens in Live Poultry Markets in China

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We analyzed eight H10N8 viruses isolated from ducks and chickens in live poultry markets from 2009 to 2013 in China. These viruses showed distinct genetic diversity and formed five genotypes: the four duck isolates formed four different genotypes, whereas the four chicken viruses belong to a single genotype. The viruses bound to both human- and avian-type receptors, and four of the viruses caused 12.7% to 22.5% body weight loss in mice.

Influenza viruses bearing the H10 subtype hemagglutinin (HA) have been detected in avian species across wide geographic areas. The first H10 isolate, an H10N7 virus, was detected in chickens in Germany in 1949 (1, 2). Since then, viruses bearing H10 HA and different neuraminidase (NA) subtypes have been widely detected in wild birds and domestic poultry around the world (3–19). Moreover, an H10N4 virus caused an outbreak of a respiratory disease in mink in Sweden in 1984 (20), and more recently, several U.S. turkey workers tested seropositive for H10 influenza virus (15). In March 2010, an H10N7 virus caused an outbreak on a chicken farm in Australia; after processing clinically normal birds from the farm, seven abattoir workers reported conjunctivitis and minor upper respiratory tract symptoms and H10 virus infection was detected in two of the seven workers (11). In 2013, H10N8 virus caused three human infections in China, two of which were fatal (21, 22). Although sequence information about the H10 viruses is increasing (23–26), the biologic properties of these viruses remain largely unknown.

A total of eight H10N8 influenza viruses were isolated from ducks and chickens during our routine surveillance from 2009 to 2013; of these eight viruses, three were isolated in Hunan province (A/duck/Hunan/S4280/2009 [DK/HuN/S4280/09], A/duck/Hunan/S3137/2009 [DK/HuN/S3137/09], and A/duck/Hunan/S1496/2011 [DK/HuN/S1496/11]) (Hunan viruses) and five were isolated in Jiangxi province (A/duck/Jiangxi/S3574/2013 [DK/JX/S3574/13], A/chicken/Jiangxi/S3581/2013 [CK/JX/S3581/13], A/chicken/Jiangxi/S3612/2013 [CK/JX/S3612/13], A/chicken/Jiangxi/S3735/2013 [CK/JX/S3735/13], and A/chicken/Jiangxi/S3755/2013 [CK/JX/S3755/13]) (Jiangxi viruses). To investigate the genetic relationships among these viruses, we sequenced the genomes of all eight viruses. The amino acid motif at the HA cleavage site of these isolates is -R-, which is a characteristic of viruses of low pathogenicity in chickens. The eight genes of the viruses showed distinct diversity, with the HA, NA, PB2, PB1, PA, NP, M, and NS genes of the eight viruses sharing 94.7 to 100, 78.4 to 99.9, 89.9 to 100, 90.1 to 100, 89.7 to 100, 90.0 to 99.9, 90.5 to 100, and 89.3 to 99.9% identity, respectively, at the nucleotide level. The HA, PA, and NS genes each formed two branches in their phylogenetic trees (Fig. 1A, E, and H), whereas the NA, PB2, PB1, and M genes formed three branches each in their phylogenetic trees (Fig. 1B, C, D, and G) and the NP gene formed four branches in its phylogenetic tree (Fig. 1F). Of note, the six internal genes of the H10N8 viruses in branch 1 were clustered with the corresponding genes of H9N2 influenza viruses (Fig. 1C to H), and the NA gene in group 1 belongs to the North American lineage (Fig. 1B).

On the basis of this genomic diversity, we divided the viruses into five genotypes (Table 1). Of note, the four duck viruses belong to four different genotypes, whereas the four chicken viruses belong to a single genotype, and the eight gene segments of the CK/JX/S3581/13 virus shared 99.2% to 99.9% identity with the human H10N8 virus A/Jiangxi-Donghu/346/2013.

A change in receptor-binding preference from α2,3-linked sialic acids (Sias) (avian-type receptors) to α2,6-linked Sias (human-type receptors) is important for an avian influenza virus to transmit among humans. Previous studies reported that over 30% of H6 influenza viruses bind to human-type receptors (27) and H7N9 viruses bind to both receptor types (28) but that the recent H9N2 viruses bind to only human-type receptors (29). In this study, we found that the HAs of the Jiangxi viruses share over 99.4% identity with each other but that the HAs of the three Hunan viruses are quite diverse. We therefore tested their receptor-binding specificity by using a solid-phase binding assay as described previously (29). All eight H10N8 viruses bound to both α2,3-linked Sias and α2,6-linked Sias, although their affinity for the α2,3-linked Sias was higher than that for the α2,6-linked Sias (Fig. 2A to H). The control H5N2 virus bound to only the α2,3-

Received 4 January 2015 **Accepted** 30 March 2015

Accepted manuscript posted online 8 April 2015

Citation Deng G, Shi J, Wang J, Kong H, Cui P, Zhang F, Tan D, Suzuki Y, Liu L, Jiang Y, Guan Y, Chen H. 2015. Genetics, receptor binding, and virulence in mice of H10N8 influenza viruses isolated from ducks and chickens in live poultry markets in China. J Virol 89:6506–6510. doi:10.1128/JVI.00017-15.

Editor: D. S. Lyles

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doi:10.1128/JVI.00017-15

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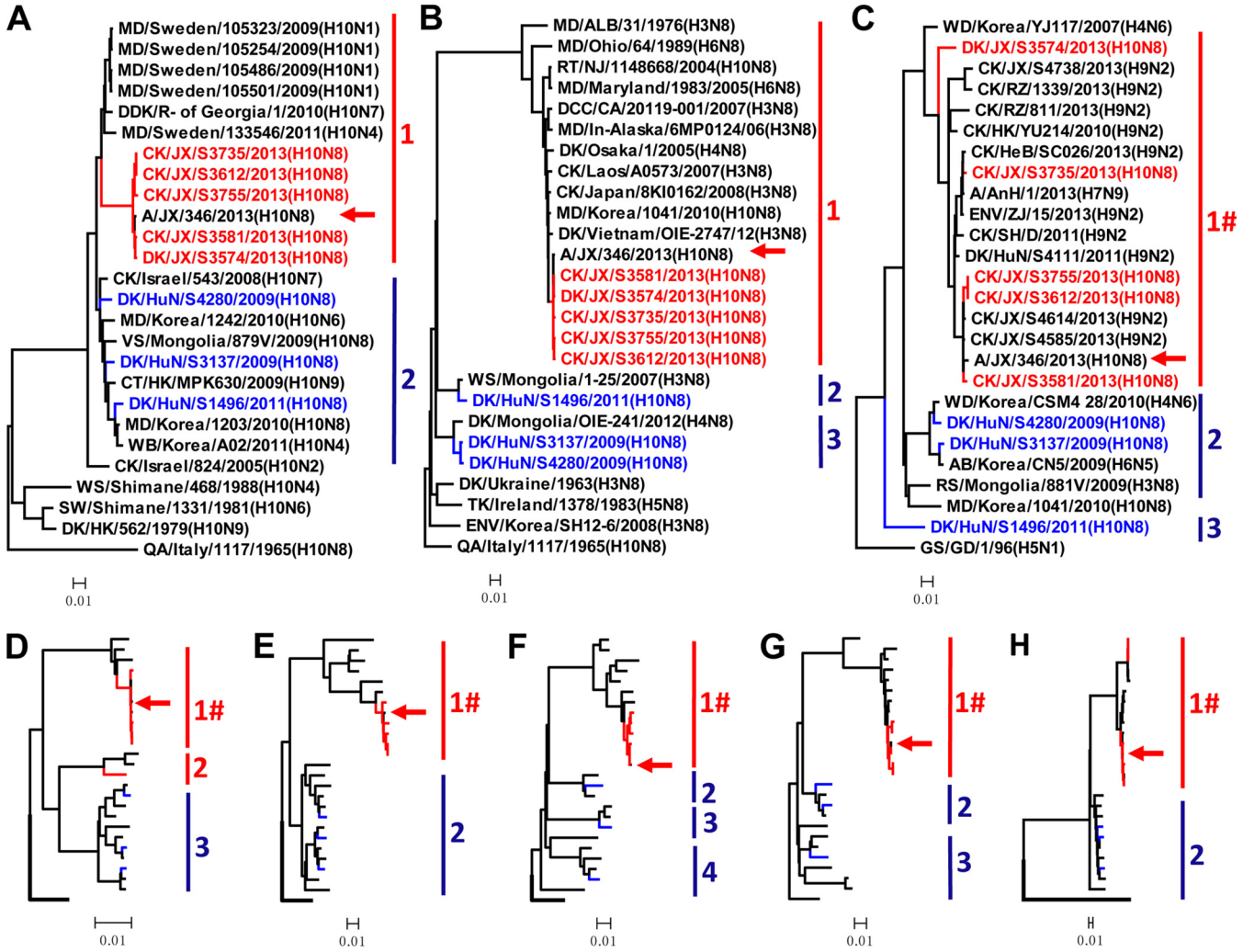

FIG 1 Phylogenetic analyses of H10N8 viruses isolated between 2009 and 2013 in China. The phylogenetic trees were generated with the PHYLIP program of the CLUSTALX software package (version 1.81). The trees were generated based on the following sequences: HA nucleotides 22 to 1704, NA nucleotides 19 to 1416, PB2 nucleotides 28 to 2307, PB1 nucleotides 25 to 2298, PA nucleotides 25 to 2175, NP nucleotides 46 to 1542, M nucleotides 26 to 1007, and NS nucleotides 27 to 864. The phylogenetic trees of HA (A) and NA (B) were rooted to A/Quail/Italy/1965(H10N8), and those of PB2 (C), PB1 (D), PA (E), NP (F), M (G), and NS (H) were rooted to A/Goose/Guangdong/1/1996(H5N1). The viruses with names in colors were characterized in this study (the viruses isolated in Jiangxi province are colored red, and the viruses isolated in Hunan province are colored blue). Sequences of viruses with names in black were downloaded from available databases. Abbreviations are as follows: AB, aquatic bird; CK, chicken; CT, common teal; DCC, double-crested cormorant; DDK, domestic duck; DK, duck; ENV, environment; GS, goose; MD, mallard; QA, quail; RS, ruddy shelduck; RT, ruddy turnstone; TK, turkey; VS, velvet scoter; WB, wild bird; WS, whistling swan; SW, swan; WD, wild duck; AnH, Anhui; ALB, Alberta; CA, California; JX, Jiangxi; HeB, Hebei; HuN, Hunan; HK, Hong Kong; In-Alaska, interior Alaska; NJ, New Jersey; R- of Georgia, Republic of Georgia; RZ, Ri Zhao; ZJ, Zhejiang; SH, Shanghai; GD, Guangdong. Groups labeled with a red "#" in the phylogenetic trees of the six internal genes contain viruses of only the H10N8, H7N9, and H9N2 subtypes, and the human H10N8 isolate is indicated with a red arrow; 96% sequence identity cutoffs were used to categorize each gene segment in the phylogenetic trees.

linked Sias (Fig. 2I), whereas the H9N2 virus and the human influenza virus A/Sichuan/1/2009 (SC/1/09) bound to only the α2,6-linked Sias (Fig. 2J and K).

There have been several reports of human infections caused by H10 viruses (11, 15, 21), but only the H10N8 virus has thus far caused fatal outcomes (21). To understand the virulence of the H10N8 viruses in mammals, we tested the replication and lethality of the eight viruses in mice. Groups of eight 6-week-old female BALB/c mice (Beijing Vital River Laboratories, Beijing, China) were anesthetized with CO_2 and inoculated intranasally (i.n.) with $10^{6.0}$ 50% egg infective doses (EID_{50}) of each test virus in a volume of 50 μl. Three mice were euthanized on day 3 postinoculation (p.i.), and the nasal turbinates, lungs, kidneys, spleens, and brains were collected for virus titration in eggs. The remaining five mice in each group were monitored daily for 14 days for weight loss and survival. (The protocol for this animal study was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences.)

Seven of the eight viruses replicated well in the lungs of mice, with mean titers ranging from 3.1 to 5.8 $\log_{10}$ EID_{50}; the eighth virus, DK/HuN/S1496/11, was detected from only two of three mice (Fig. 3A). The five Jiangxi viruses were detected in the turbinates of all of the inoculated mice, with mean titers ranging from

TABLE 1 Genotypes of H10N8 viruses

Virus	Group of each gene segment in phylogenetic tree (% similarity to human H10N8 virus)[a]								Genotype
	HA	NA	PB2	PB1	PA	NP	M	NS	
CK/JX/S3581/13	1 (99.8)	1 (99.9)	1 (99.4)	1 (99.8)	1 (99.5)	1 (99.5)	1 (99.2)	1 (99.8)	1
CK/JX/S3612/13	1 (99.4)	1 (99.8)	1 (99.3)	1 (99.9)	1 (99.6)	1 (99.9)	1 (99.6)	1 (96.3)	1
CK/JX/S3735/13	1 (99.4)	1 (99.6)	1 (97.8)	1 (99.9)	1 (99.5)	1 (99.5)	1 (100)	1 (99.8)	1
CK/JX/S3755/13	1 (99.6)	1 (99.7)	1 (99.3)	1 (99.9)	1 (99.6)	1 (99.9)	1 (99.5)	1 (96.2)	1
DK/JX/S3574/13	1 (99.9)	1 (99.8)	1 (96.8)	2 (91.3)	1 (99.5)	1 (99.3)	1 (99.3)	1 (99.8)	2
DK/HN/S1496/11	2 (94.8)	2 (78.5)	3 (89.9)	3 (90.1)	2 (90.2)	4 (91.2)	2 (91.3)	2 (90.9)	3
DK/HN/S3137/09	2 (95.6)	3 (78.7)	2 (91.5)	3 (90.4)	2 (90.1)	2 (91.1)	3 (90.8)	2 (90.7)	4
DK/HN/S4280/09	2 (95.6)	3 (78.5)	2 (91.8)	3 (90.4)	2 (90.2)	3 (90.2)	2 (91.1)	2 (90.7)	5

[a] Genome similarity was compared with the H10N8 human isolate A/Jiangxi-Donghu/346/2013. The data were generated based on the following sequences: HA nucleotides 22 to 1704, NA nucleotides 19 to 1416, PB2 nucleotides 28 to 2307, PB1 nucleotides 25 to 2298, PA nucleotides 25 to 2175, NP nucleotides 46 to 1542, M nucleotides 26 to 1007, and NS nucleotides 27 to 864.

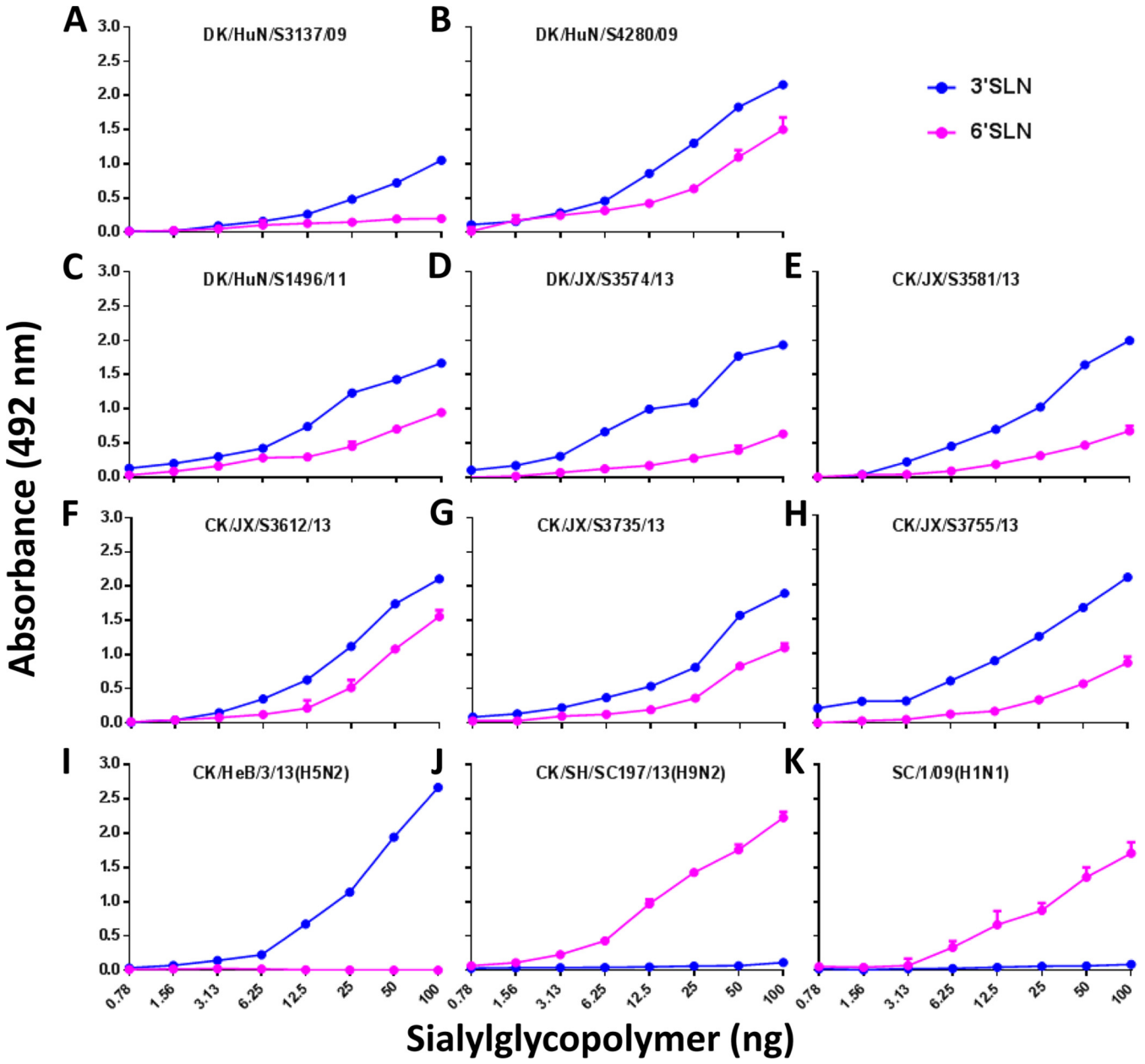

FIG 2 Characterization of the receptor-binding properties of H10N8 viruses. The binding of the viruses to two different biotinylated glycans (α2,3 glycan, blue; α2,6 glycan, pink) was tested. The data shown are the means of three repeats from one experiment; the error bars indicate standard deviations.

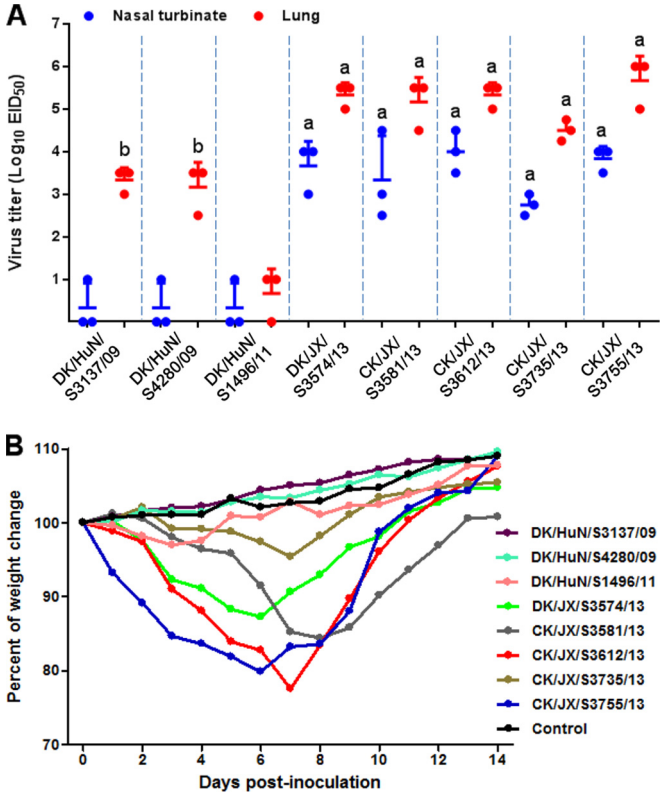

FIG 3 Replication and virulence of H10N8 viruses in mice. (A) Virus titers in organs of mice. The data shown are the means ± standard deviations for each group. Because virus was not detected from spleen, kidney, or brain of any mouse, data for these organs are not shown. a, $P < 0.01$ compared with the corresponding value for the three Hunan H10N8 virus-inoculated groups; b, $P < 0.01$ compared with the corresponding value for the DK/HuN/S1496/11-inoculated group. (B) Body weight changes of mice.

2.8 to 4.0 $\log_{10}$ EID_{50}, whereas virus was detected in the turbinate of only one of three mice inoculated with each of the three Hunan viruses (Fig. 3A). The titers in the Jiangxi virus-infected mice were significantly higher than that of the Hunan viruses (Fig. 3A). Virus was not detected in the spleen, kidneys, or brain of any mice. Mice infected with these viruses showed diverse body weight changes during the observation period: four viruses caused 12.7% to 22.5% body weight loss in mice, whereas the other four viruses did not cause apparent body weight loss (Fig. 3B). All of the mice survived during the observation period.

In summary, our genetic studies indicate that the four duck viruses belong to four different genotypes, suggesting that they were introduced into ducks independently; the four chicken viruses belong to one genotype and appear to be hybrids of a duck virus and the local H9N2 viruses (Table 1). The ability of H10N8 viruses to bind to human-type receptors facilitates their infection of humans, as occurred with the H7N9 viruses (28). The more efficient replication in mice of the viruses isolated in Jiangxi province than of the three duck viruses isolated in Hunan province suggests that the internal genes of the H9N2 viruses may have further increased the replicative ability and virulence of H10N8 viruses in mammals; of course, the surface proteins may have also contributed to the difference of the virulence. Although the viruses in our studies were all isolated from healthy birds, two H10 influenza viruses, A/turkey/England/384/79 and A/mandarin duck/Singapore/805/F-72/7/93, were reported to be highly pathogenic in chickens (3, 30). Therefore, it is important to continue monitoring the evolution of H10N8 influenza viruses and to evaluate their potential to cause disease in poultry and pandemics in humans.

Nucleotide sequence accession numbers. The nucleotide sequences of the eight viruses determined in this study have been deposited in GenBank under accession numbers KP861987 to KP862050.

ACKNOWLEDGMENTS

We thank S. Watson for editing the manuscript and Yamasa Corporation Co. Ltd. for synthesizing the sialylglycopolymer.

This work was supported by the National Science and Technology Major Project (2012ZX10004214).

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