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Highly Pathogenic Avian Influenza H5N6 Viruses Exhibit Enhanced Affinity for Human Type Sialic Acid Receptor and In-Contact Transmission in Model Ferrets

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ABSTRACT

Since May 2014, highly pathogenic avian influenza H5N6 virus has been reported to cause six severe human infections three of which were fatal. The biological properties of this subtype, in particular its relative pathogenicity and transmissibility in mammals, are not known. We characterized the virus receptor-binding affinity, pathogenicity, and transmissibility in mice and ferrets of four H5N6 isolates derived from waterfowl in China from 2013-2014. All four H5N6 viruses have acquired a binding affinity for human-like SAα2,6Gal-linked receptor to be able to attach to human tracheal epithelial and alveolar cells. The emergent H5N6 viruses, which share high sequence similarity with the human isolate A/Guangzhou/39715/2014 (H5N6), were fully infective and highly transmissible by direct contact in ferrets but showed less-severe pathogenicity than the parental H5N1 virus. The present results highlight the threat of emergent H5N6 viruses to poultry and human health and the need to closely track their continual adaptation in humans.

IMPORTANCE

Extended epizootics and panzootics of H5N1 viruses have led to the emergence of the novel 2.3.4.4 clade of H5 virus subtypes, including H5N2, H5N6, and H5N8 reassortants. Avian H5N6 viruses from this clade have caused three fatalities out of six severe human infections in China since the first case in 2014. However, the biological properties of this subtype, especially the pathogenicity and transmission in mammals, are not known. Here, we found that natural avian H5N6 viruses have acquired a high affinity for human-type virus receptor. Compared to the parental clade 2.3.4 H5N1 virus, emergent H5N6 isolates showed less severe pathogenicity in mice and ferrets but acquired efficient in-contact transmission in ferrets. These findings suggest that the threat of avian H5N6 viruses to humans should not be ignored.

On 7 May 2014, the Chinese National Health and Family Planning Commission (NHFPC) announced the first human case of avian H5N6 influenza virus infection (1). Subsequently, three more human infections with H5N6 virus cases were reported in the winter of 2014-2015 (2, 3). Between 30 December 2015 and 2 January 2016, the NHFPC notified the World Health Organization of two additional human cases of avian H5N6 virus infection. All six human infections were presented as acute respiratory distress syndrome (ARDS) of which three were fatal. Five cases had a common history of contact with or exposure to poultry or live-bird markets before disease onset (1–3), suggesting zoonotic transmission. Sequence analyses of the human H5N6 isolates indicated that the virus was derived from clade 2.3.4.4 avian H5N6 viruses that are circulating in poultry in China (1, 2, 4).

Avian H5N6 influenza virus was first isolated from mallards in North America in 1975 (5). In China, H5N6 virus first emerged in 2010 and has since been extensively circulating in both domestic and wild birds (6–9). Recent surveillance data from the Ministry of Agriculture of China indicate that H5N6 viruses have become enzootic in domestic poultry. Unlike the worldwide distribution H5N2 and H5N8 viruses (10–12), prevailing H5N6 viruses appear to be largely confined to China and Laos (13). We recently characterized the novel H5N6 viruses in poultry (14); however, their zoonotic capability and characteristics are poorly understood. In the present study, we examined the emergent H5N6 virus for its genetic characteristics, receptor binding properties, pathogenicity, and transmissibility in mice and ferrets.

MATERIALS AND METHODS

Ethical compliance. All animal work was approved by the Beijing Association for Science and Technology (approval SYXK [Beijing] 2007-0023) and conducted in accordance to the Beijing Laboratory Animal Welfare and Ethics guidelines, as issued by the Beijing Administration Committee of Laboratory Animals, and in accordance with the China Agricultural University Institutional Animal Care and Use Committee guidelines (SKLAB-B-2010-003).

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Viruses. H5N1 virus A/chicken/China/0603/2008 (CN0603) and pandemic H1N1/2009 influenza virus A/Beijing/7/2009 (BJ09) were previously isolated in our laboratory (14, 15). The four H5N6 viruses were isolated from ostensibly healthy ducks and geese in live poultry markets in China between 2013 and 2014. Virus stocks were grown in specific-pathogen-free chicken eggs. The virus isolates were A/goose/Eastern China/S0513/2013 (GS/EC/S0513/13), A/duck/Eastern China/S0711/2014 (DK/EC/S0711/14), A/duck/Eastern China/S0322/2014 (DK/EC/S0322/14), and A/duck/Eastern China/S0908/2014 (DK/EC/S0908/14). All experiments with H5 subtype viruses were performed in biosafety level 3 containment approved by the Ministry of Agriculture of the People's Republic of China.

Phylogenetic analysis and molecular characterization. Viral RNA was directly extracted from infected allantoic fluid by using an RNeasy minikit (Qiagen, Chatsworth, CA), and reverse transcription was performed using a Uni12 (5′-AGC AAA AGC AGG-3′) primer. PCR was conducted using specific virus primers as described by Hoffmann et al. (16). PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced (Beijing BGI-GBI Biotech Co., Ltd.). Sequence data were analyzed with DNAMAN5.2 (Lynnon Biosoft, USA). Phylogenetic analysis was performed by the distance-based neighbor-joining method using software MEGA 4.1 (DNAStar, Inc.).

Receptor-binding specificity assays. α-2,6 glycans (6′SLN: Neu5Aca2-6Galb1-4GlcNAcb-SpNH-LC-LC-biotin) and α-2,3 glycans (3′SLN: Neu5Aca2-3Galb1-4GlcNAcb-SpNH-LC-LC-biotin) were kindly provided by the Consortium for Functional Glycomics (Scripps Research Institute, Department of Molecular Biology, La Jolla, CA). Receptor-binding specificity was determined by a solid-phase direct binding assay as previously described (17). The receptor-binding specificities of the viruses were also determined in hemagglutinin (HA) assays using 0.5% resialylated chicken red blood cells (cRBCs). For the HA assay, sialic acid residues were enzymatically removed from cRBCs by incubation of the cells with 50 mU of *Vibrio cholerae* neuraminidase (VCNA; Roche, San Francisco, CA) at 37°C for 1 h, followed by resialylation using either with α2-6-*N*-sialyltransferase or α2-3-*N*-sialyltransferase (Sigma-Aldrich, St. Louis, MO) at 37°C for 4 h (18).

Virus binding to human airway tissues. Human respiratory tract sections were kindly provided by Jiang Gu, Shantou University Medical College. Paraffin-embedded 5-μm sections of normal human trachea and lung tissue were deparaffinized in xylene and rehydrated by alcohol. Sections were then blocked with 4% bovine serum albumin in phosphate-buffered saline (PBS), followed by virus incubation (at 64 HA units in PBS per section) at 4°C overnight. After four washes in ice-cold PBS, the sections were incubated with mouse monoclonal antibody specific for influenza nucleoprotein (NP; Abcam, Cambridge, United Kingdom) for 3 h at 4°C. Antibody binding was detected by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Abcam, Cambridge, United Kingdom) incubated for 2 h at room temperature. The samples were examined by confocal laser scanning microscopy (Leica TCS SP5 II; Leica Microsystems). As controls, tissue sections were treated with *Arthrobacter ureafaciens* sialidase (Sigma) in sodium acetate buffer (100 mM, pH 5.8) for 3 h at 37°C prior to virus incubation.

Mouse challenge studies. Groups of five 6-week-old female BALB/c mice (Vital River Laboratory) were anesthetized with Zoletil (tiletamine-zolazepam [Virbac], 20 μg/g) and intranasally (i.n.) inoculated with 50 μl of 10-fold virus serial dilutions from 10^6 to 10^1 50% egg infectious doses (EID_{50}). The mice in each group were monitored daily for 14 days for weight loss and mortality to determine the 50% mouse lethal dose (MLD_{50}). Mice that lost >20% of their body weight were euthanized. The MLD_{50} values were calculated according to the method of Reed and Muench. Virus virulence levels in mice were categorized as low ($MLD_{50} > 6.5 \log_{10} EID_{50}$), medium ($3 \log_{10} EID_{50} < MLD_{50} \leq 6.5 \log_{10} EID_{50}$), or high ($MLD_{50} \leq 3 \log_{10} EID_{50}$) pathogenicity.

To detect systemic virus spread, three mice infected with 10^6 EID_{50} of virus were euthanized on day 4 postinfection (p.i.). Tissue samples, including lung, brain, spleen, and kidney samples, were collected for virus titration.

Ferret challenge studies. All 6-month-old male Angora ferrets (Angora LTD), serologically tested by a hemagglutination inhibition (HI) assay to be negative for currently circulating influenza viruses (H1, H3, H5, H7, and H9), were >1.0 kg (range, 1.12 to 1.58 kg) in weight. Groups of two ferrets were each anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg) and i.n. inoculated with 10^6 EID_{50} of test virus in a 1-ml volume. The animals were subsequently euthanized on day 4 p.i., and nasal turbinate, tonsil, trachea, lung, and spleen samples were collected for virus titration. Lung tissues were also used for histology.

In the transmission experiment, groups of three animals were anesthetized and inoculated i.n. with 10^6 EID_{50} of virus. The next day, the three inoculated animals were individually paired by cohousing with a direct-contact ferret; a respiratory-droplet (RD)-contact animal was also housed in a wire frame cage adjacent to the infected ferret. The infected and RD-contact ferrets were 5 cm apart. To monitor virus shedding, nasal washes were collected from all animals every other day for 12 days and titrated for virus in eggs. Sera were collected from both inoculated and contacted animals at 14 days p.i. Seroconversion was analyzed by HI assay.

Statistical analyses. Statistically significant differences between experimental groups were determined by using analysis of variance with the GraphPad Prism software package (GraphPad Software, Inc., La Jolla, CA). A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. Nucleotide sequences of the gene segments of the four H5N6 viruses are available from GenBank under accession numbers KP732567 to KP732726.

RESULTS

Phylogenetic analyses of four novel avian H5N6 influenza viruses. From 2013 to 2014, four H5N6 viruses were isolated from apparently healthy ducks and geese in live bird markets in China. All eight segments of each virus were sequenced in conjunction with existing influenza A virus sequences deposited in GenBank, and phylogenetic trees were constructed which revealed that the hemagglutinin (HA) genes of all four avian H5N6 viruses are clustered in the 2.3.4.4 clade (Fig. 1A). The HA gene fragments were closely related to that of human-origin strains A/Guangzhou/39715/2014 (H5N6) and A/Changsha/1/2014 (H5N6), and A/Yunnan/0127/2015 (H5N6), since they shared 95.1 to 99.6% nucleotide homology. The four neuraminidase (NA) genes belong to the Eurasian lineage and are closely related to the H6N6 viruses isolated from waterfowl in China (19). The four H5N6 strains further form two phylogenetic groups: three viruses with 11-amino-acid deletions in the NA stalk (positions 59 to 69) and one full-length NA (Fig. 1B). Notably, the NA gene of the three human-origin H5N6 strains were with 11-amino-acid deletions in the NA stalk. All internal genes of the four H5N6 viruses were derived from highly pathogenic avian influenza (HPAI) H5N1-like viruses (see Fig. S1 in the supplemental material). Not surprisingly, the six internal segments of the four avian H5N6 viruses shared high nucleotide identity (89.5 to 99.8%) with those of human H5N6 virus A/Guangzhou/39715/2014 (4). These data suggest that the four avian H5N6 viruses share similar original ancestors with those of human-origin H5N6 viruses.

Although the four H5N6 viruses retained some molecular characteristics of clade 2.3.4 H5N1 viruses, they contained significant mutations in key amino acid sites, particularly in their HA genes (Table 1). Compared to clade 2.3.4 H5N1 viruses, the H5N6 viruses and most of other clade 2.3.4.4 viruses shared six amino acid changes within the receptor-binding site (RBS) of HA: two mutations in the 130-loop (S133L and S137A), three in the 190-

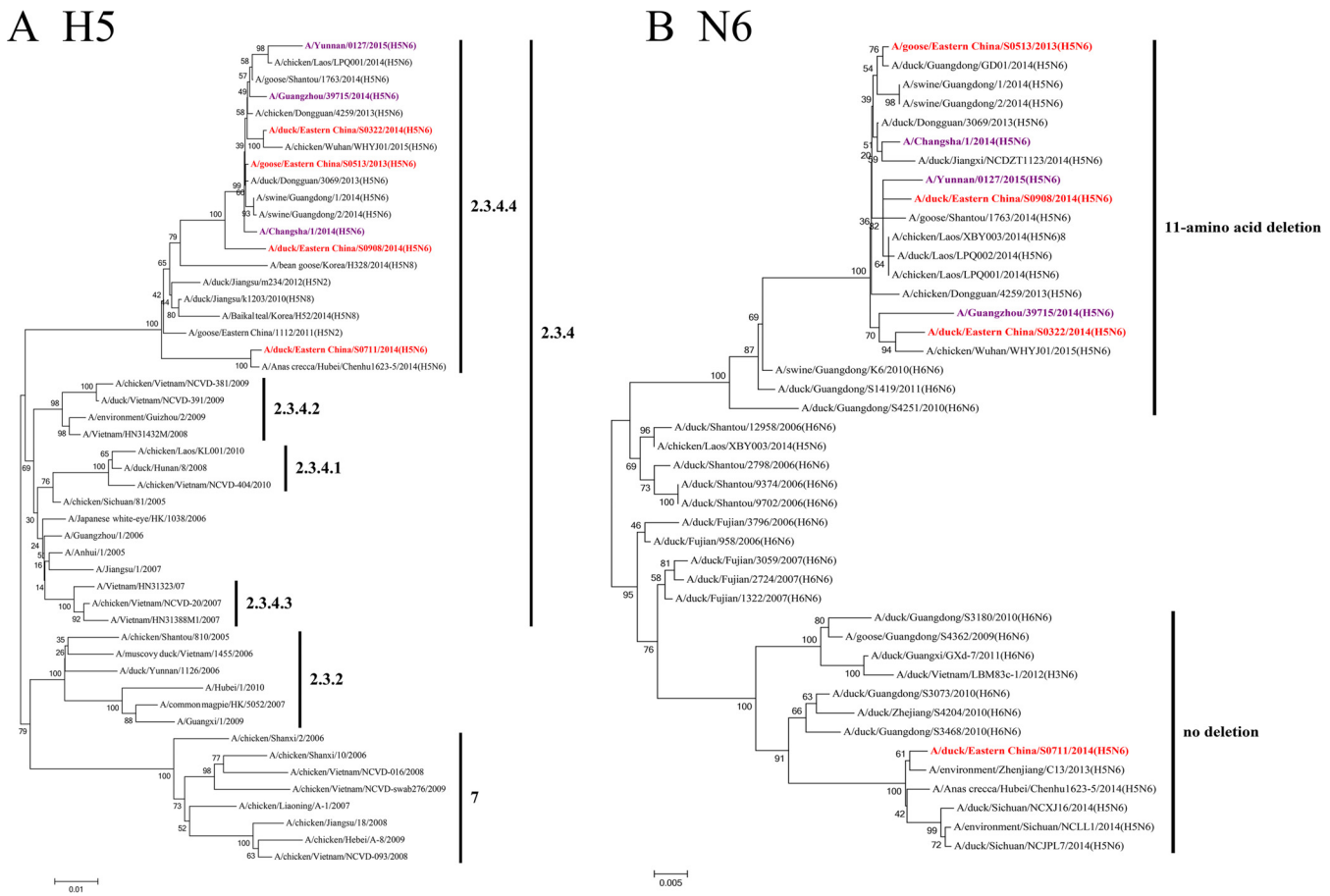

FIG 1 Phylogenetic relationships of HA and NA genes of avian H5N6 viruses. The phylogenetic trees of HA gene (A) and NA gene (B) were determined using the distance-based neighbor-joining method in software MEGA 4.1. The reliability of the tree was assessed by bootstrap analysis of 1,000 replicates. Horizontal distances are proportional to genetic distance. The H5N6 viruses isolated in the present study are indicated in red. Human H5N6 viruses are indicated in purple.

helix (D187N, K193N, and Q196K), and one in the 220-loop (S227R). Remarkably, HA genes of the clade 2.3.4.4 viruses, including the four H5N6 isolates, lacked an N-linked glycosylation site at position 158, whose absence is considered to promote receptor-binding affinity in guinea pig transmission (20). Comutation of the RBS and loss of the glycosylation site at residue 158 suggest that these H5 variants may confer receptor preference changes. Mutations related to pathogenicity in mammals (21), such as E627K and D701N in PB2, were not found in any of the four avian isolates. However, E627K of PB2 was found in human H5N6 isolates A/Guangzhou/39715/2014 and A/Yunnan/0127/2015 (4). Molecular markers of oseltamivir and amantadine resistance were not present in the NA and Matrix-2 protein sequences of the avian H5N6 viruses (Table 1).

Avian H5N6 viruses exhibited comparable binding affinity for avian and human sialic acid receptors. The binding specificity

TABLE 1 Analysis of molecular features associated with viral pathogenicity, transmissibility, and antiviral resistance[a]

		HA							NA		PB2			NS1	
				RBS					Deletion				M2	Deletion	
Virus	Clade	Cleavage site	158–160	187	193	196	133–137	225–228	(59–69)	275[b]	627	701	31[c]	(80–84)	92
H5N1	2.3.4	RERRRKR ↓ G	NNT	D	K	Q	SGVSS	GQSG							
DK/EC/S0711/14 (H5N6)	2.3.4.4	REKRRKR ↓ G	NDA[b]	N	N	K	LGVSA	GQRG	No	H	E	D	S	Yes	E
DK/EC/S0322/14 (H5N6)	2.3.4.4	REKRRKR ↓ G	NDA	N	N	K	LGVSA	GQRG	Yes	H	E	D	S	Yes	E
GS/EC/S0513/13 (H5N6)	2.3.4.4	REKRRKR ↓ G	NDA	N	N	K	LGVSA	GQRG	Yes	H	E	D	S	Yes	E
DK/EC/S0908/14 (H5N6)	2.3.4.4	REKRRKR ↓ G	NDA	N	N	K	LGVSA	GQRG	Yes	H	E	D	S	Yes	E
GD/39715/14 (H5N6)	2.3.4.4	REKRRKR ↓ G	NDA	N	N	K	LGVSA	GQRG	Yes	H	K	D	S	Yes	E

[a] H3 numbering was used. Amino acid changes from the clade 2.3.4 H5N1 viruses are indicated by underscoring. Abbreviations: HA, hemagglutinin; NA, neuraminidase; PB, polymerase basic; M, matrix; NS, nonstructural; RBS, receptor-binding site; DK, duck; EC, Eastern China; GS, goose.
[b] Molecular markers of oseltamivir resistance.
[c] Molecular markers of amantadine resistance.

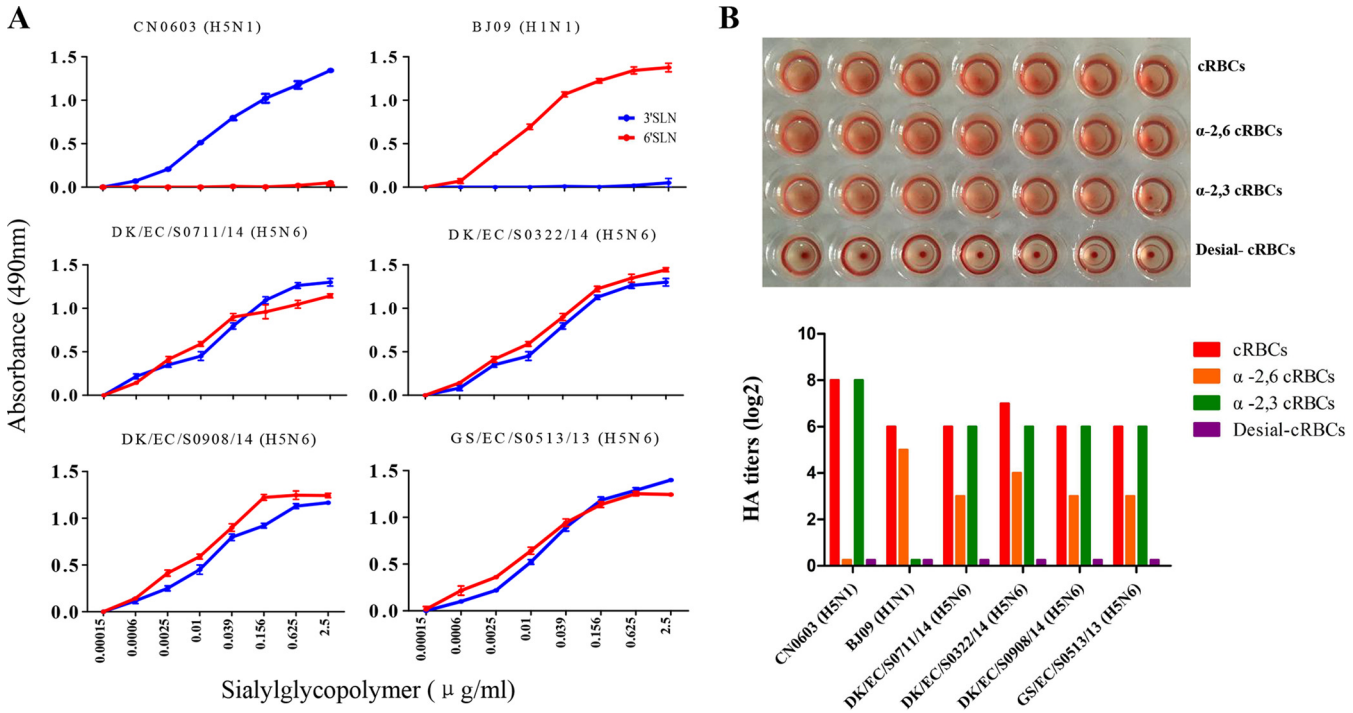

FIG 2 Avian H5N6 viruses exhibited comparable binding affinity for avian and human type of sialic acid receptors. (A) Binding affinity of inactivated viruses to α2,3-linked (blue) and α2,6-linked (red) sialic acid polymers. Each data point is the means ± the standard deviation (SD) of triplicate experiments. (B) HA assays were performed using resialylated cRBCs. The HA titers of each test virus with 0.5% cRBCs were determined as follows: cRBCs (untreated control), α-2,3 cRBCs (VCNA treated and resialylated with α-2,3 glycans), α-2,6 cRBCs (VCNA treated and resialylated with α-2,6 glycans), and desialylated (Desial) cRBCs (treated with VCNA).

of HA receptor is a critical determinant for cross-species transmission of influenza A viruses (22, 23). We determined the affinity specificity of the four H5N6 viruses by direct binding assays with SAα2,3Gal and SAα2,6Gal sialylglycopolymers. A 2009 pandemic H1N1 virus (BJ09) and the 2.3.4 clade avian H5N1 virus (CN0603) that selectively bound SAα2,6Gal and SAα2,3Gal, respectively, were used as controls. All four H5N6 viruses bound to both SAα2,6Gal and SAα2,3Gal receptors at comparable levels (Fig. 2A). Using resialyated cRBCs that expressed either SAα2,6Gal or SAα2,3Gal, we also found that the four H5N6 viruses bound both α2,6-resialylated and α2,3-resialylated cRBCs (Fig. 2B), an observation consistent with the direct virus-sialylglycopolymer binding assay results. Therefore, all four H5N6 viruses of clade 2.3.4.4, isolated from waterfowl, have maintained the affinity for avian-like SAα2,3Gal receptors and acquired considerable affinity for human-like SAα2,6Gal receptor, suggesting their potential to infect humans.

Extensive binding of avian H5N6 viruses to the upper and lower human respiratory tract. The affinity of the four H5N6 viruses for SAα2,6Gal-linked receptor was further assessed on human tracheal and lung sections. All four H5N6 viruses were found to bind tracheal epithelial and alveolar cells (Fig. 3), further affirming their potential ability to infect humans. Predictably, control avian H5N1 virus binding was restricted to the alveoli, and control 2009 pandemic H1N1 virus was able to attach to both epithelial lining of the trachea and alveoli (24).

Pathogenicity of avian H5N6 viruses in mice and ferrets. Pathogenicity of the H5N6 viruses was compared with HPAI H5N1 virus (CN0603) in mice and ferrets. All four H5N6 viruses showed relatively low pathogenicity in i.n.-inoculated BALB/c mice, with MLD_{50} values higher than 5.0 $\log_{10}$ EID_{50} (Table 2 and Fig. 4). In contrast, H5N1 virus was lethal to mice at a low dose (MLD_{50} of 1.0 $\log_{10}$ EID_{50}). To assess for systemic virus spread, various tissues were sampled for virus titration from mice at 4 days p.i. with 10^6 EID_{50} of virus. All H5N6 viruses replicated well in the lungs of mice, with titers ranging from 2.5 to 5.2 $\log_{10}$ EID_{50}. Systemically, DK/EC/S0322/14 (H5N6) virus was detected in the kidneys and DK/EC/S0908/14 (H5N6) virus was detected in the spleens of infected mice. No H5N6 virus was detected in the brain. H5N1 virus, however, caused widespread systemic infection and was readily detected in all tissues sampled, including the lungs, spleen, kidneys, and brain (Table 2).

The HA and six internal segments of the four H5N6 avian viruses shared high nucleotide identity, but their NA genes form two phylogenetic groups: three viruses with 11-amino-acid deletions in the NA stalk and one full-length NA (Fig. 1B). Since amino acids deletions in NA stalk were thought to be related to viral pathogenicity (25, 26), we selected two representative H5N6 strains GS/EC/S0513/13 and DK/EC/S0711/14 with or without amino acids deletions in the NA stalk, respectively, in ferret experiments. HPAI H5N1 virus (CN0603) was used as a control. Groups of two ferrets were i.n. inoculated with 10^6 EID_{50} of virus, and all individuals were euthanized on day 4 p.i. for virus titration and histological examination. All ferrets infected with the H5N6 viruses showed no obvious clinical signs, except transient elevation of body temperature at day 1 p.i. Ferrets infected with control H5N1 virus, on the other hand, displayed severe and early onset of clinical signs such as wheezing and coughing. H5N1 virus-infected ferrets produced generally high virus titers from all tissues sampled: nasal turbinate, trachea, lung, and spleen (Fig. 5). Prog-

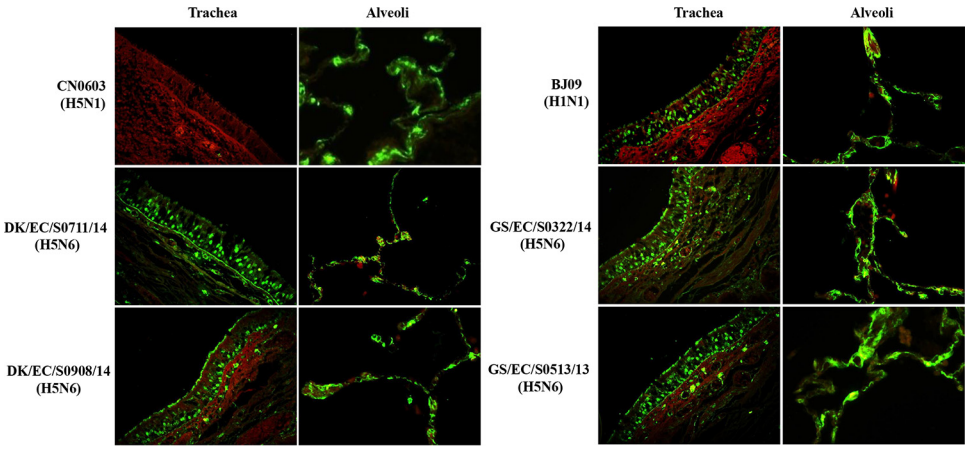

FIG 3 Extensive binding of avian H5N6 viruses to the upper and lower human respiratory tract. Avian H5N1 (control), human H1N1 BJ09 (control), and H5N6 viruses were incubated on human respiratory sections and immunostained for influenza NP. Antibody binding was detected by using FITC-labeled goat anti-mouse IgG (green). Unlike avian H5N1 virus that predictably bound only to human alveoli, avian H5N6 viruses, such as the human H1N1 virus, bound to both tracheal and alveolar linings.

eny H5N6 (DK/EC/S0711/14) virus production was significantly lower ($P < 0.05$) in the left cranial, left middle, left caudal, right cranial, and right caudal lung regions and the spleen than corresponding H5N1 virus-infected tissues (Fig. 5). The other H5N6 virus subtype (GS/EC/S0513/13) showed virus production from the lung lobes and, to a lesser extent, the spleen that was comparable to H5N1 virus infection; virus titers from nasal turbinate and trachea were, however, significantly higher ($P < 0.05$) than those from H5N1 virus infection (Fig. 5). Overall, our virus titration results from infected ferrets indicate that both H5N6 virus subtypes were capable of productive infection from the upper respiratory tract (URT); however, virus replication in the lower respiratory tract (LRT) and spleen was dependent on specific H5N6 virus subtype.

The pathological findings of H5N6 virus infection in ferrets were consistent with the relatively mild clinical response. DK/EC/S0711/14 virus-infected lungs appeared normal, but GS/EC/S0513/13 virus-infected lungs showed multifocal areas of consolidation in the diaphragmatic and intermediate lobes. H5N1 virus-infected lungs had the most severe lesions, with extensive consolidation in all lobes (Fig. 6). Microscopic lesions from DK/EC/S0711/14 virus-infected lungs showed mild bronchitis (Fig. 6C), and GS/EC/S0513/13 virus-infected lungs showed bronchopneumonia, characterized by a dropout of mucous epithelium and inflammatory cells adhering to the bronchiolar surface (Fig. 6B). H5N1 virus-infected lungs, in contrast, exhibited extensive and severe peribronchiolitis and bronchopneumonia, and interstitial pneumonia was also observed that showed interstitial edema and thickening of the alveolar walls. Moreover, the alveolar lumen was flooded with detached alveolar cells, erythrocytes, and inflammatory cells (Fig. 6A). Viral NP of H5N6 and H5N1 viruses could be readily detected in the bronchioles, terminal bronchioles, and alveoli of infected ferrets (Fig. 6D to F). In summary, based on the findings from the mouse and ferret challenge studies, H5N6 viruses are capable of extensive replication along the respiratory tract that results in significant pathological changes but, seemingly, not as severe as those caused by a clade 2.3.4 HPAI H5N1 virus.

Transmissibility of avian H5N6 viruses in ferrets. The transmissibility of the above two H5N6 viruses (DK/EC/S0711/14 and GS/EC/S0513/13) in ferrets were compared to pandemic H1N1 (BJ09) and avian H5N1 (CN0603) viruses. In the H5N6 virus groups, viruses were detected only in nasal washes from all inoculated and direct contact animals, indicating a direct contact route of H5N6 virus transmission with no aerosol spread (three animals were examined for each route) (Fig. 7 and Table 3). Pandemic H1N1 virus efficiently transmitted to all ferrets by direct contact and via aerosol (Fig. 7). Despite severe pathogenicity, there was no horizontal transmission of H5N1 virus over 10 days of infection (Fig. 7). Likewise, no H5N1 virus seroconversion was found in the direct-contact and aerosol-treated groups of animals (Table 3). These findings demonstrate that emergent avian H5N6 viruses

TABLE 2 Replication and virulence of the H5N6 viruses in mice[a]

Virus strain (subtype)	Mean virus replication (MID_{50}) ± SEM in organs[a]				MLD_{50}	Pathotype[b]
	Lung	Spleen	Brain	Kidney		
CN0603 (H5N1)	5.7 ± 0.2	3.7 ± 0.4	2.5 ± 0.6	3.2 ± 0.3	1.0	High
DK/EC/S0711/14 (H5N6)	5.2 ± 0.5	–[c]	–	–	5.3	Middle
DK/EC/S0322/14 (H5N6)	4.5 ± 0.3	–	–	1.2 ± 1.2	5.0	Middle
GS/EC/S0513/13 (H5N6)	4.6 ± 0.1	–	–	–	5.3	Middle
DK/EC/S0908/14 (H5N6)	4.6 ± 0.9	1.2 ± 0.4	–	–	5.3	Middle

[a] The virus titer in organs, MID_{50}, and MLD_{50} values are shown as the $\log_{10}$ EID_{50}.
[b] Pathotypes were determined on the basis of replication and lethality in mice. Middle, medium pathogenicity; high, high pathogenicity.
[c] –, Virus was not detected in the undiluted sample.

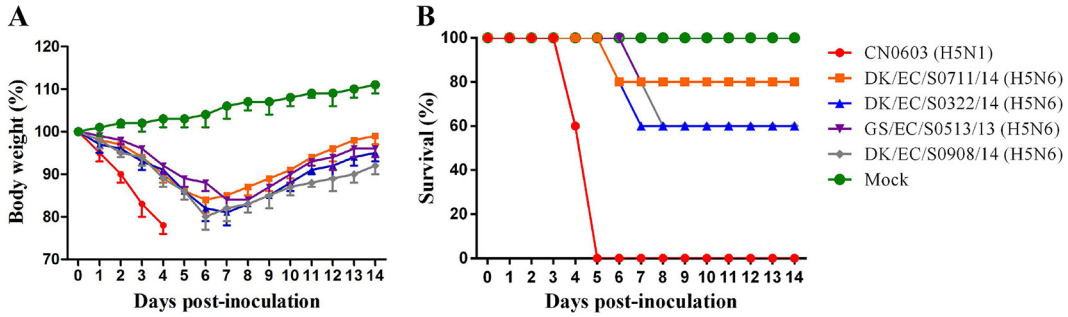

FIG 4 Weight loss and mortality of mice inoculated with avian H5N6 viruses. Six-week-old female BALB/c mice ($n = 5$ mice/group) were inoculated i.n. with 10^5 EID_{50} of virus or diluent (mock). The body weights of inoculated mice were measured daily and are represented as percentages of the weight on the day of inoculation (day 0). The averages for each group are shown, and the error bars represent the SD. (B) Survival percentages of mice infected with 10^5 EID_{50} of virus.

have acquired the ability of contact transmission but not aerosol spread in ferrets.

DISCUSSION

In the present study, we found that all four emergent avian H5N6 viruses of clade 2.3.4.4 have acquired binding affinity for the human SAα2,6Gal-linked receptor, leading to their ability to attach to human tracheal epithelia and alveoli on tissue sections. H5N6 viruses were efficiently transmitted between ferrets by direct contact but not via aerosol. They were, however, not as pathogenic in mice and ferrets as their parental clade 2.3.4 H5N1 virus.

HPAI H5N1 viruses continue to cause unprecedented outbreaks in poultry in more than 60 countries. There have been 844 human cases of H5N1 virus infection with 449 deaths (as of December 2015; http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Clade 2.3.4 H5N1 viruses emerged in chickens and waterfowl in southern China in 2005 and have become predominant in Southeast Asia (27, 28). Since 2010, its inner-clade 2.3.4.4 H5 reassortants, which include H5N2, H5N6, and H5N8 viruses, are frequently found in waterfowl and terrestrial poultry in China and other Asian countries (8, 29, 30). By 2014, H5N2 and H5N8 viruses had spread by migratory birds to poultry in multiple continents (10). Although H5N6 virus is still limited in China and Laos (6-9, 13), reports show that H5N6 virus is gradually becoming more prevalent in poultry than H5N1 virus in China (9). It is a concern that wild birds would spread the H5N6 virus to distant places to become the next potential candidate of global dissemination after H5N2 and H5N8 viruses.

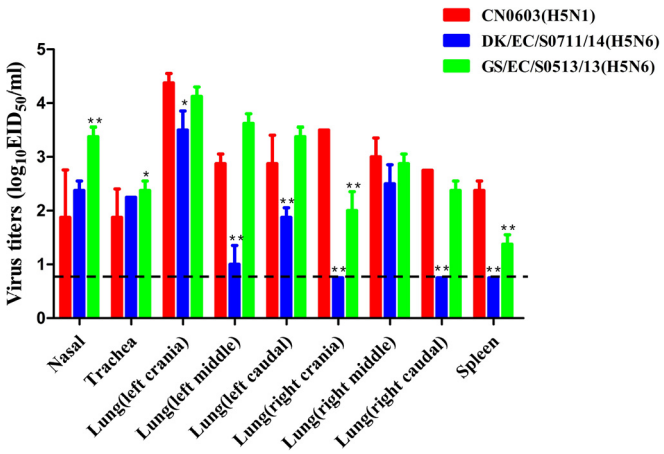

FIG 5 Productive virus replication of avian H5N6 viruses in ferrets. Each ferret, in groups of two, was i.n. inoculated with 10^6 EID_{50} of the indicated virus. The indicated tissues from control H5N1 virus (A) and two H5N6 virus subtypes DK/EC/S0711/14 (B) and GS/EC/S0513/13 (C) were collected at day 4 p.i. for virus titration. *, $P < 0.05$; **, $P < 0.01$ (H5N6 virus-infected ferrets versus H5N1 virus-infected ferrets). Dashed lines indicate the lower limit of detection.

The weak affinity of HPAI H5N1 virus for SAα2,6Gal-linked receptor is considered a key factor for limited human transmissibility (31, 32). Here, we demonstrated that the acquisition of SAα2,6Gal-linked receptor binding by emergent H5N6 viruses coincided with clear virus binding to both human URT and LRT. Previous studies showed that the loss of glycosylation at HA 158 was responsible for H5N1 virus binding to the SAα2,6Gal-linked receptor (31, 32) and transmission between guinea pigs (20). Yen et al. found that combined deglycosylation at residue 158 and S227N substitution could substantially increase the affinity of HA for SAα2,6Gal-linked receptor (33). The four emergent H5N6 viruses possess mutations in the RBS of HA and lack the N-linked glycosylation site at HA residue 158, which are characteristic of most clade 2.3.4.4 H5 viruses, including H5N2 and H5N8, indicating that these viruses have acquired other features, as yet not understood, for human-type receptor-binding affinity (34, 35).

In our ferret challenge study, pathogenicity of H5N6 viruses was relatively mild compared to the corresponding HPAI H5N1 virus infection. These results were similar to previous findings observed with clade 2.3.4.4 H5N2 and H5N8 viruses (34, 36). Nonetheless, H5N6 virus replication was detectable in ferret lungs with associated pneumonic pathology. It is noteworthy that the GS/EC/S0513/13 H5N6 virus showed virus production from lung lobes that was comparable to H5N1 virus infection and induced multifocal areas of consolidation in the diaphragmatic and intermediate lobes. Similar pulmonary changes in humans could be presented with vastly different clinical effects. The six human patients infected with H5N6 viruses were presented with fever and severe pneumonia and developed septic shock and ARDS; three of them died several days after the onset of illness (8, 13). Therefore, emergent avian H5N6 viruses, while showing less severe pathogenicity than HPAI H5N1 virus in the ferret model, can still cause severe if not fatal disease in infected humans.

We showed that H5N6 viruses were transmissible in ferrets by direct contact but not by aerosol. Other researchers have reported that clade 2.3.4.4 HPAI H5N8 viruses lack the contact transmissi-

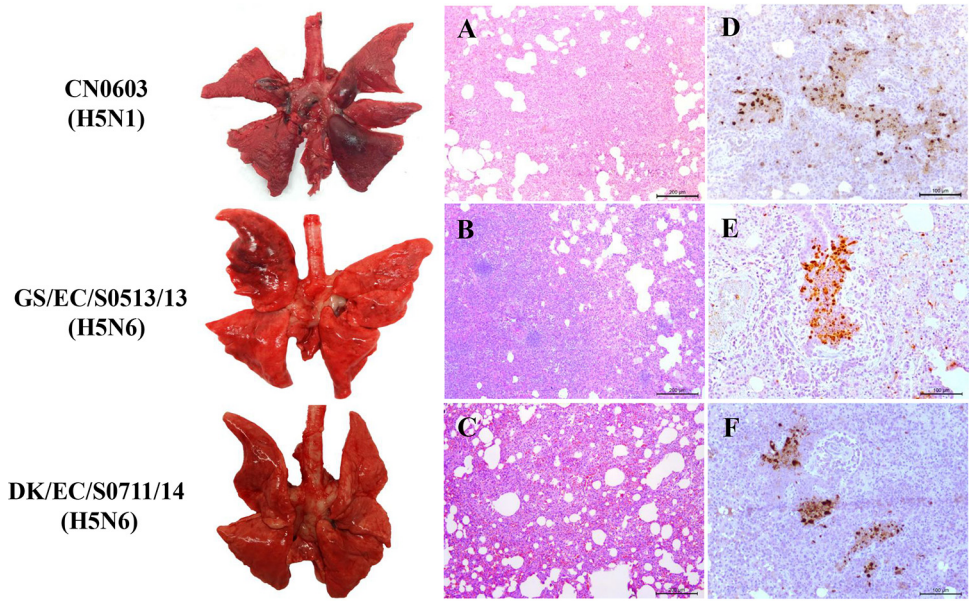

FIG 6 Gross and histopathology of lungs of ferrets infected with avian H5N6 viruses. Representative gross respiratory tracts (left column) and corresponding histological (hematoxylin and eosin staining; middle column [A to C]) and immunohistochemical (right column [D to F]) lung sections at day 4 p.i. are shown. (A) Avian H5N1 (control) infection caused severe bronchopneumonia with hemorrhage, edema, and diffuse consolidation; (B and C) GS/EC/S0513/13 (H5N6) and DK/EC/S0711/14 (H5N6) infections, respectively, produced moderate bronchopneumonia with inflammatory cell infiltrates in alveoli and interstitia. (D to F) Extensive viral NP localization to bronchioles and alveoli for all three indicated viruses.

bility in ferrets (34, 36) and guinea pigs (35). An H5N2 virus isolated in China (A/duck/Eastern China/1112/2011), however, was able to efficiently transmit between cohoused guinea pigs (35). We also found that H5N2 viruses from clade 2.3.4.4 can efficiently transmit between in-contact ferrets but not via aerosol route and that H5N8 viruses lack the ability for efficient in-contact transmission in ferrets and guinea pigs (unpublished data). Therefore, among the novel H5Nx viruses, H5N6, as well as H5N2, seem better able to infect model mammals by direct contact than are H5N8 viruses, suggesting that H5N6 and H5N2 subtypes are more infective to humans. In addition to the receptor-binding preference conferred by HA, NA and internal gene combination could

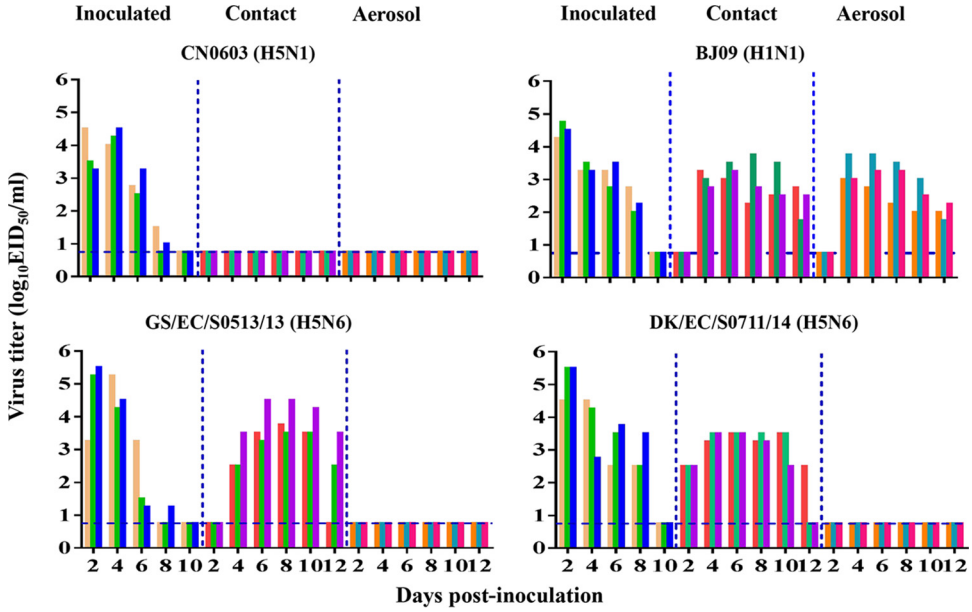

FIG 7 Horizontal transmission of avian H5N6 viruses between ferrets. Groups of three ferrets were inoculated i.n. with 10^6 EID_{50} of the indicated viruses. The next day, the inoculated animals were individually paired by cohousing with a direct-contact (DC) ferret; an RD-contact animal was also housed in a wire frame cage adjacent to the infected ferret. Nasal washes for virus shedding detection were collected every other day from all animals from day 2 of the initial infection. Each color bar represents the virus titer of an individual animal. Dashed lines indicate the lower limit of virus detection.

TABLE 3 Seroconversion of ferrets

Virus (subtype)	Seroconversion: no. positive/total no. (HI titers)[a]		
	Inoculated	Direct contact	Respiratory contact
BJ09 (H1N1)	3/3 (80, 80, 80)	3/3 (40, 40, 20)	3/3 (40, 20, 20)
CN0603 (H5N1)	3/3 (80, 80, 40)	0/3	0/3
GS/EC/S0513/13 (H5N6)	3/3 (80, 40, 40)	3/3 (20, 40, 20)	0/3
DK/EC/S0711/14 (H5N6)	3/3 (80, 80, 40)	3/3 (40, 20, 20)	0/3

[a] Sera were collected from ferrets on day 14 p.i. and treated overnight with *Vibrio cholerae* receptor-destroying enzyme. Seroconversion was confirmed by HI assay.

also play a role in moderating virus transmissibility in mammals (32). Notably, the newly isolated H5N6 virus (A/Yunnan/0127/2015, GenBank accession numbers KT245143 to KT245150) from human infections was found to be a further reassortment of internal viral genes with avian H9N2 viruses. Thus, attention should be paid to the relentless reassortment and mutational changes of these viruses which may break down the barrier of transmission between host species.

In summary, compared to the parental clade 2.3.4 HPAI H5N1 virus, the emergent H5N6 viruses have acquired an affinity for the human-like SAα2,6Gal-linked receptor to bind human tracheal epithelial and alveolar cells, with the ensuing ability for in-contact transmission in ferrets. Although their pathogenicity in model mammals was not as severe as that of the HPAI H5N1 virus, human cases of H5N6 virus infection are severe and are associated with a high death rate. The dissemination of H5N6 viruses in domestic poultry and wild birds poses a serious threat to both poultry and human health.

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