H13 Subtype Avian Influenza Viruses Are Crossing Host Species Barrier From Wild Waterfowl to Land Fowl

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Abstract

The avian influenza virus H13 subtype circulates primarily in waterfowl. To explore the ability of the H13 virus to cross the host barriers, we genetically analysed two H13 isolates from wild birds in China and evaluated the infectivity of these subtypes in chickens. Genetic and molecular analyses showed differences in the lineages and amino acid sequences between the two subtypes; A/mallard/Dalian/DZ-137/2013 (H13N6) belonged to Group I, while A/Eurasian Curlew/Liaoning/ZH-385/2014 (H13N8) belonged to Group III. The nucleotide sequence results showed high homology (approximately 96.9%-100%) to sequences in GenBank. Neither H13 isolate replicated in adult chickens or 20-day-old chicks; however, the H13N8 strain replicated in 1- and 10-day-old chicks. Viruses were recovered from the nasal turbinate, tracheal, lung and colon tissues of chicks at 1, 3 and 5 days post-inoculation. The H13N6 isolates replicated inefficiently in 1-day-old chicks and did not replicate in 10-day-old chicks. Serological surveillance results showed that domestic chickens had a 4.6%-10.4% (15/328-34/328) positive antibody titre to the H13 virus. H13N6 and H13N8 isolates replicated in mammalian cell lines, including 293T, Madin-Darby canine kidney and chicken embryo fibroblast cells. Our results suggest that the AIV H13 subtype may cross the host barrier from wild waterfowl to land fowl.

Introduction

The avian influenza virus (AIV) normally has a range of hosts[1–2], such as avians, swine, and canines[3–5]. Generally, the viruses that infect one species of animal cannot replicate efficiently in another species of animal[6], resulting in host species barriers among a variety of animals. The host species barrier of AIV is of great importance to viral evolution and human health, as AIV could adapt to other hosts by amino acid mutations in its viral proteins[7–9], giving AIV the opportunity to overcome host barriers. H5N1 AIV was first found to infect humans in 1997 and caused high human mortality[10]. Some AIV subtypes, including H7N9, H5N6, and H10N8, have caused extensive economic losses and led to human cases in recent years[11–13]. As such, we are currently facing a very large challenge, and the control and prevention of AIV have become extremely important.

AIV is often isolated from waterfowl and consists of 16 subtypes[14] that are divided on the basis of haemagglutinin and neuraminidase proteins. The AIV H13 subtype is a group of viruses with one of the 16 known HA proteins. The H13 subtype virus emerges less infrequently than the other AIVs. The low pathogenicity of H13 in avians may be one of the reasons for its low prevalence. The AIV H13 subtype was first isolated from gulls in North America in 1977 [15]. Since the 1980s, other H13 isolates have been identified worldwide. The AIV H13 subtype is divided into Eurasian and North American lineages[16] and is present in North America, Europe and Asia.

We first isolated H13N6 AIV from a mallard in China in 2013[17]and another H13N8 AIV from a Eurasian Curlew in 2014. In our preliminary study, we found some interesting results; the two stock viruses, which normally replicate in the lungs, could not infect 20-day-old chicks or adult chickens in challenge experiments, suggesting that there was a host barrier between wild waterfowl and domestic poultry.

Interestingly, there was a report on the infection of gulls and chickens with the AIV strains H13N2 and H13N8 in eastern China in 2016[18]. Therefore, we attempted to study the ability of AIV to cross the host barrier between wild waterfowl and domestic poultry.

In this study, we selected two AIV H13 subtypes that were isolated from wild waterfowl. The two stock viruses belong to distinct lineages and groups. From previous studies, we knew that these viruses were unable to infect 20-day-old chicks or adult chickens, mice or guinea pigs. However, whether they could infect young chicks was unknown. Therefore, we selected 1-day-old chicks to evaluate AIV infection in poultry hosts. Chicken serum samples from several provinces in China were collected and used to assess haemagglutination inhibition (HI) antibody titre levels. Moreover, we studied the growth curve in mammalian and chicken embryo fibroblast (CEF) cells. We expected to find possible evidence of infection by the AIV H13 subtype in domestic poultry, suggesting that the AIV H13 subtype may infect the poultry in the future.

Materials And Methods

Cells, Serum Samples and Viruses

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum (FCS). Human embryonic kidney cells (293T) and CEF cells were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, and 100 mg/ml streptomycin or 100 IU/ml penicillin. All cells were incubated at 37°Cwith 5% CO_2.

Between 2015 and 2016, 328 serum samples were collected from different regions in China, including Shandong Province, Liaoning Province and Qinghai Province. All of the serum samples originated from domestic chickens. Serum specimens were analysed to assess the antibody titres against the H13 subtype of AIV.

The H13N6 virus A/mallard/Dalian/DZ-137/2013 (abbreviated DZ137) was isolated from a mallard in Liaoning Province, China. Additionally, the H13N8 virus A/Eurasian Curlew/Liaoning/ZH-385/2014 (abbreviated ZH385) was isolated from a Eurasian Curlew in Liaoning Province, China. We propagated these viruses in 10-day-old embryonated chicken eggs, after which the allantoic fluids were collected and stored at -80°C until use.

Virus Titration in Embryonated Chicken Eggs

Virus titers of two H13 subtypes were tested in embryonated chicken eggs. The seed viruses were diluted by 10-fold dilutions and then inoculated into chicken eggs. They were inoculated at 37°Cfor 48 h. Finally, the fluids were tested by the 1% chicken erychrocytes. The 50% egg infectious dose (EID_{50}) was determined as described previously[19].

Animals and Experimental Infection

To study the difference between the two isolates, we chose 1-day-old chicks as animal models. All experimental animals were 1- or 10-day-old, pathogen-free white Leghorn chicks (from the Harbin Veterinary Research Institute, China). The animal experiments included a 1-day-old chick group and a 10-day-old chick group. The chicks were infected intranasally with $10^{6.0}$ EID_{50} of AIV H13 subtype in a volume of 50μl. Three chicks from each group were euthanized at 1 day post-inoculation (dpi), 3 dpi and 5 dpi. Tissues samples from the nasal turbinate, trachea, lungs and colon were collected and measured as described previously[20].

Virus Titration in Tissues

After inoculation with the H13 influenza subtype, the virus titre in tissues was measured. We used 10-day-old chicken embryos to isolate the influenza virus. Virus titrations were evaluated by embryonated chicken eggs. We formulated a 10-fold dilution series with DMEM in a volume of 0.1 ml and inoculated 10-day-old embryonated chicken eggs at 37°C for 48 h, and then we harvested the allantoic fluids. Virus titres were calculated in accordance with the Reed and Muench method[19].

RNA Isolation, PCR Amplification, and Sequencing

RNA was isolated from a 200μl sample in DMEM by using an RNeasy Mini kit (Qiagen, Germantown, MD). Reverse transcription of viral RNA was performed using primers specific for influenza virus. and the sequences were cloned using PCR amplification methods. The viral gene segments were sequenced by the Beijing Genomics Institute (Beijing, China), and we used the Lastergene sequence analysis software package (DNAStar, Madison, WI) to analyse the DNA sequences. Bayesian analysis was performed for the HA gene segments using BEAST version 1.8.4.

HI Assays

HI assays were conducted following the handbook of the WHO Manual on Animal Influenza Diagnosis and Surveillance. Serum samples were treated with receptor destroying enzyme (RDE,Denka Seiken Co., Ltd) and inactivated at 56℃ for 30 minutes. The results were ultimately accessed with 0.5% chicken erythrocytes.

Viral Growth Kinetics in Cell Culture

We evaluated the growth kinetics of the AIV H13 subtype in 293T, MDCK and CEF cells; 12-well plates were used to culture cells. The cells were infected with virus at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell. We cultured cells with DMEM containing 2μg/ml TPCK-treated trypsin, and the cultured cells were incubated at 37°C with 5% CO_2. At 12, 24, 36, 48, 60 and 72 h time points, we collected the supernatants and determined the virus titres in MDCK cells. All experiments were performed in triplicate.

Nucleotide Sequence Accession Numbers

In this study, the sequences of the two H13 subtype AIV accessions are available in GenBank. The GenBank accession numbers of the DZ137 viral gene segments are KJ907708-KJ907715. The GenBank

accession numbers corresponding to each of the eight ZH385 viral gene segments are KR010440-KR010447.

Statistical Analysis

The data were analysed by analysis of variance (ANOVA) using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). We evaluated the differences using two-way ANOVA. P-values of < 0.05, < 0.01, and < 0.001 were considered statistically significant.

Results

Genetic Analysis of the AIV H13 Subtype

We cloned and sequenced gene segments from the two AIV H13 subtypes and analysed the whole genomes of the two stock viruses using DNAStar software. Both the ZH385 HA and NA come from a strain infecting a species of gulls, as is DZ137 neuraminidaseNA (Table S1). However, the DZ137 HA belongs to a duck strain[17].

Phylogenetic analysis of the HA gene showed that HA genes are divided into three groups (Groups I, II and III)[21]. The HA gene of DZ137 belongs to Group I, while that of ZH385 belongs to Group III (Fig. 1). These two AIV H13 subtypes were isolated from the same location in different years. These results may be due to differences in the migration routes of wild birds.

The homology analysis of nucleotide sequences showed that the DZ137 and ZH385 gene segments shared high homology, at approximately 96.9–100% in GenBank (Table 1). The internal genes of these two H13 subtypes were related to those of different subtypes, such as H4, H9, H6 and H16. These results suggested that a reassortment had occurred in the AIV H13 subtype occurred.

Table 1
Nucleotide homology of influenza virus genes with the closest related sequences available in GenBank.

Virus	Gene	Closest related virus strain	Nucleotide identity(%)	Accession No.
H13N6	PB2	A/velvet scoter/Mongolia/883V/2009 (H4N6)	96.9%	KC986346.1
	PB1	A/duck/Hokkaido/K04/2014 (H9N2)	97.6%	LC042041.1
	PA	A/mallard/Tumuji/TMJ-748/2013(H6N2)	100%	KJ907678.1
	HA[a]	A/duck/Hokkaido/WZ68/2012 (H13N2)	98.4%	AB812744.1
	NP	A/glaucous-winged gull/ Alaska/ 414/ 2013(H13N2)	98.9%	KY131041.1
	NA	A/glaucous-winged gull/Southcentral Alaska/11JR02182/2011 (mixed)	98.0%	CY195631.1
	M	A/common gull/Altai/805/2011 (H16N3)	99.3%	KF462332.1
	NS	A/black-headed gull/Republic of Georgia/4/2011 (H13N8)	98.8%	CY185541.1
H13N8	PB2	A/black-headed gull/Republic of Georgia/4/2012 (H16N3)	98.9%	CY185592.1
	PB1	A/yellow-legged gull/Republic of Georgia/1/2013 (H13N8)	98.7%	CY185631.1
	PA	A/mallard/Republic of Georgia/13/2011 (H6N2)	98.7%	CY185582.1
	HA	A/yellow-legged gull/Republic of Georgia/1/2013 (H13N8)	98.8%	CY185625.1
	NP	A/black-headed gull/Republic of Georgia/9/2011(H13N8)	98.1%	CY185660.1
	NA	A/yellow-legged gull/Republic of Georgia/2/2013 (H13N8)	98.2%	CY185635.1
	M	A/Armenian gull/Republic of Georgia/2/2012(H13N2)	99.4%	CY185348.1
	NS	A/yellow-legged gull/Republic of Georgia/1/2010 (N2)	99.3%	CY185313.1

Molecular Analysis of the AIV H13 Subtype

The amino acids, especially those at positions 627 and 701, in the PB2 gene of the influenza virus contribute to host adaptation[22–24]; the 627 amino acid position of PB2 is glutamic acid (E), and the 701 amino acid position is aspartic acid (D) (Table 2), meaning that pathogenicity in mammals is low. These two H13 viruses maybe infect the poultry and mammals hardly.

Table 2
Amino acid signatures of the AIV H13 subtype

Protein	Amino acid position	Signatures
		H13N8 (ZH385)
PB2	526	K
	627	E
	701	D
HAa	226	S
	228	S
NA	69–73	No deletion
NS1	80–84	No deletion
	92	D

[a] The amino acid numbering in the HA protein is based on the H3 numbering.

The HA protein of AIV also plays an important role in host restriction; the amino acid sequences in these two groups are located at HA-226Q and HA-228S (Table 2). Neither the NA 69–73 nor the NS 80–84 deletion was not detected in either of these two viruses (Table 2). Position 92 in the NS1 gene was D in both viruses. These results indicate that the two AIV H13 subtypes are low-pathogenicity influenza viruses. This finding prompted differences in the characterization of the virus.

There were several amino acid differences in the internal genes. We identified changes in 17 amino acids in PB2, 13 amino acids in PB1, 4 amino acids in PA, 6 amino acids in NP, one amino acid in M1, 4 amino acids in NS1, and 4 amino acids in NS2 (Table 3). These amino acid differences may have influenced infectivity.

Table 3
Amino acid differences between DZ137 and ZH385.

Gene	Amino acid positions [a]	Identity [b]
PB2	T76I, T106A, C125L, I147T, D191E, K197R, V338I, M381L, I411V, S470N, V478I, T559I, V560I, M607L, A661T, T662N, A674S	97.8%
PB1	K54T, S59T, E172D, E178G, N213S, K214R, R215K, S257T, K386R, K391N, K430R, V591I, I667V	98.2%
PA	G316D, E327G, R353K, M441I	99.6%
NP	A27V, I33V, V67A, S247N, V408I, S482N	98.8%
M1	R95K	99.2%
NS1	T76A, A112T, N171S, T215A	98.3%
NS2	T14A, S44L, L85H, R86K	96.7%
a DZ137 is on the left and ZH385 is on the right.		
b The percent identity between DZ137 and ZH385.		

One-Day-Old Chick AIV H13 Subtype Challenge Experiments

We chose 1-day-old chicks as an animal model to evaluate infections in domestic poultry. The EID_{50} values of DZ137 and ZH385 were $10^{7.5}$ EID_{50}/ml and $10^{7.75}$ EID_{50}/ml, respectively. We established a dilution rate of $10^{6.0}$ EID_{50}/50μl for the challenge experiments, and the 1-day-old chicks were divided into DZ137 and ZH385 groups. The chicks were inoculated intranasally with the two stock viruses at $10^{6.0}$ EID_{50} in a volume of 50μl. At 1, 3 and 5 days post-infection, we euthanized the chicks and harvested their organs.

We detected the lung titres and the colon titres at 1, 3 and 5 dpi. Among the lung titres, the ZH385 strain had a higher titre than the DZ137 strain (Fig. 2a). At 3 dpi, we detected a titre of 5.07 ± 0.92 $\log_{10}$ EID_{50}/g, which was significantly different than the titres on the other collection days. The DZ137 strain showed low virus titres, and we could not consistently determine the virus titre at 1 dpi (Fig. 2a).

With respect to the colon titres, we obtained similar results. The ZH385 strain reached 4.22 ± 1.48 $\log_{10}$ EID_{50}/g at 1 dpi and 5.99 ± 0.12 $\log_{10}$ EID_{50}/g at 3 dpi (Fig. 2b), with a significant difference between the two days. The DZ137 strain showed lower titres than did the ZH385 strain.

The difference between the lung and the colon virus titres may suggest a host barrier with respect to domestic poultry.

10-Day-Old Chick AIV H13 Subtype Challenge Experiment

To compare the differences in poultry, we used 10-day-old chicks to evaluate infection. The protocol and inoculation groups were the same as those in the 1-day-old chicks. We measured titres in the nasal turbinate, tracheal, lung and colon tissues.

The results were surprising. In the nasal turbinate tissue, we determined a significant difference in the titres at 1, 3 and 5 dpi, with virus titres of 3.70 ± 0.18 $\log_{10}$ EID_{50}/g, 5.09 ± 0.22 $\log_{10}$ EID_{50}/g and 5.58 ± 0.58 $\log_{10}$ EID_{50}/g, respectively (Fig. 3a). In the tracheal tissue, the ZH385 strain had a titre of 3.71 ± 0.69 $\log_{10}$ EID_{50}/g at 3 dpi and 3.89 ± 0.88 $\log_{10}$ EID_{50}/g at 5 dpi (Fig. 3b). The lung titres of the ZH385 strain had low replication rates, and there was no significant difference between the ZH385 and DZ137 strain (Fig. 3c). The colon titres of ZH385 were 3.80 ± 3.01 $\log_{10}$ EID_{50}/g at 1 dpi, 4.49 ± 0.98 $\log_{10}$ EID_{50}/g at 3 dpi and 5.56 ± 0.45 $\log_{10}$ EID_{50}/g at 5 dpi (Fig. 3d).

This result was surprising, as we expected to detect differences in virus titres in domestic poultry through our infection experiments.

HI Serum Titre Tests against the AIV H13 Subtypes

Avian serum samples collected in China from 2015 to 2016 were serologically analysed. Fifteen samples showed detectable HI antibody titres against DZ137, and thirty-four samples had detectable HI antibody titres for ZH385 (Table 4). Serum samples from Qinghai Province had an HI antibody titre of 40 against DZ137 and ZH385 (Table 5). In Shandong Province, 14 samples had titres of 40–320 (DZ137), and 30 samples had titres of 40–320 (ZH385) (Table 6). In total, we found 15 (4.6%) samples with HI antibody titres against DZ137 and 34 (10.4%) specimens with detectable HI antibody titres against ZH385.

Table 4
Chicken and wild bird serum survey for two AIV H13 subtypes from different locations in China, 2015–2016 .

			Positive HI titre results / no. tested	
Year	Host species	Location	DZ137	ZH385
2016	Chicken	Shandong	14/189	30/189
2015	Chicken, ducks and sentinel animals	Liaoning	0/35	0/35
2016	Chicken	Qinghai	1/104	1/104
Total samples			15/328	34/328

Table 5
Chicken serum antibodies against virus subtype H13N6 (DZ137) or subtype H13N8 (ZH385) in Qinghai Province.

	HI titres against viruses	
Sample no.	DZ137	ZH385
xhj35[a]	40	40
mhj17	< 10	40
mhj16	< 10	40
mhj15	< 10	40
a The bold number means the sample contained HI titres against two H13 viruses.		

Table 6
Chicken serum antibodies against virus subtype H13N6 (DZ137) or subtype H13N8 (ZH385) in Shandong Province.

	HI titres against viruses	
Sample no.	DZ137	ZH385
10	40	< 10
25	40	< 10
51	40	< 10
57	80	< 10
64	80	< 10
69	80	< 10
84	80	< 10
127	40	< 10
136	80	< 10
145	80	< 10
99a	40	320
122	160	160
179	40	320
185	320	320
34	< 10	80
75	< 10	160
78	< 10	80
90	< 10	160
102	< 10	320
103	< 10	320
119	< 10	320
121	< 10	80
132	< 10	320
169	< 10	160

a The bold numbers indicate that the samples contained HI titres against two H13 viruses.

	HI titres against viruses	
170	< 10	160
171	< 10	80
172	< 10	80
173	< 10	40
174	< 10	160
175	< 10	160
176	< 10	80
177	< 10	80
178	< 10	320
180	< 10	160
182	< 10	160
183	< 10	80
186	< 10	80
187	< 10	160
188	< 10	160
189	< 10	80
a The bold numbers indicate that the samples contained HI titres against two H13 viruses.		

Viral Growth Kinetics in Cell Culture

We observed some differences between day-old chicks during the challenge experiments; therefore, we were interested in whether there were different characteristics in vitro. To address this question, we evaluated viral growth kinetics in MDCK, CEF and 293T cells. The DZ137 and ZH385 viruses were inoculated at an MOI of 0.01 into a variety of cell cultures. The culture supernatants were collected at different time points and measured by the 50% tissue culture infectious dose ($TCID_{50}$) for MDCK cells.

In the MDCK cells, we found only one time point, 48 h, that was significantly different from the rest (Fig. 4a), and there were no other significant differences in MDCK cells. In the MDCK cells, the DZ137 strain reached a peak viral titre of 3.83 ± 0.38 $TCID_{50}$/ml at 24 h, whereas the ZH385 strain reached a peak viral titre of 4.83 ± 0.38 $TCID_{50}$/ml at 48 h; the ZH385 strain reached its highest virus yield 24 h after the DZ137 strain (Fig. 4a). In CEF cells, both AIV H13 subtypes reached their peak titres at 36 h. The virus titre of DZ137 reached 2.58 ± 0.14 $TCID_{50}$/ml, and the titre of ZH385 reached 2.67 ± 0.14 $TCID_{50}$/ml. After 36

h, the virus titres decreased (Fig. 4b). In 293T cells, the two H13 strains reached their peak titres at 48 h (Fig. 4c). DZ137 reached 3.50 ± 0.00 $TCID_{50}$/ml, and ZH385 reached 3.42 ± 0.14 $TCID_{50}$/ml. The virus titres in CEF cells were lower than those in 293T cells. This phenomenon indicates that the same virus had different effects on different cells.

Discussion

There is an influenza host barrier between many kinds of animals species[6, 25]. AIV is widely detected in wild waterfowl and can infect domestic poultry under certain conditions. There can be highly pathogenic influenza viruses and low pathogenic influenza viruses on the basis of pathogenicity. Currently, we know that the AIV species associated with AIV polymerase contains the PB2, PB1, PA and NP genes[26–27]. When AIV develops the ability to infect one species effectively, some adaptive mutations on these proteins can often be observed [7, 20, 24]. Another study showed that the HA protein plays a key role in the receptor binding process[28–30]. However, how the influenza virus causes epidemics is less well known.

Compared to the seasonal influenza virus, the AIV H13 subtype rarely causes infection.. This subtype is a low-pathogenic influenza viral subtype and is prevalent in gulls and ducks[31–32]. In this study, we obtained two AIV H13 subtypes isolated from waterfowl in China. Previous study results showed that these isolates could not replicate in 6-week-old adult chickens[17] or 20-day-old chicks (Table S2); therefore, their host range may be restricted to waterfowl. When AIV crosses the host barrier from waterfowl to domestic poultry, the virus usually contains genetic modifications. The haemagglutinin protein is an important factor in host specificity[28, 33], and the NA deletions are often found in the stalk of the NA protein[34–36]. NS1 is a multifunctional protein in the influenza virus and may have a function in host-specific adaptation[37–38]. Our results showed that the surface glycoproteins of the two AIV subtypes belonged to different lineages : A/mallard/Dalian/DZ-137/2013 (H13N6) belonged to Group I, while A/Eurasian Curlew/Liaoning/ZH-385/ 2014 (H13N8) belonged to Group III (Fig. 1). In addition, two AIV H13 subtypes of wild bird origin were reported in 2016 in China. We found that A/black-tailed_gull/Weihai/115/2016 (H13N2) and A/mallard/Dalian/DZ-137/2013 (H13N6) belonged to the same group, while both A/black-tailed_gull/Weihai/17/2016 (H13N8) and A/Eurasian Curlew/Liaoning/ZH-385/ 2014 (H13N8) belonged to Group III. Although we just compared and analysed parts of H13 strains, we suspected that these AIV H13 subtypes were circulating frequently.

The two tested influenza viruses showed high homology to sequences in GenBank (Table 1). We sequenced the whole viral genomes and analysed key amino acids in the PB2, HA, NA and NS genes (Tables 2 and 3), and we found no deletions in the NA and NS genes. Moreover, we aligned the HA and NA sequences of the DZ137 and the ZH385 strains and found low homology. These results suggested differences at the molecular level were present. Additionally, the molecular markers of host adaptation (such as E627K and D701N) were not detected in the two H13 viruses. We also blasted the H13 subtypes in GISAID and NCBI. However, the PB2 genes of these strains showed a result of 627E and 701D. Therefore, these H13 subtypes have not obtained the ability to adapted to mammals in general.

As these AIV H13 subtypes could hardly replicate in 20-day-old chicks or adult chickens, we chose 1-day-old chicks as an experimental model. Chicks are commonly used to study vaccine efficacy and susceptibility to AIVs[39]. In this study, we divided chicks into a 1-day-old chick group and a 10-day-old chick group. The DZ137 virus replicated in only the 1-day-old chicks. Compared to the ZH385 strain, the DZ137 strain had a lower replication rate. ZH385 efficiently replicated in 1-day-old chicks. We detected the virus titres in the lung and colon tissues of 1-day-old chicks (Fig. 2). The titres in the tissues of 10-day-old chicks were evaluated in embryonated chicken eggs and presented high values (Fig. 3). These results suggested a difference in infectivity of the two AIV H13 subtypes by challenge experiments. Further study to determine the reason for these differences in infectivity is necessary. More H13 influenza viruses need to be tested in animal models. Additionally, we evaluated the transmission of A/black-tailed_gull/Weihai/17/2016 (H13N8) in 4-week-old chickens. The results showed no detectable virus shedding in oropharyngeal and cloacal swabs from the donor, direct contact and airborne contact groups (Table S3-S5) .

In a serological survey from 2015 to 2016 in China, an HI assay detected chicken serum antibody titres against the H13 subtypes ranging from 40 to 320 (Tables 4–6). Among all 328 chicken specimens, we found detectable HI titres against DZ137 in 15 (4.6%) samples and detectable HI titres against ZH385 in 34 (10.4%) samples. We did not detect the other influenza subtypes(H1, H3, H5, and so on) because of the low serum volume available. However, there has already been a report of poultry infection with H13 strains of wild bird origin. This serological evidence suggests that the AIV H13 subtype may overcome the host barrier and circulate in domestic chickens in the future.

We evaluated the differences in viral growth in mammal cells, and the virus titres in MDCK cells were higher than those in other cells (Fig. 4). The peak virus titres occurred at different time points in the three mammal cell cultures, suggesting that the two H13 subtypes can replicate in both mammal cells and chicken cells. In addition,the two H13 subtypes could infect not only CEF cells but also MDCK and 293T cells. These results show that H13 maybe infect domestic poultry and other mammals.

Conclusions

In conclusion, we analysed the genetic sequences of two AIV H13 subtypes and inoculated day-old chicks in vivo and in vitro with both subtypes. The H13N8 strain replicated in 1-day-old and 10-day-old chicks; however, the H13N6 strain replicated in only 1-day-old chicks. The serological results showed a 4.6%-10.4% HI antibody titre against two AIV H13 subtypes in chicken specimens, and both AIV strains grew in MDCK, CEF and 293T cells. The difference in virus replication between in vivo and in vitro conditions may result in a difference in the poultry host barrier. These findings suggest that the AIV H13 subtypes may cross the host barrier from wild waterfowl to land fowl.

Abbreviation

AIV: avian influenza virus; MDCK: Madin-Darby canine kidney; CEF: chicken embryo fibroblast; HI: haemagglutination inhibition; FCS: foetal calf serum; 293T: Human embryonic kidney cells; DMEM: Dulbecco's modified Eagle's medium; DZ137: The H13N6 virus A/mallard/Dalian/DZ-137/2013; ZH385: the H13N8 virus A/Eurasian Curlew/Liaoning/ZH-385/2014; EID_{50}: 50% egg infectious dose; dpi: day post-inoculation; MOI: multiplicity of infection

Declarations

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and material: All data generated or analysed during this study are included in this published article and its supplementary information files.

Authors' contributions: Weiyang Sun: Performed research, Analyzed data, Investigation, Wrote the paper. **Menglin Zhao:** Data curation, Investigation, Contributed new methods. **Zhijun Yu:** Resources. **Yuanguo Li:** Resources. **Xinghai Zhang:** Formal analysis, Investigation. **Na Feng:** Resources. **Tiecheng Wang:** Resources. **Hongmei Wang:** Resources. **Hongbin He:** Resources. **Yongkun Zhao:** Resources. **Songtao Yang:** Resources. **Xianzhu Xia:** Resources. **Yuwei Gao:** Designed study, Funding acquisition, Project administration, Supervision.

Ethics Statement and Facility: This study protocol was conducted in strict accordance with the guidelines for animal welfare established by the World Organisation for Animal Health. All experimental protocols and animal studies were approved by the Review Board of the Changchun Veterinary Research Institute of Chinese Academy of Agricultural Sciences. AIV H13 subtype experiments were conducted in a biosecurity level 2+ laboratory approved by the Changchun Veterinary Research Institute of Chinese Academy of Agricultural Sciences.

Consent to participate: All authors read and approved the manuscript.

Consent for publication: Written informed consent for publication was obtained from all participants.

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Figures

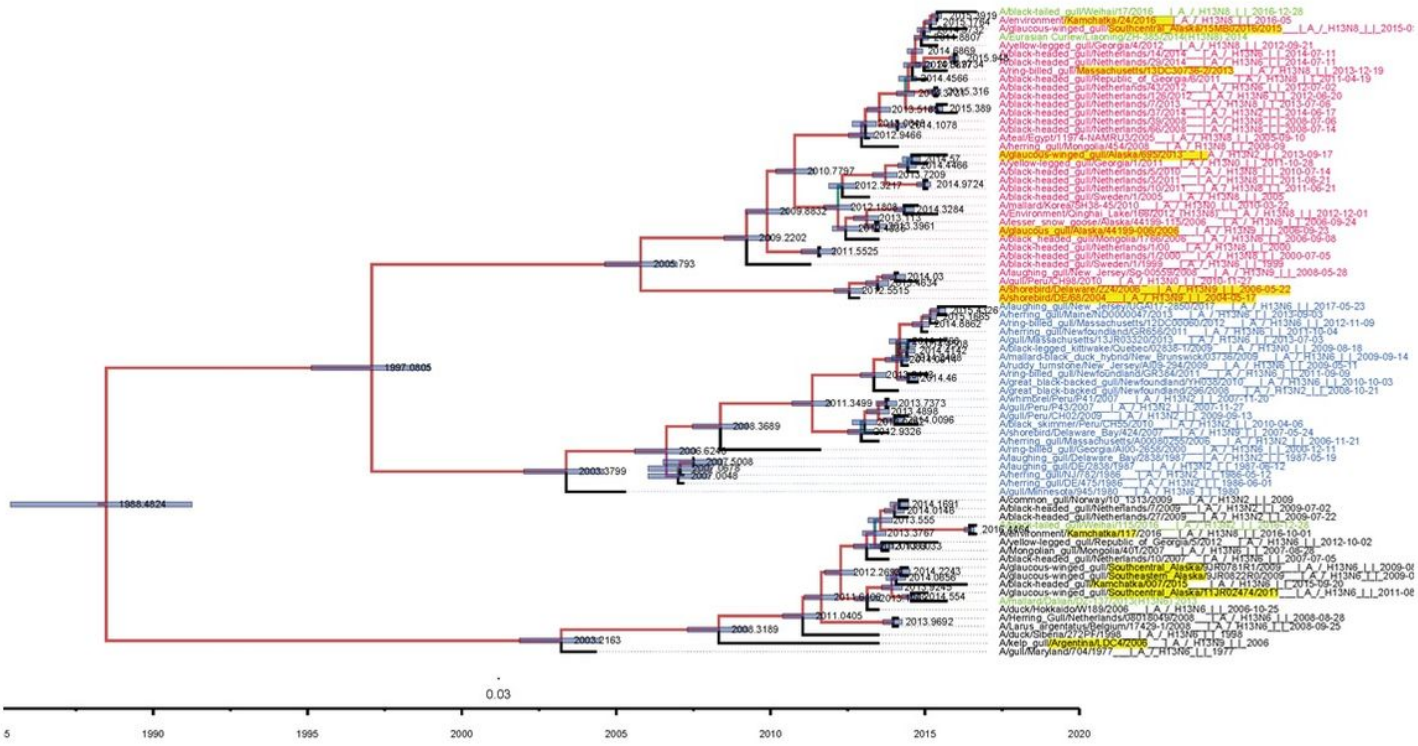

Figure 1

Temporally structured maximum-clade-credibility phylogenetic tree (years on the horizontal axis) of the haemagglutinin gene of AIV H13 subtypes. Both the isolates used in this study and isolates collected in China (A/black-tailed_gull/Weihai/115/2016 (H13N2) and A/black-tailed_gull/Weihai/17/2016 (H13N8)) are coloured green. The Bayesian posterior probabilities by which the associated taxa clustered together are shown next to the branches.

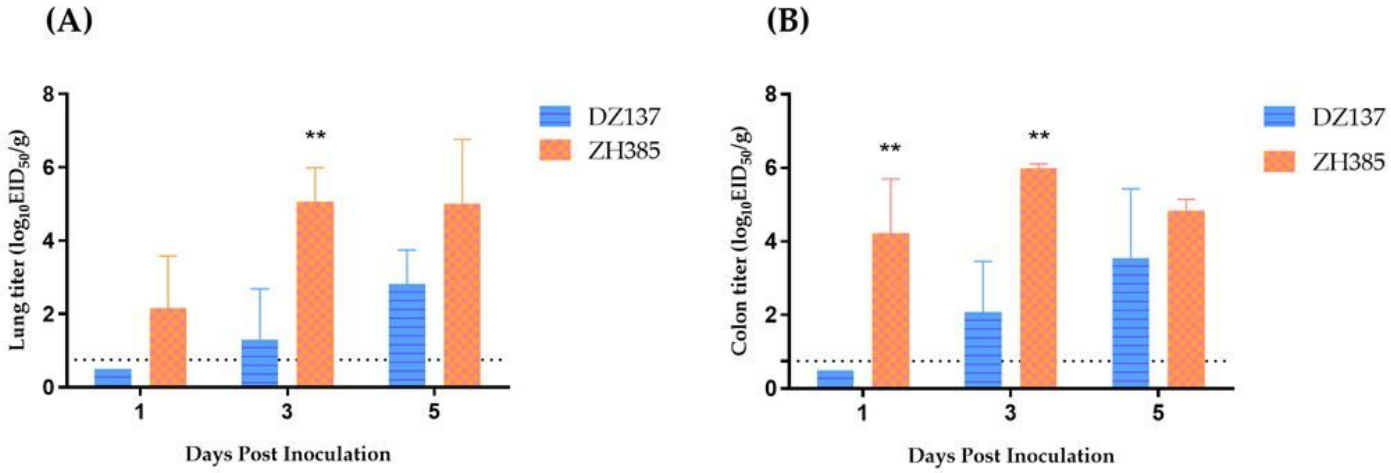

Figure 2

Replication of the AIV H13 subtype in 1-day-old chicks during the challenge experiments. Chicks were divided into two groups. Chicks (n=3) and inoculated intranasally with 50μl of AIV H13 subtype at 106.0 EID50 . (a) Virus titres in lung tissues were detected at 1, 3 and 5 dpi. (b) Virus titres in colon tissues were detected at 1, 3 and 5 dpi. The virus titres were calculated by the Reed-Muench method. The figures show the means log10EID50/g ± standard deviations (SDs). The limit of virus detection was 0.75 log10EID50/g. * indicates p<0.05. ** indicates p<0.01, *** indicates p<0.001.

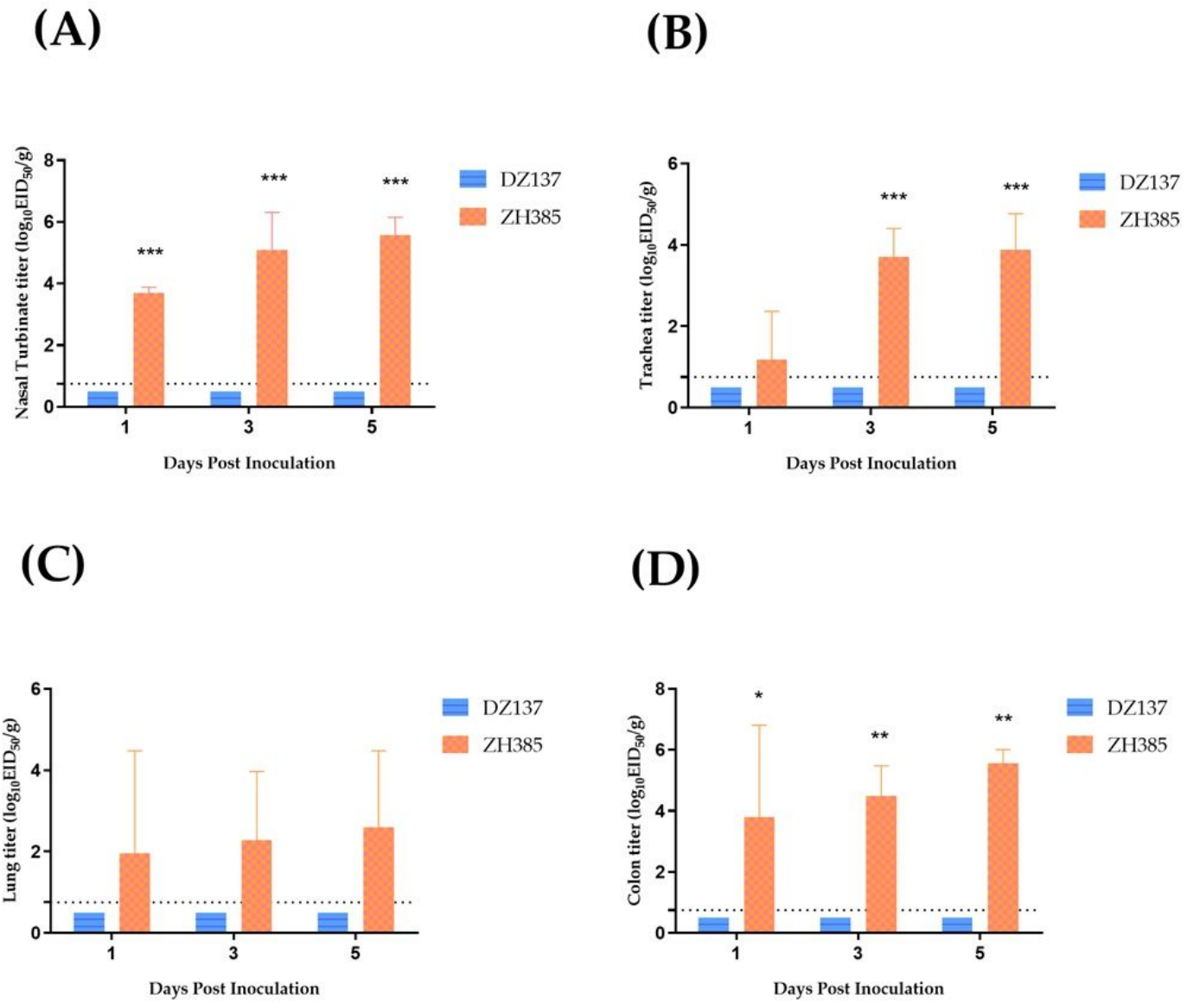

Figure 3

Results of the 10-day-old chick challenge experiment. Chicks were divided into two groups. Chicks (n=3) and inoculated intranasally with 50μl of AIV H13 subtype at 106.0 EID50. (a) Virus titres in nasal turbinate tissues were detected at 1, 3 and 5 dpi. (b) Virus titres in tracheal tissues were detected at 1, 3 and 5 dpi. (c) Virus titres in lung tissues were detected at 1, 3 and 5 dpi. (d) Virus titres in colon tissues

were detected at 1, 3 and 5 dpi. The virus titres are shown as the means log10EID50/g ±SDs. The limit of virus detection was 0.75 log10EID50/g.

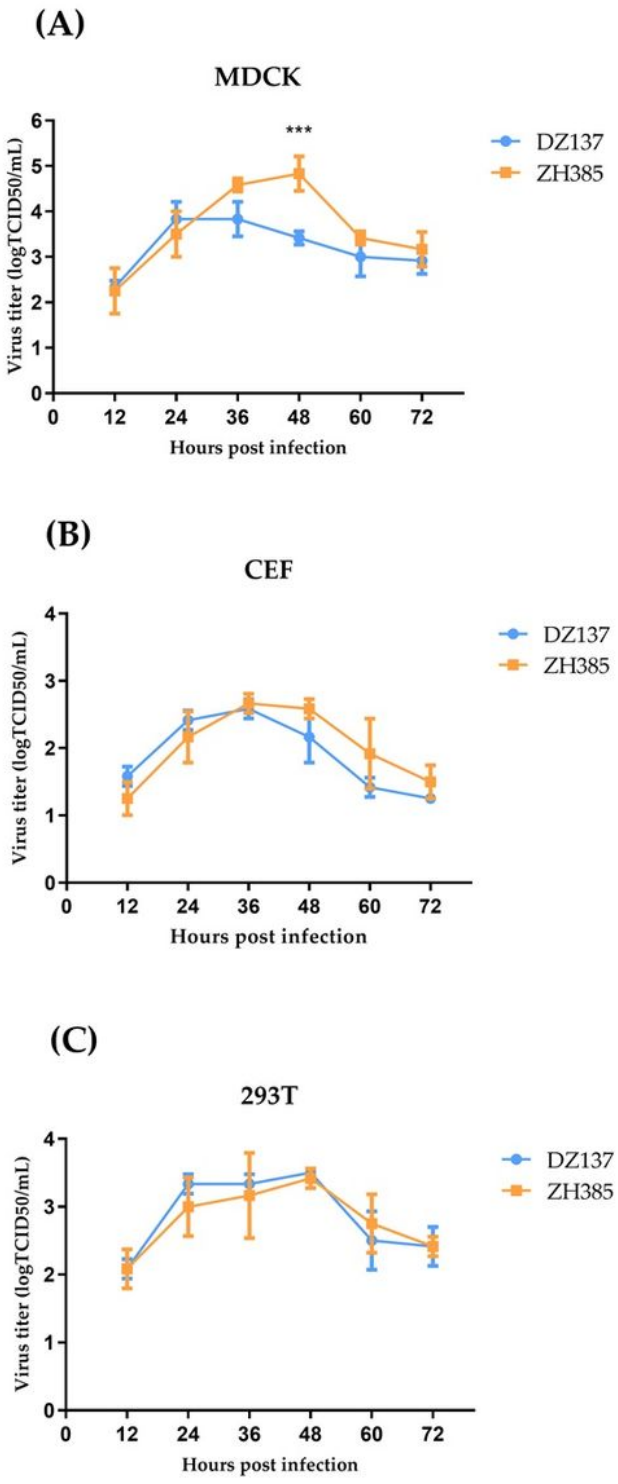

Figure 4

Viral growth kinetics of the ZH385 and DZ137 strains in MDCK, CEF and 293T cell cultures. MDCK, CEF and 293T cells were infected at an MOI of 0.01. Cells were inoculated at an MOI of 0.01 TCID50/cell with DZ137 or ZH385. The supernatant of the plates were collected at 12, 24, 36, 48, 60 and 72 h. We

determined the virus titres in MDCK cells by the TCID50. (a) MDCK cells, (b) CEF cells and (c) 293T cells. The virus titres are shown as the means log10TCID50/ml ±SDs. * indicates $p<0.05$. ** indicates $p<0.01$, and *** indicates $p<0.001$ in comparison with the values for the DZ137 virus.

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