Gene differentiation, reassortment, and evolution of H5N6 Avian influenza virus in China during 2016-2019

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Abstract

Continuous monitoring and analysis of the evolution, recombination and epidemic of avian influenza virus will help to prevent and control the emergence of new avian influenza virus. The genes of 153 H5N6 avian influenza viruses selected from our long-term surveillance data and the GISAID for the 2016-2019 period were dissected in detail. Our analysis shows that the genes of H5N6 AIVs have been divided into more lineages, and that mutations leading to amino acid replacement of hemagglutinin and neuraminidase occurred mainly between 2017 and 2018. All H5N6 viruses are differentiated into at least 20 distinct genotypes derived from different evolutionary pathways. G1 genotype has replaced other genotypes to become dominant genotype of AIV. The results of animal experiments show that H5N6 of different genotypes has high virulence to mice. which indicates that H5N6 AIVs, especially the reassortment strains, are a potential threat to public health.

Background

Influenza A viruses are RNA viruses.To date, 18 major antigenic variants of HA and 11 of NA have been detected(Fouchier et al., 2005; Tong et al., 2012). In addition, more than 20 other mammalian host species as well as various poultry species can be infected(Wille & Holmes, 2019). The first outbreak of the highly pathogenic H5N1 avian influenza was reported in Guangdong Province in 1996. The following year human infection of the H5N1 virus was reported in Hong Kong(Xu,Subbarao,Cox, & Guo, 1999). After that, the viral genes divided rapidly and produced multiple lineages (Clades 0-9)(Duan et al., 2008). Reassortments with other low pathogenic avian influenza produced numerous genotypes. Clade 2.3.2 H5N1, which is associated with the outbreak of avian flu of Lake Qinghai, China, 2005, astonished the world, in that migratory birds were observed with the infection, and over 6,000 birds died. Then, Clade 2.3.2 viruses evolved into Clade 2.3.2.1, which diversified into 2.3.2.1a, b, and c in 2009(Bi,Chen, & Zhang et al., 2016; Bi,Chen, & Zhang et al., 2016). The first isolation of Clade 2.3.3.4 was reported in Eastern China in 2010, and then spread to all parts of the world(Lee,Bertran,Kwon, & Swayne, 2017). Bi et al. reported that H5 has reassorted with H6N6 and H9N2/H7N9 from 2013 to 2016, resulting in a large number of genotypes. In China, 34 genotypes have been identified(Bi,Chen, & Wang et al., 2016). In recent years, clade2.3.4.4 and clade2.3.2.1 still can be detected in China(Bi et al., 2016; Sun et al., 2016). According to the ministry of agriculture of China, six outbreaks of highly pathogenic avian influenza have occurred in Sichuan, Xinjiang, and Hunan, China in January and February of 2020, five of which were caused by H5N6 (including four outbreaks in wild birds) and one by H5N1. A total of 6,340 poultry died, and 20,089 were culled. Two poultry outbreaks resulted in the deaths of 6, 340 poultry, and a total of 20,089 poultry were disposed of in a bio-safety way. Four outbreaks killed 48 wild birds(http://www.moa.gov.cn/gk/yjgl_1/yqfb/).

Bi et al. studied the epidemiology of H5N6 in China from 2013 to 2016, and analyzed in detail the geographic distribution and reassortment evolution of the virus in live poultry markets(Bi et al., 2016). They and many other studies have shown that live poultry trade markets play an essential role in the proliferation of viruses, and people who work, live, or have long-term contacts with the live poultry trade market (LPM) are more likely to be infected by the virus than the general population(Gao, 2014; Kim et al., 2018; Shi et al., 2018). The LPMs have played an essential role in the evolution and reassortment of avian influenza(Qi et al., 2018; Shi et al., 2018). The constant surveillance of the LPM is an important procedure in the epidemiological study of avian influenza and public health safety.With this in mind, we collected oropharyngeal and cloacal swabs from an LPM every two weeks in the Guangdong province and samples from poultry farmers in other provinces of China during 2016-2019. We used these data to analyze in detail the genetic evolution reassortment ,prevalence and pathogenicity in mice of H5N6 avian influenza virus.

Research methods

Sample collection and virus isolation

We collected oropharyngeal and cloacal swabs of poultry (including chickens, ducks, and geese) from live poultry markets in the Guangdong Province during 2016-2019, which were kept in PBS with penicillin and streptomycin. We also collected swabs or tissue samples from sick or dead poultry in farms. The collected swabs were centrifuged, and 0.2 ml of the supernatant was inoculated into nine to 10-day-old SPF chicken embryos. After 48-72 hours of culture at 37°C, the allantoic fluid was collected, and Hemagglutinin-positive samples were further confirmed.

Sequence and phylogenetic analysis

A Simply P total RNA extraction kit was used to extract RNA according to manufacturer instructions. The reverse transcription was performed in a total volume of 60 μL containing 39 μL of RNA, 3 μL of dNTPs, 3 μL of 12 bp (10 nmol), 1.5 μL of M-MLMLV, 1.5 μL of RRI, and 12 μL of 5*buffer. The liquid was mixed, then placed in a 42°C water bath for 2 to 4 hours. We used published universal primers and primers designed for different subtypes in the literature, as well as primers designed based on the downloaded sequences from the influenza database (www.gisaid.org).

PCR amplification of cDNA was performed in a total volume of 50 μL and contained 25 μL of Premix Taq, 20 μL of dd H_2O, 4 μL of upstream and downstream primers, and 1 μL of template cDNA. The PCR program comprised 34 cycles of 95°C for 50 s, 55°C for 50 s, and 72°C for 1 m and 50 s. The PCR products were separated on 1% agarose gels by electrophoresis and visualized using a UV transilluminator. The sequence was measured by generation 1 sequencing technology, and the sequence result was processed with the Lasergene 7.1 software package.

The sequences of partial H5 subtype AIVs with whole genes from 2016 to 2019 were collected from the Global Initiative on Sharing Avian Influenza Data (GISAID) database (www.gisaid.org). We used PhyloSuite data processing software for constructing phylogenetic trees(Dong Zhang & Wang, 2019) . MAFF (Katoh,Misawa,Kuma, & Miyata, 2002), which is included in the software, was used to compare the nucleotide sequences. Modefinder was used to find the best model and using maximum likelihood in IQ-tree (Kalyaanamoorthy,Minh,Wong,von Haeseler, & Jermiin, 2017; Nguyen,Schmidt,von Haeseler, & Minh, 2015), eight phylogenetic trees were constructed. ITOL was used to beautify the phylogenetic tree (https://itol.embl.de).

We calculated the timescale of avian influenza virus clades and evolutionary rates using BEAST 1.1.04 (Suchard et al., 2018). import eight sequence fragments, tag sequences of each sampling time, chose a relaxed molecular clock model, set MCMC chain length at 500000000. The acquisition interval was 50,000 and resulted in 10,000 trees eventually. The generated XML file was run by BEAST, and the log file was opened by Tracer1.7.1(Rambaut,Drummond,Xie,Baele, & Suchard, 2018) to verify that the ESS value of each parameter was > 200. We opened the generated tree file using TreeAnnotator v1.10.4 software, burn in 10% to generate the tree file, and placed it in Figtree 1.4.4 to open(http://tree.bio.ed.ac.uk/software/figtree/).

Discriminant analysis of principal components (DAPC) analysis

Year division, sequence comparison, deletion or incomplete fragments of influenza sequences by MAGE, preparation of a matched sequence file, and a file containing the classification group names, analysis the sequence using the DAPC extension package in the R language(Jombart,Devillard, & Balloux, 2010).

Animal experiment

BALB/c mice (14-16 g) were divided into five groups, four of which were the challenge group, and one was the control group, with 12 mice in each group. The H5 subtype influenza virus was diluted to 10^6EID$_{50}$. After anesthesia with isoflurane, the virus was dripped into the nasal cavity. Twelve mice in each group were observed, and we recorded the mortality and body weight for 14 days. On the third and fifth day after the attack, we took three mice from each group randomly and collected the heart, liver, spleen, lung, kidney, brain, and turbinate bone to detect the virus titer in each organ.

Results

Sample Collection

We collected samples of Oropharyngeal and cloacal swabs from live poultry markets in the Guangdong Province, including Guangzhou, Shenzhen, Foshan, and Zhanjiang cities. A small number of samples of clinically suspected avian influenza were collected from other Provinces (including Jiangsu, Guangdong, Shandong, Henan, Hebei, Guangxi, and Henan) during 2017-2019, China. All of the samples were inoculated into 9-day-old embryonated chicken eggs for virus isolation individually. The HA subtype of the viruses was confirmed using the hemagglutinin inhibition (HI) test. H5N6 strains were isolated from live poultry markets. In addition, H9N2(Jin et al., 2020), H7N9(Qi et al., 2018), H6N6, H6N2, H3N2, H3N8(Li et al., 2019), H1N1, and other subtypes were also isolated. In this study, ten strains of the H5 subtype avian influenza were selected to sequence the full gene sequence, and four strains were selected to determine the virulence by developing animal experiments[EPI_ISL_402100, EPI_ISL_422383-EPI_ISL_422396].

Phylogenetic Analysis

We performed a phylogenetic analysis of the H5N6 viruses sequenced in this study, along with 144 complete H5N6 genomes between 2016 and 2019 (available from GISAID [The GISAID Initiative, 2016]) (Table S1). The HA phylogeny revealed that nearly all of the H5N6 sequences fell within Clade 2.3.4.4 [Figure 1(A)]. The HA gene of the H5N6 strains can be further divided into two groups (major and minor). The time of differentiation of the two groups of the HA gene was calculated by BEAST during May 2014 (Figure S2). Strains from 2018 to 2019 were mostly distributed in the minor group from the collection time analysis of the sequence. Except for one strain A/duck/guangdong/17460/2017 in Clade H6N6, the neuraminidase (NA) phylogeny showed that all of the N6 genes from the Chinese poultry belong to the Eurasian lineage [Figure 1(B)]. The N6 genes could be further classified into two groups, whereas the H5N6 NA genes belong to two groups: H5N6-N6/Major and H5N6-N6/Minor.

Interestingly, 69 of 153 H5N6 viruses (45.1%) had HA, and NA genes from the H5N6-H5/Minor and H5N6-N6/Major lineages (H5/Minor:: N6/Major), 49 of 153 (32%) have the H5/Major:: N6/Major combination and 35 of 153 (22.9%) have the H5/Major:: N6/Minor combination[Figure 1(A)][Table S1]. All of H5/Major: N6/Minor viruses were isolated during 2016, the H5/Major::N6/Major virus were mainly isolated during 2016-2017, and only two of the H5/Major::N6/Major viruses were isolated in January 2018. The H5/Minor::N6/Major viruses were isolated during 2016-2019[Table S1]. Our results showed that there was a distinct time scale distribution of the HA-NA gene combinations and that the H5/Minor:: N6/Major H5N6 viruses were prevalent in China [Table S1][Figure 2]. Both HA and NA genes showed three gene differentiation events between 2016 and 2019 by ML and BL phylogenetic tree analysis results. The evolution of the H5 influenza virus is similar to that of seasonal influenza, which typically only divides into a few small branches and evolves in one direction. These data illustrate the rapid evolution of influenza viruses in the current vaccine immune environment.

Genetic Reassortment and Virus Evolution

We performed phylogenetic analyses of the internal genes of H5N6 viruses and classified them into different lineages according to tree topology and Bayesian phylogenetic analyses [Figure S1][Figure S2]. Each internal gene belongs to at least two lineages, one from circulating H5 AIVs and the other from LPAIVs, including H6 and H9N2/H7N9. In general, the H5/Minor::N6/Major viruses and H5/Major::N6/Major viruses have nearly all of the internal genes derived from H5N6 viruses, 23 of 34 of the H5/Major::N6/Minor H5N6 had H9N2/H7N9-derived internal genes [Figure 1(A)]. Some H5N6 isolates contained internal gene(s) from LPAIVs circulating in waterfowl. According to lineage classification of phylogenetic trees and Bayesian phylogenetic analyses, we classified all of the H5N6 viruses into 20 genotypes [Figure 2][Table S1]. These genotypes are G1(53), G1.1(4), G1.2(4), G1.3(1), G1.4(4), G1.5(3), G2(9), G2.1(30), G2.2(3), G3(1), G3.1(17), G3.2(3), G3.3(3), G3.4(2), G3.5(4), G3.6(3), G3.7(1), G3.8(1), G2.1.1(6), and G2.1.2(1)[Figure 2]. 81 of 153 comprised 15 genotypes have been isolated since 2016, 39 of the viruses were classified into six genotypes (G1(28), G1.1(2), G1.2(1), G1.3(1), G2.1(7)) and had been isolated during 2017, and 27 of 153 virus comprised six genotypes (G1(17), G1.1(2), G1.2(3), G1.5(3), and G2.1(2)) and had been isolated during 2018. Only six viruses (G1(6)) were isolated during 2019 [Figure 2]. The number of genotypes decreased between 2016 and 2019 in terms of the genetic diversity of the viruses, which means that the strain may have evolved to become more stable. The G1 genotype HA-NA and the combination of internal genes may be best suited to the current adaptation of the strains.

Bi et al. showed that, from 2013 to 2016, a large number of H5N6 reassortment strains emerged(Bi et al., 2016). Our research confirmed that the H5N6 reassortment strain types in 2016 were very numerous. By contrast, the genotypes of this strain gradually decreased from 2017 to 2019 [Figure 2]. Influenza mainly occurs in winter, and we set July 2015 to June 2016 as one influenza season. It can be seen that all of the strains of the G3 series mostly (30/33) occurred in this winter. Many genotypes (G3.1, G3.2, G3.3, G3.4, G3.5, G3.6, G3.7, and G3.8) emerged by the G3 strain reassortment with H9N2 and other subtypes of AIVs[Table S1]. The frequency of G3.1 posed a major advantage. Only three strains of G3 were isolated during the subsequent influenza season. The G2 and G2.1 strain appeared in early 2016 and the winter of 2016. In the winter of 2016, two reassortment genotypes, G2.1.1 and G2.1.2, emerged by the reassortment of G2.1 and H9N2 with other subtype strains and G2 with H9N2. Four genotypes (G1.1, G1.2, G1.3, and G1.4) emerged by G1 reassorted with H6 and other subtypes of the AIVs during July 2017 and June 2018. Only the G1.4 genotype and the new G1.5 genotype were isolated in the winter of 2018 [Figure 2][Table S1].

DAPC and Mutation of Surface Genes

The HA and NA proteins of AIVs play a critical role in virulence, transmissibility, and antigenicity. The variation and combinations between HA and NA also primarily determined the pandemic strain of the virus. We first divided the 2016-2019 strain into epidemic seasons (that is, 2015.7-2016.6 as a popular season, and 2016.7-2017.6 as the next popular season). We calculated the changes of HA and NA in four popular seasons from 2016 to 2019 through DAPC analysis. The horizontal axis represents the first principal component, and the vertical axis represents the second principal component. It can be seen that the strains are clustered in each epidemic season [Figure 3].

The HA principal component analysis showed a significant difference in the first principal component in 2016, 2017, and 2018. The 2019 strain showed a greater distance from the 2018 strain in the second principal component, while the difference in the first principal component was smaller [Figure 3(A)]. This means that the variation of the strain varies significantly from 2016 to 2018 yearly, in contrast to the smaller variation in 2019. This variation also largely represents the evolution and antigenicity of the strain. The principal component analysis of NA was similar to that of HA. The difference in the first principal component between 2016 and 2017 is increasing, There is no significant difference in the first principal component between the 2018 strain and the 2019 strain, but there are some significant differences in the second principal component [Figure 3(B)].

A total of 22 amino acid sites in the HA gene and 13 amino acid sites in the NA gene with obvious variation were identified from 2016 to 2019 and were compared and analyzed [Figure 4(A)(B)]. The results showed that the amino acid sites changed regularly. The HA amino acid changes were mainly focused on the HA1

area, positions 83, 115, 120, 123, 126, 127, 138, 140, 141, and 269 have been proven to cause antigenic drift in other strains of research (Beato et al., 2013; Velkov et al., 2013), which may cause significant changes in the antigenicity of the virus.

In summary, we believe that the virus has undergone a rapid evolution from 2016 to 2018, that the amino acid substitutions were basically completed in 2017-2018 [Figures 3, 4], and that the strain has basically evolved to adapt itself to the environment. Consequently, the strain changed considerably less in 2019. We suppose that the reason for the big difference in strains from 2016 to 2018 may be related to the current vaccination environment. Vaccines are constantly being updated. From 2017, the H5-H7 dual vaccine was mainly used for H5 and H7N9. Research by Shi et al. showed that the vaccine controlled the outbreak of H7N9 to a large extent and greatly reduced the cases of human infection with the virus(Shi et al., 2018). We speculate that H5N6 has evolved to adapt to the current vaccine immune environment.

H5N6 Has Become Highly Virulent in Mice

To understand the pathogenicity of the different epidemic H5N6 of avian influenza to mice, we selected three H5N6 strains (A/duck/jiangsu/18012/2018, A/duck/guangdong/18217/2018, A/chicken/guangdong/18231/2018) and one H5N1 strain (A/duck/shandong/17771/2017) as control, hereinafter referred to as 18012,18217,18231,17771 respectively) to infect in mice and detect the EID_{50} in the different organs.

Three strains of H5N6 showed different virulence in mice.18012 had the strongest virulence among the four strains as 50% of the mice died within three days of challenge, and all of the mice died in four days of challenge [Figure 5 A, B]. 18012 maintained a high virus concentration in the lungs and brain in the 3-5 dpi period. The strain 17771 was slightly less virulent than 18012,all of the mice died in 5 days of post-challenge.18217 showed a moderate virulence in mice, which caused partial death at the 7 dpi, 30% survived in 14 dpi. 18231 showed a weak virulence in mice, as 100% were alive 14 dpi [Figure 5A, B] and only showed decreased body weight. Four strains had detected different viral levels in different organs. And the concentration was highest in the lungs. Strains 17772 and 18012 were detected in the brain at 3 dpi, whereas strains 17772, 18012, and 18217 were detected in the brain at 5 dpi [Figure 5C, D]. This suggests that 17772, 18012, and 18217 can cross the blood-brain barrier.

Discussion

Highly pathogenic AIVs have been affecting the world's poultry industry and also brought enormous challenges to the public health security since they accidentally moved from animals to human, causing a first outbreak in Guangdong, China, in 1996(Shortridge et al., 1998; Webster & Govorkova, 2014; Xu et al., 1999). Fortunately, there is no evidence of human-to-human transmission of the avian influenza virus. The H5 avian influenza virus has evolved rapidly from Clade 0 to the present Clade 2.3.4.4 and Clade 2.3.2.1(Group, 2012), and frequent reassortments of AIVs have been the leading cause of several epidemics(Bi et al., 2016; Karo-karo et al., 2019; Qi et al., 2018; Shi et al., 2018). The highly pathogenic avian influenza virus prevalence in China is an extraordinarily complicated problem to solve. We focused on the prevalence, gene differentiation, and reassortment of H5N6 in China, based on the available data for 2016-2019. We gathered evidence on a new reassortment virus complex diversity produced by frequent reassortment of different subtypes AIVs. Cases of both poultry and human infections are largely related to the complexity of the reassortment.

The rate of evolution of avian influenza also deserves close attention. The rate of evolution of H5N6 HA and NA genes is 7.3262E-03 and 6.9073E-03, respectively. And the rate of evolution of H9N2 HA and NA is 2.19E-03-2.83E-03, and is 3.3E-03-3.7E-03, respectively(Jin et al., 2020). H5N6 compared to H9N2 has a faster rate of evolution in HA and NA genes.This has a lot to do with the clade of HA and NA genes rapid differentiation is closely linked. And internal gene is relatively complicated, due to the restructuring of the LAIVs, especially the part of basic internal genes from H6N6, H6N2, H9N2, most notably the two H6N6 viruses from which the immune pressure is relatively small. According to the study of Bi et al(Bi et al., 2016), the PB1 gene of H5N6 is basically derived from H5N1, and PB2 is partly derived from H6N6, which may be the reason why our study showed that PB1 has significant gene differentiation compared with other genes.

In this study, a total of 16 gene sequences from humans [Figure 6 A], including eight strains isolated during 2016 (six isolates from January to April and two from November and December), two isolated since November, seven of eight strains were new reassortment H5N6 viruses that contain the internal genes fragments from H9N2 [Figure 6 A], two strains isolated during 2017 which are of the G1 genotype, and six strains isolated during 2018. These last six strains comprise three strains with the G1 genotype and three with the G1.4 genotype, and differed from G1 due to a PA gene from other AIVs. We propose that the virus can move from poultry to humans after new reassortment. The LPAIV provides the gene segments for the reassortment event, which is a problem that cannot be ignored. The current epidemic strain is of the G1 genotype, which is of great importance because G1 can also infect humans. From our mice experimental data, all of the strains are H5N1, except one, three H5N6 belong to the G1, G1.2, and G1.4 genotypes, and show differences virulence in mice. Apparently, one strain is unable to represent all of the genotype fully, but what caught our attention is that H5N6 shows high virulence, which potentially increases the risk of infection to humans.

Waterfowl have long been considered the natural reservoir of avian influenza, and our research results show that the duck, goose, and wild bird waterfowl contain numerous genotypes, respectively 8, 3, and 3 [Figure 6B]. Two major epidemic genotypes, G1 and G2.1, which are distributed in the three kinds of waterfowl. There were no G2.1 genotypes in human and chicken sources, and we only found H9N2 gene reassortment strains isolated from chicken, people, and the environment. We speculate that the virus could have spread from ducks to chickens with H9N2 reassorting and the production of the G2.2, G3.1, and G3.3 new genotypes. The virus could then have spread to people through chickens or the environment. G1.4 and G3.2 are distributed only in ducks, humans, and the environment, and from ducks or the environment can spread to people [Figure 6B]. The pandemic genotypes G1 and G2 are distributed in wild birds, which probably means that the virus continues to circulate in wild birds and waterfowl. The contact between waterfowl and landfowl mainly occurs in LPMs, which are the main place for virus reassortment and cross-host transmission of avian influenza. Both previous research and the work of our group confirm this scenario.

H5N1 and H5N6 can be highly pathogenic and induce high mortality in chicken. Vaccination is one of our main measures against the highly pathogenic avian influenza(Alarcon et al., 2018; Arai et al., 2019; Shi et al., 2018). The Chinese vaccine is upgraded continuously, following the evolution of the influenza virus, and the vaccine can protect poultry effectively, reducing the virus isolation rate greatly. However, there are still outbreaks of HPAIV every year, according to statement from the Ministry of Agriculture. Our laboratory has received from the farm of clinically suspected cases of AIV. Through analysis, we confirmed that the part of the clinically suspected case really is HPAIV, and most isolated strains from the clinically suspected cases are reassortment viruses, which may explained why there are still bits of outbreaks of HPAIV. Some may unuseed vaccines,and these reassortment strains may escape the vaccine's immune response. A large number of reassortment viruses can be detected each year. And the vaccine obviously cannot resist all of the new reassortment viruses. Even so, occasional outbreaks can be addressed by rapid culling, disinfection, and landfills, and the effect of preventing the spread of the virus is also significant. Thus, we need to raise enough awareness about the occasional outbreak, and only quick and effective prevention measures can prevent a possible outbreak.

Conflict of interest

The authors declare not conflict of interest.

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Author Contributions

Xiao Li, Jianglin Chen, Jing liu, Lezi Yin, Shumin Xie, Mengmeng Zhang, Siru lin, Yiqi liao, Meifeng liang, Xuanjiang Jin, Jingkai Hu, Jinfeng Wang and Yifan Wu. conducted the experiments. Xiao Li and Weixin Jia analyzed the data; Xiao Li, Wenbao Qi, Ming Liao and Weixin Jia wrote the paper.

Data Availability Statement

Used in this study data are derived from the Global Initiative on Sharing Avian Influenza Data (GISAID's EpiFlu Database), Moreover, the sequence data generated in this study has been uploaded to GISAID's EpiFlu Database[EPI_ISL_402100, EPI_ISL_422383-EPI_ISL_422396].

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The South China agricultural university's guidelines for the Care and Use of Laboratory Animals were followed.

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Figure 1. Phylogenetic analysis of H5N6 Influenza Viruses.

1. Phylogenetic tree of hemagglutinin genes of H5N6 viruses sequenced in this study; the clade origins of each gene segment are indicated by different colored bars.
2. Phylogenetic tree of neuraminidase genes of H5N6 viruses sequenced in this study.

Figure 2. Reassortment of the H5N6 AIV.

The eight gene segments are PB2, PB1, PA, HA, NP, NA, M, and NS (horizontal bars starting from top to bottom of the virion). Different colors represent different virus lineages.

Figure 3. Scatterplots resulting from the DAPC.

Individual isolates from the same AVIs season are depicted as unique color shapes and surrounded by 95% inertia ellipses. The PCA and DA eigenvalues inset panels show the overall variability among individuals and the relative capture of variance for each discriminant function, respectively. The y- and x-axes indicate the first and second discriminant principal components, respectively, which best summarize the differences between clusters while neglecting within-cluster variation. (A) and (B) represent HA and NA genes, respectively.

Figure 4. SeqLogo analysis of amino acid substitutions in H5N6 viruses.

, (B) HA and NA (H5 numbering) are shown.(A) The first three sites (9, 12, 16) in HA signal peptide,The last site (169) in HA2,others in HA1.

Figure 5. Replication and virulence of the H5N6 viruses in mice.

Weight changes in mice after challenge, (B) Survival rate of mice after challenge. (C) The virus content in different organs in the mice on the third day of the challenge. (D) The virus content in various organs in mice on the fifth day of the challenge.

Figure 6. Genotype of H5N6 viruses in different hosts.

Schematic for the genetic Source of human H5N6 isolates. (B) Genotypes of H5N6 viruses isolated in humans, domestic ducks, domestic chickens, domestic geese, environment, and wild birds. The eight gene segments are PB2, PB1, PA, HA, NP, NA, M, and NS (horizontal bars starting from top to bottom of the virion). Different colors represent different virus lineages.