Characterization of Four Novel H5N6 Avian Influenza Viruses with the Internal Genes from H5N1 and H9N2 Viruses and Experimental Challenge of Chickens Vaccinated with Current Commercially Available H5 Vaccines

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Abstract:

Since 2014, highly pathogenic avian influenza H5N6 viruses have been responsible for outbreaks in poultry. In this study, H5N6 avian influenza virus strains were isolated from fecal samples of sick white ducks and dead chickens in Shandong in 2020. These H5N6 viruses were triple-reassortant viruses that have not been previously characterized. Their HA genes were derived from the H5 viruses and were closely related to the vaccine strain Re-11. Their NA genes all fell into the N6-like lineage and the internal gene were derived from H5N1 and H9N2 viruses. They all showed high pathogenicity in mice and caused lethal infection with high rates of transmission in chickens. Moreover, the SPF chickens inoculated with the current used vaccine in China were completely protected from these four H5N6 viruses. Our study indicated the necessity of continued surveillance and the importance of timely update of vaccine strains in poultry industry.

Keywords: H5N6 influenza virus; phylogenetic analysis; Pathogenicity; Protective efficacy

Introduction

Influenza A viruses (IAVs) belong to the family of *Orthomyxoviridae,* members of which have segmented, negative sense, single stranded RNA genom further categorized into 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes based on the serological glycoproteins (Alexander, 2007; Tong 2013). IAVs are widely distributed in nature and can be isolated from a wide variety of birds and mammals, including poultry, humans, pigs, horses, dogs, cats, tigers, and sea mammals (Lang, Mistry, Haslam, & Barclay, 2019; Taubenber 2010). IAVs display different pathogenicity and transmissibility depending on virus strain. Avian IAVs exclusively subtypes H5 and H7 subtypes, were highly pathogenic avian influenza virus (HPAIV), that usua contagious systemic disease with significant morbidity and mortality in susceptible populations, resulting in severe economic losses (Alexander, 2007).

The Gs/Gd-lineage (prototype strain A/goose/Guangdong/1/96) of H have caused continuous outbreaks in poultry and wild birds and have been reported in more than 70 countries in the world since 1996. The first outbreak occurred goose farm in Guangdong province, China (WHO, 2016). Since then, the Gs/Gd-lineage has undergone significant genetic diversification and antigenic drift, and has evolved into 10 distinct clades (0–9) with subclades (Wood et al., 2017). Since 2008, multiple novel H5 subtypes (named H5Nx HPAIVs) of Gs/Gd lineage belonged to subclade 2.3.4.4, especially the viruses bearing various NA subtypes li H5N3, H5N5, H5N6, H5N8, and H5N9, caused cumulative waves unprecedented magnitude among avian species accompanied by severe losses to the poultry industry around the world(Song et al., 2019; WHO, 2020). In addition, H5Nx HPAIVs could sporadically infect humans and may cause severe respiratory diseases and fatalities (Pan et al., 2016; Z. F. Yang, M 2015). From February 2014 to February 2020, there had been 24 confirmed cases of

humans infections, including (WHO, 2020). With continued incidence of avian influenza infection due to domestic and novel influenza A (H5) vi poultry, there is a necessity to remain vigilant in animal and public h surveillance should be warrant to detect human cases transmissibility and pathogenicity of these viruses.

China has been a dedicated leader in H5 avian influenza vaccine development and application. A series of inactivated vaccines (with seed viruses generated by plasmid-based reverse genetics) have been widely used to control H5 influenza viruses poultry in China and countries (C. Li, Bu, & Chen, 2014). To increase the efficacy of poultry vaccine, H5/H7 trivalent inactivated vaccines have been developed by using the viruses from clade 2.3.2.1 and clade 2.3.4.4 and H7 seed virus HPAIVs. The vaccines have been extensively evaluated for safety against challenges with different H5 and H7 viruses in the laboratory and field.

In this study, we performed phylogenetic analysis and assessed the replication and pathogenic potential of four H5N6 HPAIVs in chickens and mice. We also tested the commercial vaccines harboring HA proteins derived from clade 2.3.4.4 H5 AIV in specific pathogen free white leghorn chickens against the challenges with the four H5N6 HPAI viruses. The elucidation of the characters of the H5N6 AIVs will b helpful to disease control and surveillance, and the protection experiment of vaccine to novel H5N6 viruses prompts to update the vaccine strain in a timely manner.

Material and Methods

Ethics Statements

Six-week-old SPF female BALB/c mice were purchased from the Gua Medical Laboratory Animal Center in Guangzhou, China. Three-week-old and six-week-old chickens and 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs were purchased from were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd., China. All experiments were carri facilities in compliance with biosafety committee of Sou University approved protocols (SCAUABSL2019-006). The handling of chickens and mice were performed in accordance with experimental animal administratio

ethics committee of South China Agriculture University approved guideline.

Viruses and vaccine

The H5N6 viruses A/duck/Shandong/SD02/2019 (SD02), A/chicken/Shandong/SD03/2019 (SD03), and A/chicken/Shandong/SD04/2019 (SD04) were isolated from fecal samples of white ducks and dead chickens in Shandong, Eastern China in 2019. All isolated viruses were purified by three rounds of limiting dilution in specific-pathogen-free (SPF) chicken embryos. Virus aliquots were stored at -80 °C after collection. Values of 50% egg infective doses (EID_{50}) and 50% egg lethal doses (ELD_{50}) were calculated by the Reed–Muench method (Reed and Muench 1981).

The commercially available reassortant avian influenza virus (H5+H7) trivalent inactivated vaccine (cell culture-based vaccine, H5N2 Re-11 strain+Re-12 H7N9 H7-Re-2 strain) was provided by Guangdong Dahuanjie Biotechnology co. LTD (lot number 2020003).

Phylogenetic analysis

The full genomes of H5N6 viruses used in this study were sequenced by Shanghai Invitrogen Biotechnology Co., Ltd. DNA sequences were assembled and translated using Lasergene 7.1 (DNASTAR). Phylogenetic trees were generated by the distance-based neighbor-joining method using software MEGA 4.0 (Sinauer Associates, Inc., Sunderland, MA). The reliability of the tree was assessed by bootstrap analysis with 1000 replicates. Horizontal distances are proportional to genetic distance. The nucleotide sequences obtained in the present study are available from GenBank under the accession numbers (pending).

Animal experiment

Experimental infection of mice

To evaluate the morbidity and mortality of mice infected with four H5N6 viruses, the mice were randomly divided into four groups with twelve mice each. The mice were inoculated intranasally with 10^6 EID_{50} of virus in a 50 µl volume after light anesthesia with CO_2. Additionally, twelve mice inoculated with 50 µl PBS served as negative controls. Three mice in each group were euthanized at 3 and 5 days post-inoculation (DPI) to determine virus titers in brain, spleen, kidney, and lung. Briefly,

the collected organs were homogenized in pho
supplemented with antibiotics (a final concentration of 2,000 units/ml penicillin , and 2,000 units/ml streptomycin,) and were centrifuged at 1000 g for 30 minutes at 4°C to isolate supernatant fluids. The supernatant fluids of tissue were collected and titrated for virus infectivity in 10-day-old specific-pathogen-free (SPF) chicken embryos. The remaining mice were monitored for clinical signs, weight loss, and mortality for 14 days.

Experimental infection of chickens

Eight six-week-old SPF white leghorn chickens were inoculated intranasally with 100μl allantoic fluid containing 10^6 EID_{50}/100μl of the SD01, SD02, SD03, and SD04 viruses, respectively. At 24h post-infection, three contact chickens inoculated intranasally with 100μl phosphate buffered saline (PBS) were housed together with the inoculated chickens. At 3 days post infection (DPI), three infected chickens were euthanized to test for the virus replication in different organs, including hearts, livers, spleens, lungs, kidneys, brains, and tracheas. The remaining infected chickens in each group were observed for clinical symptoms for 14 days.

Oropharyngeal and cloacal swabs were taken from the chickens at 1, 3, 5, 7, 9, 11 and 14 DPI, and suspended in 1 ml PBS. All of the tissues and swabs were collected and titrated for virus infectivity in 10-day-old SPF chicken embryos. Seroconversion of the surviving birds on 14 DPI was confirmed by hemagglutination inhibition (HI) test.

Immunogenicity and efficacy of the vaccine in chickens against the four H5N6 viruses

Two groups of eighty 3-week-old white Leghorn SPF chickens were intramascular (i.m.) with 0.3 ml PBS or reassortant avian influenza virus (H5+H7) trivalent inactivated vaccine. At 28 days post-vaccination (p.v.), sera were obtained from all chickens to monitor the HI antibody against Re-11 standard antigen (Harbin Weike Biotechnology Development Company) using the methods described in OIE standard protocols. Meanwhile, ten chickens from the PBS group and ten chickens from the vaccinated group were challenged with 10^6 EID_{50} of four H5N6 viruses, SD01, SD02, SD03, and SD04, respectively. Oropharyngeal and cloacal swabs of all chickens were collected on days 3 and 5 post challenge (p.c.) for virus isolation. All

chickens were observed for clinical signs and survival for 2 weeks after challenge.

Statistical analysis

Statistical analyses were used by the GraphPad Prism 5.0 software (Gra
Software Inc., San Diego, CA, USA). Statistical analyses for virus titer in organs of
chickens and mice were performed by using a two-way ANOVA.
< 0.05 were considered significant.

Results

Genetic and phylogenetic analysis of the four H5N6 viruses

To understand the origin of the four H5N6 viruses, we performed blast analyses
and constructed eight phylogenetic trees using th
representative viruses available in the NCBI database.

The results demonstrated that the HA gene of the four H5N6 virus
nucleotide sequence similarities
A/chicken/Vietnam/HU9-847/2018(H5N6). The NA genes of the SD01 vi
SD02 virus, and the SD03 virus wer
A/duck/Fujian/3242/2007 (H6N6), with 91.5% to 92.
similarities. The PB1, PA, NP, M, and NS genes of the four H5N6 viruses
closely related to those of A/Muscovy duck/Vietnam/LBM636/2014(H5N1). The PB2
genes of these H5N6 viruses shared sequence similarities ranging from 98.4
98.5% with that of A/chicken/Qingyuan/zd201601/2016(H
Table S1).

Phylogenetic analysis of the HA gene showed that all of the H5N6 viruses in the
present study belonged to the clade 2.3.4.4 of the Asian HPAI H5 virus (Figure 1).
They fell into the same clade with the Re-11 vaccine strain. The NA genes were likely
originated from the H6N6 viruses of the Eurasia lineage (Figure S1A). The
genes of these viruses were uniquely derived from H9N2 viruses of the G
lineage (Figure S1B). The PB1, PA, NP, M, and NS genes of the four viruses a
originated from the H5N1 viruses, which circulated in Vietnam and China in 2
(Supplementary Figure S1B, S1C, S1D, S1E, S1F, and S1G).

Thus, the results suggested these H5N6 viruses were novel tri
viruses which bear genes from H5N1 viruses, H6N6 viruses, and H9N2

(Figure 2).

To identify possible determinants of host adaptation and virulence, the deduce amino acid sequences were analyzed. The HA cleavage sites of these H5N6 viruses were all RERRRKR↓GLF, meeting the criteria of HPAI viruses in chickens. T160A in the HA protein of these four H5N6 viruses might increase a binding specificity to human-like receptors(Gao et al., 2018). Some amino acid substitutions that may play a role in increasing the virulence in mammalians were shared by these four H5N6 viruses. These substitutions included a deletion residue (position 56-68) in the stalk region of the NA protein, N30D and T215A substitutions in the M1 protein, and P42S and D92Esubstitutions and amino acid deletion (position 80-84) in the NS1 protein (Table S2).

Pathogenicity studies in mice

To investigate the potential pathogenicity of these H5N6 viruses in humans, female SPF BALB/c mice, which are used as mammalian surrogates for humans in influenza research. The mice in the control group didn't show any clinical sympt weight loss during the course of the observation. SD01 virus, SD02 virus, S virus, and SD04 virus all caused obvious weight loss, and all mice died on 10 DPI, 9 DPI, 12 DPI and 8 DPI, respectively (Figure 3A and 3B). Three mice fro infected group and the control group were euthanized on 3 and 5 DPI to monitor viral replication in different organs. As expected, no virus was detected in the lungs from the control group. As a comparison, robust replication was observed in the lungs from mice infected with the four avian-origin H5N6 viruses. The mean titers of the SD01, SD02, SD03, and SD04 viruses in the lungs reached 6.3, $\log_{10}EID_{50}$/0.1ml on 3 DPI, respectively, a $\log_{10}EID_{50}$/0.1ml on 5 DPI, respectively. To determine if the H5N6 viruses co reach other organs of the mice after intranasal infection, we collected tissue samples of brains, spleens and kidneys from the control and the infected mice on 3 and 5 DPI. We found no evidence of viral replication in any of the organs tested in the control mice, however, H5N6 viruses were detected in the brains of the infected mice on both days. However, SD01, SD02, and SD03 viruses replicated to lower titers in brains comparing with SD04 virus on 5 DPI, with tite $\log_{10}EID_{50}$/0.1ml. The SD01, SD02, SD03, and SD04 viruses

efficiently in the mouse spleens. The mean titers reached 3.9, 2.8, 2
log10EID50/0.1ml on 3 DPI, respectively, a
log10EID50/0.1ml on 5 DPI, respectively. These four H5N6 viruses could be
detected in kidneys, with the mean tite
log10EID50/0.1ml on 3 DPI, respectively, a
log10EID50/0.1ml on?, respectively (Figure 3C and 3D).

These results suggested that the H5N6 viruses showed high virulence in mice and could establish replication in multiple organs. Therefore, these viruses m ability to infect other mammals including humans.

Pathogenicity studies in chickens

To investigate the pathogenicity and transmissibility o chickens, we inoculated SPF chickens intranasally with the fou inoculated chickens in each group showed typical clinical sympto depression, inappetence/reduction in food and water in discharges, dyspnoea and/or conjunctivitis, incoord neurological dysfunction. Chickens inoculated with 10^6 EID_{50} of the SD01 virus, the SD02 virus, the SD03 virus and the SD04 virus showed 100% (8/8) mortality within 4 and 5 DPI, respectively (Figure 4A). Viruses could be detected from the infected chickens in each in all tested organs at 3 DPI, including the hearts, livers, spleens, lungs, kidneys, brains, and tracheas (Table 1). All four H5N6 viruses efficiently in lungs; the mean titers reached 8.67 log10EID50/0.1ml, 8.50 log10EID50/0.1ml and 9.67 log10EID50/0.1ml, respectively. The four viruses could also replicate in the brains; the mean titers ranged fr log10EID50/0.1ml to 8.00 log10EID50/0.1ml. These H5N6 viruses also repl efficiently in the hearts, livers, spleens, kidneys, and tracheas of infected chickens. The mean titers were 7.67–8.67 $log_{10}EID_{50}$/0.1ml, 5.75–7.42 $log_{10}EID_{50}$/0.1ml, 6.67–7.50 $log_{10}EID_{50}$/0.1ml, 8.08–8.17 $log_{10}EID_{50}$/0.1ml, and 5.58–6.42 $log_{10}EID_{50}$/0.1ml, respectively.

Additionally, shedding of the four H5N6 viruses from the inoculated chickens was detected in oropharyngeal and cloacal swabs at 1, 3, 5, 7, 9, 11 and 14 DPI (Table 2). At 1 DPI, virus shedding could be detected in 7out of 8 inoculated chickens in the SD01 group from oropharyngeal and cloacal swabs. All of the 8 chickens in the SD02

group exhibited virus shedding observed from oropharyngeal swabs and 5 chickens from cloacal swabs. The SD03 virus was recovered from oropharyngeal swabs of all inoculated chickens, and from cloacal swabs of 5 out of 8 chickens. Virus shedding was detected in all the chickens evidenced by oropharyngeal and cloacal swabs. At 3 DPI, the four viruses were recovered from oropharyngeal and cloacal swabs from all chickens inoculated.

To understand the transmission of these H5N6 viruses, three naïve chickens were housed with the inoculated animals. During the observed period, contact chicke showed the clinical symptoms similar to those of infected chickens. All the contact chickens of the SD01, SD02, SD03, and SD04 viruses died within 6 DPI, 7 DPI, 8 DPI, and 6 DPI, respectively (Figure 4B).

Additionally, 2/3 of the contact chickens could be detected shedding the SI virus from oropharyngeal swabs and cloacal swabs at 1 DPI. All contact chickens could be detected the SD01 virus from oropharyngeal swabs and cloacal swabs at both 3 DPI and 5 DPI. No contact chicken could be detected shedding the SD02 virus from oropharyngeal swabs and cloacal swabs at 1 DPI. All contact chickens could be detected the SD02 virus from oropharyngeal swabs and cloacal swabs at 3 DPI, 5 DPI and 7DPI.

There was no contact chicken shedding the SD03 virus from oropharyngeal swabs and cloacal swabs at 1 DPI. All contact chickens could be detected the SD03 virus from oropharyngeal swabs and cloacal swabs at 3 DPI, 5 DPI and 7DPI. 2/3 of the contact chickens could be detected shedding the SD04 virus from oropha swabs and cloacal swabs at 1 DPI. All contact chickens could be detected the SD04 virus from oropharyngeal swabs and cloacal swabs at both 3 DPI and 5 DPI.

Overall, our results indicated that the tested H5N6 viruses were highly pathogenic to chickens, and could be transmitted among chickens by contact.

Protective efficacy of the current vaccine against the challenge of the four H5N6 viruses

To evaluate if the current vaccine could provide protection for the chickens against these four H5N6 isolates, chickens were vaccinated with reassortant avian influenza virus (H5+H7) trivalent inactivated vaccine (cell culture-based vaccine, H5N2 Re-11 strain+Re-12 strain, H7N9 H7-Re-2 strain) and challenged with th

viruses (Table 3).

At 28 days post-immunization, sera of all chickens were collected to monitor the HI titer H5. The results demonstrated that the mean HI antibody titers of the PBS group were 0 log2 and was therefore considered negative. The mean HI antibody of the chickens in the vaccination group from the different vaccination groups ranged from 9.4 log2 to 9.9 log2 (Figure 5).

At 28 days post-immunization, both vaccinated and co challenged with 10^6EID$_{50}$/0.1ml of the SD01 virus, SD02 virus, SD03 virus, or SD04 virus. Chickens in the control groups shed virus from both oropharynx and cloaca at day 3 after challenge and all died at day 5 after challenge. All vaccinated chickens were asymptomatic and survived during the observation perio recovered from oropharyngeal and cloacal swabs from the vaccinated chickens.

Therefore, the results indicated that the current vaccine, reassortant avian influenza virus (H5+H7) trivalent inactivated vaccine, could provide complete protectio chickens from the HPAI H5N6 viruses.

Discussion

The antigenic shift is an important evolutionary mechanism which can result in modification of host range, pathology, and transmission of the IAVs and generate the influenza virus s potential(Urbaniak & Markowska-Daniel, 2014). Since 2005, clade 2.3.4 HPAI H5N1 viruses had been introduced into and est China(Y. Li et al., 2010). In 2007, clade 2.3.4.4 was identified (L. Yang et al., 2017). Since 2009, the clade 2.3.4.4 H5 viruses reassorted with viruses of different NA subtypes, generating the HPAI H5 viruses(Gu et al., 2013). Since 2011, there have been three kinds of the reassortant H5N6 viruses found(L. Yang et al., 2017). One kind of the reassortant H5N6 virus bears the HA gene from H5N2 viruses reassorting H6N6 with the full-length NA gene and clade 2.3.2.1c H5N1 viruses. Another kind of the reassortant H5N6 v generated by reassorting HA gene from H5N8 viruses, and the NA gene from H6N6 viruses with the deletion from positions 59 to 69 in the stalk region, and six internal genes from clade 2.3.2.1c H5N1 viruses. Since 2015, consecutive reassortment

H5N6 viruses with six internal genes from chicken H9N2 viruses generated the novel reassortant H5N6 viruses. In our study, the results demonstrated that SD02, SD03, and SD04 viruses are all novel triple-reassortant viruses. The HA gene of the four H5N6 viruses belonged to the clade 2.3.4.4 of the Asian HPAI H5 virus. The NA genes originated from the H6N6 viruses of the Eurasia lineage. The PB2 genes of these viruses were uniquely derived from H9N2 viruses of the G lineage. And the PB1, PA, NP, M, and NS genes of the four viruses all originated from the H5N1 viruses, which circulated in Vietnam and China. Our results suggested a possible existence of a different kind of the reassortant H5N6 v Therefore, it is important to monitor the ecology and evolution of the potent zoonotic avian influenza viruses in order to prepare the public health responses to the threat posed by emerging and re-emerging influenza viruses timely.

It is well known that the RNA-polymerase in IAVs lack the ability of proofreading (Ahlquist, 2002; Chen & Holmes, 2006). As a result, mutations (antigenic drift) may generate during virus replication. Significant mutations evolution, the host spec vir(uCsaersrat & Flahault, 2007; Shao, L 2017.) Similar to the previous (sKwdin et al., 2018; Lee, Bertran, Kwon, & Swayne, 2017; Mei et al., 2019; Mine et al., 2019; Qu et al., 2019; Song et al., 2019; Sun et al., 2018; Uchida et al., 2019), all of the four H5N6 viruses in our study were highly pathogenic to chickens, which contained a series of multiple basic amino acids in the HA cleavage, and they also could transmit to contact chickens. However, our viruses exhibited high virulence in mice and could replicate lungs, brains, spleens, and kidneys. We observed mutations and deletions in the HA, NA, PB1, M1, and NS genes. For example, although amino acid residues in the 226 and 228 still were Q and G, T160A changes in the four H5N6 viruses earmarked a binding specifici human-like (rHeceeptsor et al., 2012; Linster et al., 2014; Y 2020). Some studies have demonstrated that N30D, and T215A mutations in the M1 and P42S, D92E mutations in the NS1 could increase the pathogenicity of the avian influenza virus in mice(Jiao et al., 2008; Seo, Hoffmann, & Webster, 2002; Yamaji et al., 2020). Additional investigation is required to determine if these mutations could influence the virulence of IAVs in mammals.

Vaccination is an important way to control and prevent the outbreaks of H5 HPAI in poultry in endemic countries. In China, inactivated vaccines are widely used in poultry industry. The conventional inactivated vaccines are generated genetics. The seed virus always bears the HA and NA genes of the epidemic virus and the six internal genes of the high-growth A/Puerto Rico/8 virus (Horimoto & Kawaoka, 2006; Luke & Subbarao, 2006; Wood & Robertson 2004). In general, the antigenic match between a vaccine and circulating viruses is one of the most important factors to determine protective efficacy. If the vaccine does not match with the circulating viruses antigenically, the seed virus of the vaccine should be then updated (C. Li et al., 2014). Since 2004, the HA gene of the vaccine strains used in China have been updated several times (Zeng et al., 2018). In 2018, in response to the new emerging highly pathogenic avian influenza virus, a new H5/ H7 bivalent inactivated vaccine was authorized by the Ministry of Agricultural and Rural Affairs of the People's Republic of China. Given these four H5N6 viruses used in this study belonging to the clade 2.3.4.4, we evaluate the protection of the current vaccine against these H5N6 isolates. The results demonstrated that these H5N6 viruses have slightly antigenic drifted away from Re-11, however, the current used H5/H7 bivalent inactivated vaccine could provide complete protection to chickens from the HP H5N6 viruses. Mutations in the HA gene often happen and may alter antigenicity of avian influenza viruses. As a result, the currently used vaccine may not be able to provide solid protection. Therefore, active surveillance still needs to be enforced and any newly detected viruses must be carefully evaluated.

In summary, our results demonstrated that the four H5N6 HPAI viruses were novel triple-reassortant viruses which bear genes from H5N1, H6N6 and H9N2 viruses. All of the four viruses were highly pathogenic to chickens tested and could be effectively transmitted among chickens via direct or indirect contact. They also caused leth infections in mice. More importantly, some amino acid substitutions indicated that these H5N6 viruses possessed the ability to infect humans. Therefore, more effective control measures should be taken to prevent the circulation and evolution H5N6 avian influenza virus.

Date Availability: The data used to support the findings of this study are included

within the article.

Acknowledgments: This work was supported by the National Key and Development Plan of China (Grant NO. 2017YFD0502300), Science Foundation Youth Fund (31802201).

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 potential conflict of interest.

References

Ahlquist, P. (2002). RNA-dependent RNA polymerases, viruses, and R
s i *S* e n(e5 n5 c7 g1
doi:10.1126/science.1069132

Alexander, D. J. (2007). An overview of the epidemiology of avian influenza. *Vaccine, 25*(30), 5637-5644. doi:10.1016/j.vaccine.2006.10.051

Carrat, F., & Flahault, A. (2007). Influenza vaccine: the challenge of antigenic d r *V* i *a* f (*c* t 3 *c* . 9 *i* - *n* 4
doi:10.1016/j.vaccine.2007.07.027

Chen, R., & Holmes, E. C. (2006). Avian influenza virus e evolutionary dynamics. *Mol Biol Evol, 23*(12), 2336-2341. doi:10.1093/molbev/msl102

Gao, R., Gu, M., Liu, K., Li, Q., Li, J., Shi, L., . . . Liu, X. (2018). T16 mutation-induced deglycosylation at site 158 in hemagglut critical determinant of the dual receptor binding properties of 2.3.4.4 H5NX subtype av*V* a h *M* n i t l r w e b n i z a, *217*, 158-166. doi:10.1016/j.vetmic.2018.03.018

Gu, M., Zhao, G., Zhao, K., Zhong, L., Huang, J., Wan, H., . . . Liu, X. (2013). Novel variants of clade 2.3.4 highly pathogen

A (H5N1) virus. *Emerging Infectious Diseases, 19*(12), 2021-2024. doi:10.3201/eid1912.130340

Herfst, S., Schrauwen, E. J., Linster, M., Chutinimitkul, Munster, V. J., . . . Fouchier, R. A. (2012). Airborne transmission of influenza A/H5N1 virus between ferrets. *Science, 336*(6088), 1534-1541. doi:10.1126/science.1213362

Horimoto, T., & Kawaoka, Y. (2006). Strategies for developi against H5N1 influenza A viruses. *Trends Mol Med, 12*(11), 506-514. doi:10.1016/j.molmed.2006.09.003

Jiao, P., Tian, G., Li, Y., Deng, G., Jiang, Y., Liu, C., . . . Chen, H. (2008). A single-amino-acid substitution in the NS pathogenicity of H5N1 avian *J Virol, 82*(3), 1146-1154. doi:10.1128/JVI.01698-07

Kwon, H. I., Kim, E. H., Kim, Y. I., Park, S. J., Si, Y. J., Lee, I. W., . . . Choi, Y. K. (2018). Comparison of the pathogenic po pathogenic avian influenza (HPAI) H5N6, and H5N8 viruses isolated in South Korea during t *Emerg Microbes Infect, 7*(1), 29. doi:10.1038/s41426-018-0029-x

Lee, D. H., Bertran, K., Kwon, J. H., & Swayne, D. E. (2017). Evol global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. *Journal of Veterinary Science, 18*(S1), 269-285. doi:10.4142/jvs.2017.18.S1.269

Li, C., Bu, Z., & Chen, H. (2014). Avian influenza vaccines against H5N1 'bird flu'. *Trends in Biotechnology, 32*(3), 147-156. doi:10.1016/j.tibtech.2014.01.001

Li, Y., Shi, J., Zhong, G., Deng, G., Tian, G., Ge, J., . . . Chen, H. (2010). Continued evolution of H5N1 influenza viruses in wild birds, domestic poultry, and humans in C *J Virol, 84*(17), 8389-8397. doi:10.1128/JVI.00413-10

Linster, M., van Boheemen, S., de Graaf, M., Schrauwen, E. J. A., Lexmond, P., Manz, B., . . . Herfst, S. (2014). Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1

virus. *Cell, 157*(2), 329-339. doi:10.1016/j.cell.2014.02.040

Long, J. S., Mistry, B., Haslam, S. M., & Barclay, W. S. (2019). Host and viral determinants of influen *Nat Rev Microbiol, 17*(2), 67-81. doi:10.1038/s41579-018-0115-z

Luke, C. J., & Subbarao, K. (2006). Vaccines for pand *Emerg Infect Dis, 12*(1), 66-72. doi:10.3201/eid1201.051147

Mei, K., Guo, Y., Zhu, X., Qu, N., Huang, J., Chen, Z., . . . Jiao, P. (2019). Different Pathogenicity and Transmissibility of Goose- Avian Influenza Viruses. *Viruses, 11*(7). doi:10.3390/v11070612

Mine, J., Uchida, Y., Nakayama, M., Tanikawa, T., Tsunekuni, R., Sharshov, K., . . . Saito, T. (2019). Genetics and pathogenicity of H5N6 highly pathogenic avian influenza viruses isolated from wild b chicken in Japan during winter 2017-2018. *Virology, 533*. doi:10.1016/j.virol.2019.04.011

Pan, M., Gao, R., Lv, Q., Huang, S., Zhou, Z., Yang, L., . . . Shu, Y. (2016). Human infection with a novel, highly pathogenic avian influe (H5N6) virus: V *J Infect, 72*(1), 52-59. doi:10.1016/j.jinf.2015.06.009

Qu, N., Zhao, B., Chen, Z., He, Z., Li, W., Liu, Z., . . . Jiao, P. (2019). Genetic characteristics, pathogenicity and tran pathogenic avian influen *Transbound Emerg Dis, 66*(6), 2411-2425. doi:10.1111/tbed.13299

Seo, S. H., Hoffmann, E., & Webster, R. G. (2002). Lethal H5N1 influenza viruses escape hos *Nat Med, 8*(9), 950-954. doi:10.1038/nm757

Shao, W., Li, X., Goraya, M. U., Wang, S., & Chen, J. L. (2017). Evolution of Influenza A Virus by *Int J Mol Sci, 18*(8). doi:10.3390/ijms18081650

Song, Y., Li, W., Wu, W., Liu, Z., He, Z., Chen, Z., . . . Jiao, P. (2 Phylogeny, Pathogenicity, Transmission, and Host Immune Responses of Four H5N6 Avian Influenza Viruses in Ch

Viruses, 11(11). doi:10.3390/v11111048

Sun, W., Li, J., Hu, J., Jiang, D., Xing, C., Zhan, T., & Liu, X. (2018). Genetic analysis and biological characteristics of different internal gene origin H5N6 reassortment avian influenza v *Vet Microbiol, 219*, 200-211. doi:10.1016/j.vetmic.2018.04.023

Taubenberger, J. K., & Kash, J. C. (2010). Influenza virus evolution, adaptation, and pandemic formation. *Cell Host Microbe, 7*(6), 440-451. doi:10.1016/j.chom.2010.05.009

Thakur, A. K., & Fezio, W. L. (1981). A computer program for estimating LD50 and its confidence limits using modified B cumula mD tr u mg e tC hh oe (dm3.) T, o 2 9 c7 o doi:10.3109/01480548109018136

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D. A., Chen, L. M., . . . Donis, R. O. (2012). A distinct lineage of influenza A virus from bats. *Proc Natl Aca* (d 1 1S)c ,i 4U 2 6S 9 doi:10.1073/pnas.1116200109

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., . . . Donis, R. O. (2013). New world bats harbor divers *PLoS Pathog, 9*(10), e1003657. doi:10.1371/journal.ppat.1003657

Uchida, Y., Mine, J., Takemae, N., Tanikawa, T., Tsunekuni, R., & Saito, T. (2019). Comparative pathogenicity of H5N6 subtype highly pathogenic avian influenza viruses in chicken, Pekin duck and Muscovy *Transbound Emerg Dis, 66*(3), 1227-1251. doi:10.1111/tbed.13141

Urbaniak, K., & Markowska-Daniel, I. (2014). In vivo reassortment of influenza viruses. *Acta Biochim Pol, 61*(3), 427-431.

WHO. (2016). Weekly epidem https://www.who.int/wer/2016/wer9111.pdf

WHO. (2020). Antigenic and genetic characteristics of zoonotic influenza A viruses and development of candidate vaccine viruses for pandemic prepa https://www.who.int/influenza/vaccines/virus/202002_zoonotic_vaccine virusupdate.pdf?ua=1

Woo, C., Kwon, J. H., Lee, D. H., Kim, Y., Lee, K., Jo, S. D., . . . Jeong, J. (2017). Novel reassortant clade 2.3.4.4 avian influenza A (H5N8) virus in a grey heron in South Korea in 2017. *Arch Virol, 162*(12), 3887-3891. doi:10.1007/s00705-017-3547-2

Wood, J. M., & Robertson, J. S. (2004). From lethal virus to l vaccine: developing inactivated vaccines for pa *Nat Rev Microbiol, 2*(10), 842-847. doi:10.1038/nrmicro979

Yamaji, R., Saad, M. D., Davis, C. T., Swayne, D. E., Wang, D., Wong, F. Y. K., . . . Zhang, W. (2020). Pandemic potential of highly pathogen avian influenza clade 2.3.4.4 A(H5) viruses. *Rev Med Virol, 30*(3), e2099. doi:10.1002/rmv.2099

Yang, L., Zhu, W., Li, X., Bo, H., Zhang, Y., Zou, S., . . . Shu, Y. (2017) Genesis and Dissemination of Highly Pat Influenza Viruses. *J Virol, 91*(5). doi:10.1128/JVI.02199-16

Yang, Z. F., Mok, C. K., Peiris, J. S., & Zhong, N. S. (2015). Human Infection with a Novel Avian Influenza A(H7N9) Virus. *N Engl J Med, 373*(5), 487-489. doi:10.1056/NEJMc1502983

Zeng, X., Tian, G., Shi, J., Deng, G., Li, C., & Chen, H. (2018). Vaccination of poultry successfully eliminated human infection with H7N9 China. *Sci China Life Sci, 61*(11), 1465-1473. doi:10.1007/s11427-018-9420-1

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Table 1. Virus loads in different organs in chickens inoculated intranasally [a] with the A/duck/Shandong/SD01/2019(H5N6), A/duck/Shandong/SD02/2019(H5N6), A/chicken/Shandong/SD03/2019(H5N6), and A/chicken/Shandong/SD04/2019(H5N6).

Strains	Virus replication on 3 DPI ($log_{10}EID_{50}$/0.1ml) [b] in						
	Heart	Liver	Spleen	Lung	Kidney	Brain	Trachea
SD01	8.25±0.43	7.42±0.14	7.42±0.14	8.50±0.25	8.17±0.58	7.00±0.66	6.42±0.14
SD02	7.67±0.14	7.17±0.58	7.50±0.25	8.67±0.14	8.08±0.52	6.75±0.50	6.42±0.14
SD03	8.42±0.14	7.17±0.58	7.42±0.14	8.50±0.25	8.17±0.58	7.33±0.52	6.42±0.14
SD04	8.67±0.38	5.75±0.66	6.67±1.04	9.67±0.52	8.17±0.38	8.00±0.50	5.58±0.88

a Six-week-old SPF chickens were inoculated intranasally (i.n.) with 106EID50 of SD01, SD02, SD03 and SD04 viruses in a volume of 0.1 ml, respectively; three chickens in each group were euthanized on 3 DPI, and virus titer was determined in samples of heart, liver, sp kidney, brain and Trachea in SPF eggs.

b For statistical analysis, a value of 1.5 was assigned if the virus was not detected from the undiluted sample in three embryonated hen eggs (Sun et al.,2011). Virus titers are expressed as means ± standard deviation in log10EID50/0.1 ml of tissue.

Table 2 Virus shedding in oropharyngeal and cloacal swabs from SPF chickens

Strain	Infection sample	1 DPI		3 DPI		5 DPI		7 DPI		9 DPI		11 DPI		14 DPI	
		T	C	T	C	T	C	T	C	T	C	T	C	T	C
SD01	Inoculated	7/8 [a]	7/8	4/4	4/4	- [b]	-	-	-	-	-	-	-	-	-
	Contacted	2/3	2/3	3/3	3/3	1/1	1/1	-	-	-	-	-	-	-	-
SD02	Inoculated	8/8	5/8	3/3	3/3	-	-	-	-	-	-	-	-	-	-
	Contacted	0/3	0/3	3/3	3/3	2/2	2/2	1/1	1/1	-	-	-	-	-	-
SD03	Inoculated	8/8	5/8	2/2	2/2	-	-	-	-	-	-	-	-	-	-
	Contacted	0/3	0/3	3/3	1/3	3/3	3/3	1/1	1/1	-	-	-	-	-	-
SD04	Inoculated	8/8	8/8	5/5	5/5	-	-	-	-	-	-	-	-	-	-
	Contacted	2/3	2/3	3/3	3/3	3/3	3/3	-	-	-	-	-	-	-	-

Abbreviations: DPI, day post-inoculation; T, oropharyngeal swab; C, cloacal swab.

[a]virus positive birds/tested birds

[b]all of the chickens died at the end of the observation.

Table 3 Protective efficacy of Reassortant Avian Influenza Virus (H5+H7) Trivalent Vaccine, Inactivated (Cell source, H5N2 Re-11 strain+ Re-12

597 strain, H7N9 H7-Re-2 strain) against the four H5N6 viruses challenge in chickens

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Challenge virus	Group	Virus isolation from swabs (shedding/total) on day 5 p.c				No. protection/total
		Day 3		Day 5		
		oropharyngeal	cloacal	oropharyngeal	cloacal	
SD01	Vaccinated	0/10	0/10	0/10	0/10	10/10
	Control	6/6[a]	6/6	0/0[6]	0/0	0/10
SD02	Vaccinated	0/10	0/10	0/10	0/10	10/10
	Control	7/7	7/7	3/3[c]	3/3	0/10
SD03	Vaccinated	0/10	0/10	0/10	0/10	10/10
	Control	6/6	6/6	2/2[c]	2/2	0/10
SD04	Vaccinated	0/10	0/10	0/10	0/10	10/10
	Control	4/4	4/4	3/3[c]	3/3	0/10

599 [a] some chickens died before day 3 p.c

600 [b] all the chickens died.

601 [c] some chickens died on day 4 p.c

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Figure Legends

Figure 1. Phylogenetic tree of HA. The tree was generated by using the neighbor joining method with the Maximum Composite likelihood model and MEGA version 4.0. Viruses highlighted with black triangles (▲) were the H5N6 viruses isolated in our study.

Figure 2. Reassortant patterns of the four HPAI H5N6 virus and their potential donor viruses. The eight gene segments of the virus, represented by horizontal bars, from top to bottom, are PB2, PB1, PA, HA, NP, NA, M, and NS.

Figure 3. Weight change lethality and replication of BALB/c mice during the 14 days postinoculation. Mice were inoculated intranasally with the H5N6 virus in a volume of 50 µl. Mice inoculated with PBS served as a control group.

Figure 4. Lethality of the infected chickens (A) and contact chickens (B) in each group.

Figure 5. HI antibody duration induced by inactivated vaccine in SPF chickens. Three-week-old white Leghorn SPF chickens were injected intramuscularly (i.m.) with 0.3 ml of reassortant avian influenza virus (H5+H7) trivalent vaccine, and sera were collected from chickens on 28 days post-immunization for HI antibody detection. The bars indicated the standard deviation.