Novel Reassortant Avian Influenza A(H5N6) Virus, China, 2021

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Although reports of human infection with influenza A(H5N6) virus were identified in China in 2014, during 2014–2020, a total of 26 cases of human infection were laboratory confirmed (1,2). Sporadic cases did not attract widespread attention until 2021 (3,4). During February–October 2021, China reported 24 laboratory-confirmed cases of human infection with H5N6 virus and 5 deaths (Figure 1, panel A); the number of human infections within only 8 months was close to the total for the previous 7 years.

The policy of compulsory poultry immunizations in China was adopted to prevent and control infection with highly pathogenic avian influenza (HPAI) subtype H5Nx (5). Although vaccination can reduce the likelihood of severe clinical disease and reduce shedding of virus in poultry, it cannot prevent sporadic infections with H5N6 virus in waterfowl. Because it is difficult to achieve a qualified 100% rate of H5N6 virus antibodies in waterfowl (6), these birds have become a weak link in prevention and control of the virus. In the context of selection pressure for vaccines and the absence of immunity in waterfowl, antigenic drift causes the H5N6 virus to continuously evolve (7), making currently available H5N6 vaccines ineffective.

On November 27, 2020, an outbreak of influenza A(H5N8) virus infections among wild swans was reported in China, resulting in the death of 2 swans (8,9). Since then, H5N8 clade 2.3.4.4b virus has spread throughout China, resulting in co-endemicity of H5N6 clade 2.3.4.4h/b and H5N8 clade 2.3.4.4b viruses. This 2020 outbreak was not the first outbreak of H5N8 virus in China; the earliest introduction of the virus into China was reported in Liaoning on September 12, 2014 (10,11). Because of China’s immunization policies for poultry, H5N8 virus was quickly eliminated, only to reemerge in 2020.

The reappearance and spread of H5N8 virus is a serious threat to the poultry industry. The ecologic environment of the virus has been altered, given the increasing number of influenza A(H5N6) cases in humans. The current prevalence and mode of virus reassortment is of great concern. We discovered a novel H5N6 virus that has spread throughout the poultry industry, and similar viruses caused a sharp rise in human infections.

The Study

In June 2021, we isolated an H5N6 virus from a sick duck on a live poultry farm in Chengdu, Sichuan, China. In July 2021, we isolated another H5N6 virus from a dead chicken in the backyard of a human patient with confirmed infection in Chongqing. In August 2021, we detected an H5N6 virus on a chicken farm in Maoming City and another on a goose farm in Huizhou City (both cities in Guangdong, China). In September 2021, we detected an H5N6 virus on a chicken farm in Qinzhou, Guangxi. Last, in October 2021, we detected 5 H5N6 viruses at live poultry
markets in Dongguan City of Guangdong Province (3 from ducks, 1 from a goose, and 1 from the environment) (Figure 1, panel B).

To examine the genetic relationships of the viruses, we sequenced the genomes of the 10 H5N6 viruses and constructed maximum-likelihood phylogenetic trees divided into H5N6 epidemic clades (12), according to the protocol established by the World Health Organization (Figure 2, panel A). The H5N6 virus fell within 8 hemagglutinin (HA)
The similarity between the HA genes of all 10 viruses was 99.1%–100%, and all belonged to clade 2.3.4.4b. The HA genes of all the strains had the typical HPAI virus amino acid sequence RRKR|GLF at the cleavage site (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/28/8/21-2241-App1.pdf). All viruses contain the S137A and T192I mutations in the HA gene, which enable it to bind to the human α-2, 6-linked sialic acid receptor, thereby increasing human susceptibility to the virus (8,13). Mammalian adaptive mutations, such as T33K, L89V, and G309D (14), were detected in the polymerase basic (PB) 2 gene of all strains, which increases the virulence of H5 viruses in mice (Table 1). Those variants are uncommon in previously circulating H5N6 (clade 2.3.4.4b) viruses.

Because there was an epidemiologic correlation between the avian virus strains from Chongqing and the human infections in Chongqing, we used Chongqing avian strains as a representative virus for the control analysis with human-origin virus sequences (Table 2). We deemed this approach to be the appropriate way to reveal links between the human-origin and avian-origin viruses. We found many similarities between the PB2, polymerase acidic, and nonstructural genes of the Chongqing avian-origin strain and those in influenza A(H3N2) virus, which suggests that the novel virus reassorted with H3N2 virus. The PB1 gene is derived from influenza A/H1N2 virus. The HA gene of the Chongqing H5N6 virus was 99.2% similar to that of the H5N8 virus and the matrix genes were 99.9% similar to those of the H5N8 virus, which leads us to believe that both genes originated from the H5N8 virus. The neuraminidase gene was derived from the H5N6 virus, however, and we speculate that the novel H5N6 virus is a reassortment of the H5N8 and H5N6 viruses. The HA and matrix genes of all viruses were derived from H5N8, which suggests that the novel H5N6 virus may use the gene backbone of the H5N8 virus. Other low-pathogenicity avian influenza viruses have been found to be involved in reassortment, which makes the internal genes of all strains appear complex but inconsistent (Appendix Tables 2–11). Notably, >1 pattern of reassortment seems to be present. Some human-derived strains have internal genes that are close to known H5N6 HPAI virus genes, and others are less closely related (Figure 2, panel B).

Bayesian analysis indicated that the viruses in the Pearl River Delta region (Guangdong), the upper reaches of the Yangtze River (Sichuan/Chongqing), and the middle and lower reaches of the Yangtze River (Hunan/Hangzhou) formed 3 subclades according to geographic characteristics (Appendix Figure 1). Compared with the study of Gu et al. (15), we found that the domestic novel H5N6 virus initially formed 3 subclades and an additional 7 types of genomes (Figure 2, panel B). Current vaccine strains lack protection (hemagglutination inhibition test) against novel H5N6 circulating virus strains (Appendix Table 12). This result differs from that reported by Gu et al. (15), which may result from isolation of the virus from different regions. Our study suggests that the virus poses a high risk for further transmission, which can be reduced or avoided by updating the vaccine strains. In January 2022, the Chinese government introduced

| Strain | HA gene | Function | PB2 gene | Function | Host |
|--------|---------|----------|----------|----------|------|
| A/Chongqing/00013/2021/H5N6 | S137A/T192I | Increased α-2,6 sialic acid receptor affinity | T33K/L89V/G309D | Enhanced virulence of H5 viruses in mice | Human |
| A/Sichuan/06681/2021/H5N6 | | | | | Human |
| A/Sichuan/06689/2021/H5N6 | | | | | Human |
| A/Hunan/09285/2021/H5N6 | | | | | Human |
| A/Hunan/09911/2021/H5N6 | | | | | Human |
| A/Chongqing/02/2021/H5N6 | | | | | Human |
| A/chicken/Chongqing/001/2021/H5N6V† | | | | | Avian |
| A/goose/Guangdong/210520/2021/H5N6V† | | | | | Avian |
| A/chicken/Guangdong/210518/2021/H5N6† | | | | | Avian |
| A/chicken/Dongdu/210462/2021/H5N6† | | | | | Avian |
| A/goose/Dongguan/D48/2021/H5N6† | | | | | Avian |
| A/duck/Dongguan/LD9/2021/H5N6† | | | | | Avian |
| A/ENV/Dongguan/ED3/2021/H5N6† | | | | | Environment |
| A/duck/Dongguan/IBC54/2021/H5N6† | | | | | Avian |
| A/duck/Dongguan/IBC59/2021/H5N6† | | | | | Avian |
| A/chicken/Guangxi/210539/2021/H5N6† | | | | | Avian |
| A/Whooper swan/Xinjiang/13/2020/H5N6‡ | 137S/192T | None | | | Avian |
| A/chicken/Suzhou/6/2019/H5N6‡ | 137S/192T | None | | | Avian |
| A/China/Original/2018/H5N6‡ | 137S/192T | None | | | Human |

*HA, hemagglutinin; PB2, polymerase basic 2.
†Avian and environmental strains isolated in study of novel reassortant avian influenza A(H5N6) virus, China, in 2021. The remaining reference strains were downloaded from GISAID (http://www.gisaid.org). All strains isolated from humans in 2021 were novel A(H5N6) viruses.
‡Reference strain (clade 2.3.4.4b) that did not have HA gene mutation before 2021.
new vaccine strains H5-Re14 and rHN5801 to prevent an epidemic of poultry infection with H5N6 virus. Because reported cases in humans were concentrated in southern China within a short time and most of these cases happened during the noninfluenza season, we suspect that transmissibility or viral load of the novel H5N6 viruses has increased. Furthermore, the virus that was isolated from environmental swab specimens (sewage ditch swab in a live poultry market) in Dongguan indicates that the virus is already present in the surrounding environment, which could increase the likelihood that the virus will infect humans.

**Conclusions**

At the peak of human cases, we isolated a total of 10 novel reassortment H5N6 virus strains from local poultry and the environment that were highly similar to the H5N6 (human-origin) virus reported during the same period. The human and avian viruses belong to clade 2.3.4.4b. The initial epidemic strains clustered into 3 geographically characterized subclades, and each avian strain had the same mammalian susceptibility mutation. The apparent antigenic differences between the virus and vaccine antisera suggest further transmission risk.

**Acknowledgments**

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Appendix

Methods

Sample collection and virus isolation

For the farm poultry, we collected tissues from the organs, larynxes, and lungs, and we collected throat and cloacal swabs for the birds in the LPM. We isolated the viruses in SPF chicken embryos and conducted RT-PCR amplification and sequencing. Throat and cloacal swab samples of poultry from live poultry markets were collected in Guangdong and Guangxi provinces, China. Disease tissues were from Chongqing and Chengdu provinces of China, where Chongqing disease samples were from dead chickens at the rear of confirmed cases. Each sample was placed in 2 ml of the PBS supplemented with penicillin (5000 U/ml) and streptomycin (5000 U/ml). All the samples were inoculated in the allantoic cavities of 10-day-old SPF embryonated chicken egg at 37°C. The allantoic fluid was collected and tested for hemagglutinin (HA) assay with 1% chicken red blood cells and then used in this study.

RNA extraction, RT-PCR, and DNA sequencing

RNA was extracted from the suspension of virus isolates with the RNeasy Mini Kit (Fastagen) as directed by the manufacturer. Two-step RT-PCR was conducted with universal primers as previously described (1), and the genome of H5N6 was amplified under standard conditions. PCR products were purified with a QIAamp Gel extraction kit (Qiagen) and sequenced with an ABI 3730 DNA Analyzer (Applied Biosystems).
**Phylogenic analysis**

All the available genomic sequences with the complete coding regions of influenza A(H5N6) viruses were downloaded from GISAID (http://www.gisaid.org/). The genome of H5N6 sequences data set (sequences alignment was available on request) was then created. The downloaded genomic sequences together with new 10 H5N6 strains were aligned using the MAFFT (version 7.149) program (2). The ML (Maximum likelihood) phylogenetic tree of each gene fragment was constructed by Phylosuite software (v 1.2.2) (3). Number of bootstrap with 5000 and Modle with GTR. The phylogenetic tree was visualized in the iTOL (version 6) website (https://itol.embl.de/).

**Statistical analyses**

Fluctuation curves of photoreceptors were generated by GraphPad Prism 5.0.

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### Appendix Table 1. Information on the influenza A(H5N6) viruses in this study

| Name                                             | Location | Host | Date       | Subtype | Cleavage site sequence |
|--------------------------------------------------|----------|------|------------|---------|------------------------|
| A_Duck_Chengdu_210462_2021_H5N6                  | Chengdu  | Duck | 2021/6/30  | H5N6    | RRKR↓GLF               |
| A_Chicken_Chongqing_001_2021_H5N6                | Chongqing| Chicken | 2021/7/29  | H5N6    | RRKR↓GLF               |
| A_Goose_Guangdong_210520_2021_H5N6               | Guangxi  | Chicken | 2021/8/24  | H5N6    | RRKR↓GLF               |
| A_Chicken_Guangdong_210518_2021_H5N6             | Guangdong| Chicken | 2021/8/24  | H5N6    | RRKR↓GLF               |
| A_Chicken_Guangxi_210539_2021_H5N6               | Guangdong| Chicken | 2021/9/23  | H5N6    | RRKR↓GLF               |
| A_Goose_Dongguan_D48_2021_H5N6                   | Guangdong| Chicken | 2021/9/2   | H5N6    | RRKR↓GLF               |
| A_Duck_Dongguan_LD9_2021_H5N6                    | Guangdong| Chicken | 2021/9/15  | H5N6    | RRKR↓GLF               |
| A_ENV_Dongguan_ED3_2021_H5N6                     | Guangdong| Chicken | 2021/9/1   | H5N6    | RRKR↓GLF               |
| A_Duck_Dongguan_IBC54_2021_H5N6                  | Guangdong| Chicken | 2021/9/7   | H5N6    | RRKR↓GLF               |
| A_Duck_Dongguan_IBC59_2021_H5N6                  | Guangdong| Chicken | 2021/9/9   | H5N6    | RRKR↓GLF               |

### Appendix Table 2. A_Duck_Dongguan_IBC59_2021_H5N6 genomic similarity

| Gene | Name                                                       | Similarity | Subtype |
|------|------------------------------------------------------------|------------|---------|
| HA   | A/goose/China/21FU008/2020(H5N8)                           | 99.06%     | H5N8    |
| NA   | A/China/Original/2018(H5N6)                                | 98.91%     | H5N6    |
| PB2  | A/Cygnus columbianus/Hubei/52/2020(H5N8)                   | 100.00%    | H5N8    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)                           | 100.00%    | H1N2    |
| PA   | A/Cygnus columbianus/Hubei/116/2020(H5N8)                  | 99.69%     | H5N8    |
| NP   | A/chicken/Ganzhou/GZ43/2016(H3N2)                          | 97.42%     | H3N2    |
| NS   | A/duck/Mongolia/543/2015(H4N6)                             | 97.90%     | H4N6    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8)                   | 100.00%    | H5N8    |

### Appendix Table 3. A_ENV_Dongguan_ED3_2021_H5N6 genomic similarity

| Gene | Name                                                       | Similarity | Subtype |
|------|------------------------------------------------------------|------------|---------|
| HA   | A/goose/China/21FU008/2020(H5N8)                           | 99.59%     | H5N8    |
| NA   | A/goose/Fujian/3.15_FZHX0007-C/2018(H5N6)                  | 97.93%     | H5N6    |
| PB2  | A/duck/Guangxi/293D21/2017(H1N2)                           | 96.71%     | H1N2    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)                           | 97.42%     | H1N2    |
| PA   | A/wild bird/ Shandong/11706/2019(H9N2)                     | 97.95%     | H9N2    |
| NP   | A/duck/Hunan/5.29_YYGK903-OC/2018(mixed)                   | 95.97%     | H5N6    |
| NS   | A/Duck/China/B1_NS/2018(H5N6)                              | 99.15%     | H5N6    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8)                   | 100.00%    | H5N8    |
### Appendix Table 4. A_Duck_Dongguan_IBC54_2021_H5N6 genomic similarity

| Gene | Name                                          | Similarity | Subtype |
|------|-----------------------------------------------|------------|---------|
| HA   | A/goose/China/21FU008/2020(H5N8)              | 99.47%     | H5N8    |
| NA   | A/China/Original/2018(H5N6)                   | 98.91%     | H5N6    |
| PB2  | A/Cygnus columbianus/Hubei/52/2020(H5N8)     | 99.79%     | H5N8    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)              | 100.00%    | H1N2    |
| PA   | A/Cygnus columbianus/Hubei/116/2020(H5N8)    | 99.69%     | H5N8    |
| NP   | A/chicken/Ganzhou/GZ43/2016(H3N2)            | 97.42%     | H3N2    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8)     | 99.81%     | H5N8    |

### Appendix Table 5. A_Goose_Dongguan_D48_2021_H5N6 genomic similarity

| Gene | Name                                          | Similarity | Subtype |
|------|-----------------------------------------------|------------|---------|
| HA   | A/goose/China/21FU008/2020(H5N8)              | 99.59%     | H5N8    |
| NA   | A/duck/China/FJ1904/2019(H5N6)                | 98.48%     | H5N6    |
| PB2  | A/Cygnus columbianus/Hubei/52/2020(H5N8)     | 99.69%     | H5N8    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)              | 97.61%     | H1N2    |
| PA   | A/Cygnus columbianus/Hubei/116/2020(H5N8)    | 99.87%     | H5N8    |
| NP   | A/duck/Mongolia/543/2015(H4N6)                | 99.10%     | H4N6    |
| NS   | A/chicken/Ganzhou/GZ43/2016(H3N2)            | 97.30%     | H3N2    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8)     | 99.81%     | H5N8    |

### Appendix Table 6. A_Duck_Dongguan_LD9_2021_H5N6 genomic similarity

| Gene | Name                                          | Similarity | Subtype |
|------|-----------------------------------------------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8)     | 99.47%     | H5N8    |
| NA   | A/China/Original/2018(H5N6)                   | 98.77%     | H5N6    |
| PB2  | A/Cygnus columbianus/Hubei/52/2020(H5N8)     | 99.70%     | H5N8    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)              | 97.65%     | H1N2    |
| PA   | A/Cygnus columbianus/Hubei/116/2020(H5N8)    | 99.60%     | H5N8    |
| NP   | A/duck/Mongolia/543/2015(H4N6)                | 99.16%     | H4N6    |
| NS   | A/duck/Zhejiang/10.26_HZBX001-C/2018(mixed)   | 97.09%     | H5N6    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8)     | 99.71%     | H5N8    |
## Appendix Table 7. A_Chicken_Chongqing_001_2021_H5N6 genomic similarity

| Gene | Name                              | Similarity | Subtype |
|------|-----------------------------------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8) | 99.18%     | H5N8    |
| NA   | A/Muscovy duck/China/H5N6/2020(H5N6) | 99.28%     | H5N6    |
| PB2  | A/chicken/Guangxi/165C7/2014(H3N2)  | 96.88%     | H3N2    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)   | 97.82%     | H1N2    |
| PA   | A/duck/China/322D22/2018(H3N2)     | 96.51%     | H3N2    |
| NP   | A/Muscovy duck/China/H5N6/2020(H5N6) | 95.19%     | H5N6    |
| NS   | A/chicken/Ganzhou/GZ43/2016(H3N2)  | 97.85%     | H3N2    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8) | 99.81%     | H5N8    |

## Appendix Table 8. A_Goose_Guangdong_210520_2021_H5N6 genomic similarity

| Gene | Name                              | Similarity | Subtype |
|------|-----------------------------------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8) | 99.55%     | H5N8    |
| NA   | A/Muscovy duck/China/H5N6/2020(H5N6) | 99.13%     | H5N6    |
| PB2  | A/Cygnus columbianus/Hubei/52/2020(H5N8) | 99.70%     | H5N8    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)   | 97.39%     | H1N2    |
| PA   | A/Cygnus columbianus/Hubei/116/2020(H5N8) | 99.55%     | H5N8    |
| NP   | A/duck/Mongolia/543/2015(H4N6)     | 99.22%     | H4N6    |
| NS   | A/chicken/Ganzhou/GZ43/2016(H3N2)  | 97.53%     | H3N2    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8) | 99.71%     | H5N8    |

## Appendix Table 9. A_Chicken_Guangdong_210518_2021_H5N6 genomic similarity

| Gene | Name                              | Similarity | Subtype |
|------|-----------------------------------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8) | 99.77%     | H5N8    |
| NA   | A/duck/China/FJ1904/2019(H5N6)     | 97.97%     | H5N6    |
| PB2  | A/Duck/China/B6_PB2/2019(H5N6)     | 98.33%     | H5N6    |
| PB1  | A/duck/Mongolia/MN18-1/2018(H3N6)  | 99.05%     | H3N6    |
| PA   | A/duck/Laos/XBY004/2014(H5N6)      | 98.22%     | H5N8    |
| NP   | A/duck/Guangdong/8.30_DGCP030-C/2017(mixed) | 98.40%     | H5N6    |
| NS   | A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6) | 98.74%     | H5N6    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8) | 99.32%     | H5N8    |
### Appendix Table 10. A_Chicken_Guangzhou_210539_2021_H5N6 genomic similarity

| Gene | Name                                      | Similarity | Subtype |
|------|-------------------------------------------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8)  | 99.55%     | H5N8    |
| NA   | A/Muscovy duck/China/H5N6/2020(H5N6)     | 99.13%     | H5N6    |
| PB2  | A/chicken/Guangxi/165C7/2014(H3N2)       | 96.71%     | H3N2    |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2)        | 97.74%     | H1N2    |
| PA   | A/duck/China/322D22/2018(H3N2)           | 97.76%     | H3N2    |
| NP   | A/duck/Hunan/4.26_YYGK90R3-OC/2018(mixed)| 98.66%     | H5N6    |
| NS   | A/chicken/Ganzhou/GZ43/2016(H3N2)       | 98.09%     | H3N2    |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8) | 99.71%     | H5N8    |

### Appendix Table 11. A_Chicken_Guangzhou_210539_2021_H5N6 genomic similarity

| Gene | Name                                      | Similarity | Subtype |
|------|-------------------------------------------|------------|---------|
| HA   | A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)| 97.46%     | H5N6    |
| NA   | A/duck/Hainan/12.29_HKPL002-C/2017(H5N6)  | 97.82%     | H5N6    |
| PB2  | A/chicken/Qingyuan/zd201601/2016(H9N2)   | 98.85%     | H9N2    |
| PB1  | A/Muscovy duck/Vietnam/HN4856/2018(H5N6)  | 97.82%     | H5N6    |
| PA   | A/goose/Fujian/3.15_FZH0007-O/2018(H5N6)  | 97.98%     | H5N6    |
| NP   | A/goose/Fujian/3.15_FZH0010-O/2018(H5N6)  | 98.78%     | H5N6    |
| NS   | A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)| 98.40%     | H5N6    |
| M    | A/Muscovy duck/Vietnam/HN5135/2018(H5N6)  | 99.42%     | H5N6    |
Appendix Table 12. Hemagglutinin inhibition tests of antigen differences between influenza A (H5N6) virus and vaccine in China in 2021

| Antigen                                      | Antibody | Re-11 | Re-12 | rFJ56 | rSD57 |
|----------------------------------------------|----------|-------|-------|-------|-------|
| A_Chicken_Chongqing_001_2021_H5N6            |          | 2     | 0     | 2     | 0     |
| A_Goose_Guangdong_210520_2021_H5N6           |          | 2     | 0     | 2     | 0     |
| A_Chicken_Guangxi_210539_2021_H5N6           |          | 2     | 0     | 2     | 0     |
| A_Goose_Dongguan_D48_2021_H5N6               |          | 5     | 3     | 3     | 2     |
| A_Duck_Dongguan_LD9_2021_H5N6                |          | 3     | 2     | 3     | 0     |
| A_ENV_Dongguan_ED3_2021_H5N6                 |          | 0     | 0     | 0     | 0     |
| A_Duck_Dongguan_IBC54_2021_H5N6              |          | 1     | 0     | 0     | 0     |
| A_Duck_Dongguan_IBC59_2021_H5N6              |          | 3     | 0     | 3     | 1     |
| Re-11                                        |          | 8     | -     | -     | -     |
| Re-12                                        |          | -     | 9     | -     | -     |
| rFJ56                                        |          | -     | -     | 10    | -     |
| rSD57                                        |          | -     | -     | -     | 9     |

Re-11, Re-12, rFJ56 and rSD57 are vaccine strains widely used in China. Both antigen and antiserum of Re-11 and Re-12 were purchased from the Harbin Weike Biotechnology Development Company (www.hvriwk.com). Both antigen and antiserum of rFJ56 and rSD57 were from Guangzhou South China Biologic Medicine (http://www.gzscbm.com).

Appendix Table 13. A_Chongqing_00013_202_H5N6 genomic similarity

| Gene | Name                                      | Similarity | Subtype |
|------|-------------------------------------------|------------|---------|
| HA   | A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)| 97.46%     | H5N6    |
| NA   | A/duck/Hainan/12.29_HKPL002-C/2017(H5N6)   | 97.82%     | H5N6    |
| PB2  | A/chicken/Qingyuan/zd201601/2016(H9N2)    | 98.85%     | H9N2    |
| PB1  | A/Muscovy duck/Vietnam/HN4856/2018(H5N6)   | 97.82%     | H5N6    |
| PA   | A/goose/Fujian/3.15_FZHX0007-O/2018(H5N6)  | 97.98%     | H5N6    |
| NP   | A/goose/Fujian/3.15_FZHX0010-O/2018(H5N6)  | 98.78%     | H5N6    |
| NS   | A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)| 98.40%     | H5N6    |
| M    | A/Muscovy duck/Vietnam/HN5135/2018(H5N6)   | 99.42%     | H5N6    |
### Appendix Table 14. A_GX-guilin_11151_202_H5N6 genomic similarity

| Gene | Name | Similarity | Subtype |
|------|------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8) | 99.66% | H5N8 |
| NA   | A/Muscovy duck/Vietnam/HN6606/2020(H5N6) | 99.02% | H5N6 |
| PB2  | A/duck/Guangxi/293D21/2017(H1N2) | 96.50% | H1N2 |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2) | 97.18% | H1N2 |
| PA   | A/duck/Vietnam/HN5958/2019(H3N8) | 97.58% | H3N8 |
| NP   | A/chicken/Shandong/2.28_TAWM016-C/2017(H5N6) | 98.72% | H5N6 |
| NS   | A/Muscovy duck/Vietnam/HN6610/2020(H5N6) | 99.54% | H5N6 |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8) | 99.61% | H5N8 |

### Appendix Table 15. A_Hangzhou_01_202_H5N6 genomic similarity

| Gene | Name | Similarity | Subtype |
|------|------|------------|---------|
| HA   | A/Cygnus columbianus/Hubei/53/2020(H5N8) | 99.16% | H5N8 |
| NA   | A/Muscovy duck/Vietnam/HN6606/2020(H5N6) | 98.77% | H5N6 |
| PB2  | A/duck/Guangxi/293D21/2017(H1N2) | 96.53% | H1N2 |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2) | 97.05% | H1N2 |
| PA   | A/wild bird/Shandong/11706/2019(H9N2) | 97.77% | H9N2 |
| NP   | A/chicken/Anhui/8.28_YHZGS017-O/2018(H5N6) | 98.19% | H5N6 |
| NS   | A/Muscovy duck/Vietnam/HN6609/2020(H5N6) | 99.03% | H5N6 |
| M    | A/eurasian coot/Shandong/W5611/2020(H5N8) | 99.49% | H5N8 |

### Appendix Table 16. A_Sichuan_06681_2021_H5N6 genomic similarity

| Gene | Name | Similarity | Subtype |
|------|------|------------|---------|
| HA   | A/environment sample/China/TZ001/2021(H5N8) | 99.44% | H5N8 |
| NA   | A/duck/Hainan/12.29_HKPL002-C/2017(H5N6) | 97.54% | H5N6 |
| PB2  | A/chicken/Guangxi/165C7/2014(H3N2) | 96.63% | H3N2 |
| PB1  | A/duck/Guangxi/293D21/2017(H1N2) | 97.86% | H1N2 |
| PA   | A/duck/China/322D22/2018(H3N2) | 97.76% | H3N2 |
| NP   | A/duck/Hunan/4.26_YYGK90R3-OC/2018(mixed) | 98.66% | H5N6 |
| NS   | A/chicken/Ganzhou/GZ43/2016(H3N2) | 98.09% | H3N2 |
| M    | A/Cygnus columbianus/Hubei/56/2020(H5N8) | 99.90% | H5N8 |
**Appendix Table 17. A_GX_hechi_01_2021_H5N6 genomic similarity**

| Gene | Name                                                      | Similarity | Subtype |
|------|-----------------------------------------------------------|------------|---------|
| HA   | A/environment/Anhui/11.29_YHZGS003-E/2018                | 97.74%     | H5N8    |
| NA   | A/Muscovy duck/Vietnam/HN6606/2020(H5N6)                 | 99.23%     | H5N6    |
| PB2  | A/goose/Fujian/3.15_FZH0011-O/2018(H9N2)                 | 92.18%     | H9N2    |
| PB1  | A/duck/China/322D22/2018(H3N2)                           | 94.79%     | H3N2    |
| PA   | A/duck/Vietnam/HN5958/2019(H3N8)                         | 97.04%     | H3N8    |
| NP   | A/Muscovy duck/Vietnam/HN6611/2020(H5N6)                 | 99.62%     | H5N6    |
| NS   | A/duck/Vietnam/HN6610/2020(H5N6)                         | 99.43%     | H5N6    |
| M    | A/chicken/Hunan/4.26_YYGK37R3-OC/2018(mixed)             | 98.64%     | H5N8    |

**Appendix Table 18. The accession numbers in GISAID of the influenza A(H5N6) viruses in this study**

| Name                                           | ID sequence   | Time   |
|------------------------------------------------|---------------|--------|
| A_Duck_Chengdu_210462_2021_H5N6               | EPI_ISL_5797454 | 2021/6/30 |
| A_Chicken_Chongqing_001_2021_H5N6             | EPI_ISL_5797453 | 2021/7/29 |
| A_Goose_Guangdong_210520_2021_H5N6           | EPI_ISL_5797280 | 2021/8/24 |
| A_Chicken_Guangdong_210518_2021_H5N6         | EPI_ISL_5797279 | 2021/8/24 |
| A_Chicken_Guangxi_210539_2021_H5N6           | EPI_ISL_5797272 | 2021/9/23 |
| A_Goose_Dongguan_D48_2021_H5N6               | EPI_ISL_5797238 | 2021/9/2  |
| A_Duck_Dongguan_LD9_2021_H5N6                | EPI_ISL_5797237 | 2021/9/15 |
| A_ENV_Dongguan_ED3_2021_H5N6                 | EPI_ISL_5797236 | 2021/9/1  |
| A_Duck_Dongguan_IBC54_2021_H5N6              | EPI_ISL_5797235 | 2021/9/7  |
| A_Duck_Dongguan_IBC59_2021_H5N6              | EPI_ISL_5795871 | 2021/9/9  |
Appendix Figure 1. Phylogenetic tree of HA gene sequences. The total HA genes (n=342) of H5 viruses collected from 2000-2021 in China were analyzed. The tree is rooted to A/Guizhou/1/2012/H5N6. The red color indicates the novel H5N6 isolates in this study.
Appendix Figure 2. Maximum confidence tree of the HA gene sequence. The total number of HA genes (n = 174) from H5N6 viruses collected in 2014-2021 was analyzed. The red circle represents the human origin H5N6 isolate in this study, and the Red Pentagram represents the avian origin isolate in this study. The MCMC chain was run for 100 million steps with tree sampling every 1000 steps, and a 10% burn-in fraction was used when calculating the final MCC tree with common ancestor node heights in TreeAnnotator v.1.10.4.
Appendix Figure 3. Phylogenetic tree of NA gene sequences. The total NA genes (n=242) of H5 viruses collected from 2000-2021 in China were analyzed.
Appendix Figure 4. Phylogenic tree of PB2 gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.
Appendix Figure 5. Phylogenetic tree of PB1 gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.
Appendix Figure 6. Phylogenic tree of PA gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.
Appendix Figure 7. Phylogenetic tree of NP gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.
Appendix Figure 8. Phylogenetic tree of NS gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.
Appendix Figure 9. Phylogenetic tree of M gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.