Evolution and pathogenicity of H6 avian influenza viruses isolated from Southern China during 2011 to 2017 in mice and chickens

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H6 subtype avian influenza viruses spread widely in birds and pose potential threats to poultry and mammals, even to human beings. In this study, the evolution and pathogenicity of H6 AIVs isolated in live poultry markets from 2011 to 2017 were investigated. These H6 isolates were reassortant with other subtypes of influenza virus with increasing genomic diversity. However, no predominant genotype was found during this period. All of the H6N2 and most of the H6N6 isolates replicated efficiently in lungs of inoculated mice without prior adaptation. All of the H6N2 and two H6N6 isolates replicated efficiently in nasal turbinates of inoculated mice, which suggested the H6N2 viruses were more adaptive to the upper respiratory tract of mice than the H6N6 viruses. One of H6N2 virus caused systemic infection in one out of three inoculated mice, which indicated that H6 avian influenza virus, especially the H6N2 viruses posed a potential threat to mammals. Five H6 strains selected from different genotypes caused no clinical signs to inoculated chickens, and their replication were limited in chickens since the viruses have been detected only from a few tissues or swabs at low titers. Our study strongly suggests that the H6 avian influenza virus isolated from live poultry markets pose potential threat to mammals.

Avian influenza virus (AIV), belonging to influenza A virus in the family of Orthomyxoviridae, contains eight negative stranded RNA segments. Influenza A viruses are subdivided into 18 different hemagglutinin (HA) and 11 neuraminidase (NA) subtypes based on the surface proteins1–4. Subtype H6 is one of the widespread AIV subtypes though it is easy to be neglected for its low pathogenicity in birds5–7.

The H6 AIV was isolated first from a turkey in the United States in 19658,9. Since then, H6 AIVs have been detected frequently from wild aquatic birds and domestic poultry throughout the world10–13. During 2000–2002, H6N2 AIVs were isolated from chickens from 12 different locations in California. The pathological changes observed in the early cases were primarily associated with mild respiratory infections, but yolk peritonitis had become the main feature of all the subsequent cases through 2001 and 200213. In 2009, H6N1 AIV was isolated once again from turkey exhibiting typical signs associated with AIV infection in Israel14.

Domestic ducks act as an important reservoir for influenza viruses and have also facilitated the establishment of multiple H6 influenza virus lineages15. Among those, the H6N2 and H6N6 were the main epidemiological subtypes recently circulated in Southern China16,17,18. Though clinical disease caused by H6 in chicken farms has not been reported in China, H6 AIVs were isolated continually from chickens in the live poultry markets (LPMs) and might pose a huge threat on the poultry industry. Previous studies have shown that the H6 AIVs have a broad host range, and the H6 viruses might have crossed the species barrier and infected mammals, including humans, without adaptation17,18. In 2010, an avian-origin H6N6 swine influenza virus was isolated from sick pigs in Southern China19. Three years later, an H6N1 virus was isolated from a woman with flu-like symptoms in Taiwan20. In addition, an H6N1 virus with molecular characteristics closely related to the human isolates, was found to cause infections in dogs21. H6 AIVs have the potential to cross the species barrier and infect mammals, including humans18,20. Around 34% of H6 viruses isolated from Southern China could bind to the human-like receptor17. Specially, a single amino acid change from glutamine to leucine at position 226 of hemagglutinin causes a switch in receptor-binding preference from avian-like to mammalian-like21. In addition,
H6 AIVs provided internal genes for other subtype viruses and generated novel viruses frequently, such as H5N1, H9N2 and H5N6 AIVs which were detected in humans. Recently, a large amount of H6 AIVs were isolated in Southern China through routine surveillance and their phylogenetic characters were analyzed comprehensively. However, the pathogenicity of those H6 AIVs on chickens and mammals are unclear. In this study, the evolution and pathogenicity of 7 H6N2 and 15 H6N6 viruses, isolated from LPMs in Southern China from 2011 to 2017, were evaluated in mice and chickens.

Materials and methods

Virus isolation. Oropharyngeal swabs were collected at different LPMs in Hunan, Jiangsu, Zhejiang, Guangdong and Fujian provinces from 2011 to 2017. Each swab was soaked in phosphate-buffered saline (PBS) and tested by reverse transcription and polymerase chain reaction (RT-PCR) using influenza-specific HA primers for H6 subtype as described previously. To isolate the viruses, the H6-positive samples were filtered through 0.22 μm filters and inoculated into allantoic cavity of 9-day-old specific pathogen free (SPF) embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China). The viruses were purified and propagated by 3 rounds of limiting dilution in embryonated SPF chicken eggs. The viral titers were determined in SPF embryonated chicken eggs and calculated using the Reed and Muench method.

Genetic analysis and phylogenetic analysis. Viral RNAs were extracted from allantoic fluid containing H6 viruses with the QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The first-strand cDNAs were synthesized using reverse transcriptase M-MLV (Takara Bio Inc., Dalian, China) with universal primer for influenza A viruses (5’ - AGC RAA AGC AGG-3’) following the manufacturer's protocol. The eight gene segments of each virus were amplified by PCR with universal primers for influenza A viruses (5`- AGC RAA AGC AGG-3`) following the manufacturer's instructions. The PCR products were purified using DNA Gel Extraction Kits (Axygen, Hangzhou, China) and sequenced by GENEWIZ Biotechnology Company (Suzhou, China). The sequences were edited with Seqman module of the DNAStar package. Phylogenetic trees were generated by the distance-based neighbor-joining method using Clustal W. The reliability of the trees was assessed by bootstrap analysis with 1000 replications. Reference sequences were cited from GenBank of NCBI and GISAID.

Studies with mice. To determine the pathogenicity of the H6 AIVs in mice, each of eight 4-week-old female BALB/c mice (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) were anesthetized and inoculated intranasally (i.n.) with 10^6.0 EID50 in 30.0 μl PBS of each virus in the 22 H6 isolates. Eight control mice were inoculated intranasally with 30.0 μl PBS. At five days post-inoculation (dpi), three mice in each group were euthanized, and their lungs, nasal turbinates, hearts, livers, spleens and brains were collected for virus titration. Each organ was homogenized in chilled PBS and centrifuged at 2500g for 10 min. The viruses in the supernatants were titrated using the methods described previously. The body weight and clinical signs of remaining five mice in each group were recorded until 14 dpi.

Studies with chickens. The pathogenicity of 5 H6 viruses from different genotypes, was evaluated in SPF chickens. Each of six 4-week-old SPF chickens were inoculated i.n. with 10^6.0 EID50/bird of each virus in a volume of 100.0 μl, respectively. One day later, three naïve chickens were introduced into each of these isolates to evaluate the transmission of viruses among chickens. Six control birds were inoculated i.n. with the same amount of PBS. Three inoculated chickens in each group were euthanized, and their tracheas, lungs, kidneys, spleens and duodenums were collected for virus isolation at 3 dpi. Cloacal and oropharyngeal swabs were collected from the other 3 chickens at 2, 4 and 6 dpi. All of the tissues were homogenized in PBS (0.1 g per 1.0 ml), and the swabs in 1.0 ml PBS were stirred on vortex, and then all samples were centrifuged at 2500 g for 10 min. Each supernatant was collected, filtered through 0.22 μm filters, and inoculated into allantoic cavities of three 9-day-old embryonated chicken eggs. At 14dpi, blood samples were drawn from the remaining inoculated chickens and three contact chickens for detection of H6 subtype specific antibodies.

Ethics statement and statistical analysis. All animal studies in this study were conducted in accordance to the guidelines of the Animal Care and Use Committee of Shanghai Veterinary Research Institute, and all animal studies protocols are approved by Shanghai Veterinary Research Institute (Permit number: SHVRI-Po-0120). Good living environment with sufficient food and water were available for all the animals. Comparisons of the weight changes between two groups were determined by nonparametric t-tests using GraphPad (Version 6.0, GraphPad Prism). The differences were considered statistically significant if P values < 0.05(*), P < 0.01(**), P < 0.001(***)

Results

Virus isolation and identification. A total of 22 H6 AIVs were isolated from ducks and geese without any obvious clinical signs in LPMs during 2010–2017. All of viruses were propagated in SPF chicken embryonated eggs and their virus titers ranged from 10^6.7 to 10^9.5 EID50/100 μl. Full genomes of 7 H6N2 and 15 H6N6 viruses were sequenced and deposited to the NCBI database, the accession number and abbreviations of viruses used in this study have been listed in Table 1.
Molecular characterization. According to sequence analysis, all of the H6 isolates shared the same amino acids (PQIETR/GLF) with single basic amino acid at the cleavage site between HA1 and HA2, which displayed a low pathogenic feature. The receptor-binding sites in HA protein possessed the amino acid residues Q226 and G228 (H3 HA numbering, which is used throughout the manuscript), suggesting that all H6 isolates would have a higher affinity to α-2,3-linked sialic acid which is predominant in the upper respiratory tract of avian species. Several mutations were found in the receptor-binding area in the HA proteins and listed in Table 2, including the amino acids from 169 to 171 which related to a potential glycosylation site, and the amino acid at 190 which was found to determine the binding capability of H9N2 viruses to lung epithelial cells of mouse and human. The amino acid changes of A138S and P186T/I were appeared in ZJ/B1994/H6N6, FJ/D3480/H6N6 and JS/F336/H6N6. Those 22 viruses contained 119E, 274H and 294 N on the NA proteins (N2 numbering), which suggested that all viruses are sensitive to oseltamivir. There were 11 amino acids deletion in the stalk region of three H6N6 isolates (ZJ/B1994/H6N6, FJ/D3480/H6N6 and JS/F336/H6N6), which might be associated with increased virulence in mammals. It is notable that S31N substitutions in the M2 protein, which are associated with amantadine resistance of influenza virus were observed in GD/E3503/H6N2 and GD/E3891/H6N2 viruses. Amino acid residues were E and D at positions 627 and 701 of the PB2 protein in all those H6 isolates, which have been associated with the pathogenicity of avian influenza viruses in mammals. Previous reports showed

Table 1. Abbreviations used and GenBank accession numbers for H6 Avian Influenza virus isolates. *Viruses whose PB2, PB, PA, HA, NP, NA, M and NS genes were sequenced in the present study.

| Virus Abbreviation | Virus titer | GenBank accession No. for gene |
|-------------------|-------------|-------------------------------|
| A/duck/Hunan/A729-2/2011(H6N6) | HN/A729 | MT828554 MT828845 MT829178 MT826249 MT828279 MT827972 MT827847 MT828310 |
| A/goose/Guangdong/1268-1/2011(H6N2) | GD/1268 | MT828555 MT828846 MT829179 MT826250 MT828280 MT827973 MT827848 MT828311 |
| A/goose/Guangdong/1127-1/2011(H6N2) | GD/1127 | MT828556 MT828847 MT829180 MT826251 MT828281 MT827974 MT827849 MT828312 |
| A/duck/Hunan/A2282-1/2011(H6N6) | HN/2282 | MT828557 MT828848 MT829181 MT826252 MT828282 MT827975 MT827850 MT828313 |
| A/duck/Zhejiang/B2039-2/2012(H6N6) | ZJ/B2039 | MT828558 MT828849 MT829182 MT826253 MT828283 MT827976 MT827851 MT828314 |
| A/duck/Zhejiang/B2044-2/2012(H6N6) | ZJ/B2044 | MT828559 MT828850 MT829183 MT826254 MT828284 MT827977 MT827852 MT828315 |
| A/duck/Zhejiang/B1994-2/2012(H6N6) | ZJ/B1994 | MT828560 MT828851 MT829184 MT826255 MT828285 MT827978 MT827853 MT828316 |
| A/duck/Zhejiang/B2028-1/2012(H6N6) | ZJ/B2028 | MT828561 MT828852 MT829185 MT826256 MT828286 MT827979 MT827854 MT828317 |
| A/duck/Guangdong/D1501-1/2014(H6N6) | GD/D1501 | MT828562 MT828853 MT829186 MT826257 MT828287 MT827980 MT827855 MT828318 |
| A/duck/Fujian/D3480-1/2014(H6N6) | FJ/D3480 | MT828563 MT828854 MT829187 MT826258 MT828288 MT827981 MT827856 MT828319 |
| A/duck/Jiangsu/E1201-2/2015(H6N6) | JS/E1201 | MT828564 MT828855 MT829188 MT826259 MT828289 MT827982 MT827857 MT828320 |
| A/duck/Guangdong/E3415-2/2015(H6N6) | GD/E3415 | MT828565 MT828856 MT829189 MT826260 MT828290 MT827983 MT827858 MT828321 |
| A/duck/Guangdong/E3503-2/2015(H6N2) | GD/E3503 | MT828566 MT828857 MT829190 MT826261 MT828291 MT827984 MT827859 MT828322 |
| A/duck/Guangdong/E3724-1/2015(H6N6) | GD/E3724 | MT828567 MT828858 MT829191 MT826262 MT828292 MT827985 MT827860 MT828323 |
| A/duck/Guangdong/E3742-2/2015(H6N2) | GD/E3742 | MT828568 MT828859 MT829192 MT826263 MT828293 MT827986 MT827861 MT828324 |
| A/duck/Guangdong/E3780-1/2015(H6N2) | GD/E3780 | MT828569 MT828860 MT829193 MT826264 MT828294 MT827987 MT827862 MT828325 |
| A/duck/Guangdong/E3798-1/2015(H6N6) | GD/E3798 | MT828570 MT828861 MT829194 MT826265 MT828295 MT827988 MT827863 MT828326 |
| A/duck/Guangdong/F1473-2/2016(H6N2) | GD/F1473 | MT828571 MT828862 MT829195 MT826266 MT828296 MT827989 MT827864 MT828327 |
| A/duck/Jiangsu/F336-2/2016(H6N2) | JS/F336 | MT828572 MT828863 MT829196 MT826267 MT828297 MT827990 MT827865 MT828328 |
| A/duck/Guangdong/F3891-1/2016(H6N2) | GD/F3891 | MT828573 MT828864 MT829197 MT826268 MT828298 MT827991 MT827866 MT828329 |
| A/duck/Jiangsu/G91-1/2017(H6N6) | JS/G91 | MT828574 MT828865 MT829198 MT826269 MT828299 MT827992 MT827867 MT828330 |
| A/duck/Jiangsu/G93-1/2017(H6N6) | JS/G93 | MT828575 MT828866 MT829199 MT826270 MT828300 MT827993 MT827868 MT828331 |
that the substitution D92E in the NS1 protein associated with reducing its phosphorylation and increasing the virus resistance to interferon41, but this substitution was not detected in any of the isolates. In addition, no amino acid changes associated with increased virulence in mammals were detected in the PA or PB1 proteins.

Phylogenetic analysis of HA and NA genes. The nucleotide and amino acid similarity of the HA from 22 isolates in this study were 82.0% to 99.9% and 84.0% to 99.8%, respectively, as shown in Table 3. The phylogenetic tree of HA gene (Fig. 1a) indicated that all of the H6 isolates were divided into two clades under Eurasian lineage. All the H6N2 isolates were clustered in A/duck/Shantou/339/2000(H6N2)-like (ST/339-like) virus clade except GD/F1473/H6N2. Together with all of the H6N6 viruses, GD/F1473/H6N6 was derived from A/wild duck/Shantou/2853/2003(H6N2)-like (ST/2853-like) virus clade. Additionally, 3 H6N6 strains (ZJ/B1994/H6N6, FJ/D3480/H6N6 and JS/F336/H6N6) belonged to A/swine/Guangdong/K6/2010(H6N6)-like (GD/K6-like) subclade.

The NA genes of 7 H6N2 isolates separated into two clades (ST/339-like and ST/2853-like clades). Only GD/F1473 clustered in the ST/2853-like clade, in which H3N2, H4N2, and H6N2 viruses were clustered. The other H6N2 viruses fell into ST/339-like clade, in which the viruses were isolated from ducks, goose and chickens (Fig. 1b). The NA genes of 15 H6N6 isolates were divided into two clades as well. The majority of those viruses were clustered together with the clade of ST/192-like viruses. Only the JS/E1201/H6N6 strain clustered on the clade of A/Muscovy duck/Fujian/FZ01/2008(H5N6)-like (FJ/FZ01-like) virus (Fig. 1c).

Phylogenetic analysis of the internal genes. Phylogenetic analysis of the six internal genes showed that all those 22 H6 AIVs clustered in the Eurasian lineage. The PB1, PB2, PA and NS genes were divided into two clades, and the NP and M genes were divided into 3 clades (Fig. S1-6). Phylogenetic tree of PB1 genes showed that nine isolates were clustered in the ST339-like clade, in which the viruses were isolated from different birds including chicken, duck, goose and wild bird. The other thirteen isolates were closely related to A/duck/Guangdong/S3180/2010(H6N6) (GD/S3180) (Fig. S1). In the phylogenetic trees of the PB2 and NS genes, only GD/F1473/H6N2 located in a separated clade (Fig. S2 and S6). PB2 gene of GD/F1473/H6N2 was closely related to that of the early H5N1 AIV isolate A/goose/Guangdong/1/96 in the BJ/BJ/1/94-like clade. The NS gene of GD/F1473/H6N2 was closely related to that of H3N2, H3N8, and H6N2 AIVs isolated from different waterfowl. As for the PA genes, all of the H6N6 viruses and one H6N2 virus GD/F1473/H6N6 were clustered in the ST339-like clade (Fig. S3). The other 5H6N2 viruses were located in the Gs/GD196-like clade. The NP gene of the virus JS/E1201/H6N6 was clustered in a separated A/duck/Hunan/573/2002(H6N2)-like (HN/573-like) clade, while other isolates were divided into ST339-like clade A/duck/Mongolia/54/2001(H5N2)-like (Mongolia/54-like) virus groups (Fig. S4). On the NP tree, most of the H6N6 and GD/F1473/H6N2 were related to a H5N2 virus A/duck/ Mongolia/54/2001. In the PA tree, the clade, the NP genes of 6H6N2 isolates clustered into a A/chicken/Guang-
**Table 3.** Nucleotide and amino acid identity of the HA genes among the twenty-two H6 subtype isolates from Southern China, 2011–2017.

| Virus   | Nucleotide identity (%) | Amino acid identity (%) |
|---------|-------------------------|-------------------------|
| JS/F336 | 85.9 85.5 91.7 91.5 82.8 90.3 90.4 | 82.6 86.8 85.5 91.7 91.5 82.8 90.3 90.4 |
| GD/F358 | 86.2 97.5 97.9 85.4 85.4 86.4 86.4 86.9 82.8 82.9 82.8 90.3 90.4 |
| GD/1268 | 86.9 99.5 82.6 82.2 82.9 82.4 83.1 82.9 82.1 82.8 82.4 98.3 82.3 97.9 97.9 82.4 82.7 82.1 97.0 82.1 82.2 |
| ZJ/B2039 | 86.9 99.3 82.4 82.0 82.7 82.3 83.0 82.7 82.0 82.7 82.3 98.1 82.2 97.8 97.8 82.3 82.5 82.0 96.9 82.1 82.2 |
| ZJ/B2044 | 95.4 86.2 86.2 94.4 98.6 92.2 99.2 99.4 92.1 92.2 95.0 82.6 94.9 82.9 82.5 92.4 92.5 91.6 83.1 94.4 94.5 |
| ZJ/B1994 | 92.4 86.2 86.2 91.2 92.9 94.4 92.3 91.9 97.2 90.8 91.5 82.2 91.5 82.6 82.1 90.5 90.4 97.6 83.0 90.9 91.1 |
| GD/D1501 | 94.9 85.9 85.9 94.0 97.7 99.1 93.5 98.9 92.1 92.0 94.8 82.6 94.8 82.9 82.5 92.2 92.2 91.3 83.0 94.4 94.5 |
| FJ/D3480 | 92.4 86.2 86.2 91.2 92.6 94.0 97.9 93.8 93.5 90.8 91.7 81.9 91.7 82.3 81.8 90.7 90.5 96.3 82.5 91.1 91.1 |
| JS/E1201 | 98.6 87.1 87.1 96.6 93.1 94.5 91.9 94.7 94.0 91.9 91.5 82.6 91.4 82.8 82.7 93.5 93.4 90.2 82.9 91.4 91.5 |
| GD/E3724 | 93.7 84.7 84.7 92.6 95.1 96.5 92.1 96.6 95.6 91.4 93.1 99.6 84.3 82.8 82.4 91.8 91.9 91.2 83.1 96.8 96.9 |
| GD/E3742 | 86.4 97.9 98.2 85.2 85.2 86.2 86.2 86.4 85.9 86.2 86.9 85.2 98.6 84.8 97.9 82.5 82.7 82.4 98.9 82.5 82.6 |
| GD/E3780 | 86.1 97.5 97.9 84.8 84.3 85.4 85.0 85.5 85.2 85.0 86.2 84.3 98.9 84 98.2 82.4 82.0 97.2 82.1 82.2 |
| GD/E3798 | 97.2 86.4 86.4 96.1 93.1 94.4 91.9 94.5 93.8 91.9 96.1 93.5 85.7 93.1 |

**Pathogenicity in mice.** To evaluate the pathogenicity in mice, groups of white mice were inoculated i.n. with each H6 isolates (10⁶ EID₅₀/mice). All of the mice used for observation survived till 14 dpi. None of H6N2 viruses, except GD/1268/H6N2, caused significant body weight loss (P < 0.05) in mice compared with the control group (Fig. 3). As for H6N6 viruses, only HN/A729/H6N6 and ZJ/B1994/H6N6 caused significant growth retardation. In the nasal turbinates, only GD/E3503/H6N6 and FJ/D3480/H6N6 could be detected in all of the three mice, and the average virus titers were 10¹¹ and 10¹² EID₅₀/ml, replicated efficiently in the lungs of inoculated mice. In the nasal turbinates, only GD/E3415/H6N6 and FJ/D3480/H6N6 could be detected in all of the three mice, and the average virus titers were 10¹¹ and 10¹² EID₅₀/ml, respectively. Except ZJ/B2039/H6N6 and ZJ/B2044/H6N6 which were not recovered from the nasal turbinates...
Figure 1. Phylogenetic trees of H6 AIVs isolated from 2011 to 2017 in Southern China. The phylogenetic trees of the H6 HA (a), N2 NA (b) and N6 NA (c) genes were generated by using the neighbor-joining method using MEGA 6.0. The genomic sequences of the viruses listed in black were downloaded from available databases; the viruses listed in red were evaluated in this study. The scale bar represents the distance between sequence pairs, and horizontal distances are proportional to genetic distance.
Figure 1. (continued)

| Year | N6 | H6N2 | H6N6 |
|------|----|------|------|
| 2011 |    |      |      |
| 2012 |    |      |      |
| 2013 |    |      |      |
| 2014 |    |      |      |
| 2015 |    |      |      |
| 2016 |    |      |      |
| 2017 |    |      |      |

Figure 2. Genotypes of H6 AIVs isolated from 2011 to 2017 in Southern China. The genotypes of H6 influenza viruses were determined by the clades of each of their gene segments on the phylogenetic trees.
of all three mice, the remaining H6N6 viruses were detected in some inoculated mice. All of the H6N6 isolates were detectable in the hearts of some to all of the three inoculated mice, except ZJ/B2039/H6N6, GD/E3780/

Figure 3. Bodyweight changes of BALB/c mice infected with the H6 AIVs. 4-week-old female BALB/c mice were inoculated i.n. with $10^{6.0}$EID$_{50}$ of virus in a volume of 30.0 μl. The body weight of 5 mice were measured daily until 14 dpi. Weight changes of the BALB/c mice infected with H6N2 (Fig. 3a) and H6N6 (Fig. 3b) were shown respectively. The data were graphed using the Prism 6.0 software (Vision 6.0, GraphPad Prism). Comparisons of the weight changes between two groups were determined by nonparametric t-tests using GraphPad. (*P < 0.05, **P < 0.01, ***P < 0.001).
H6N6 and JS/G91/H6N6. Lower viral titers of some H6N6 viruses were detected in livers, spleens and brains in some inoculated mice, suggesting that those viruses replicate in mice (Table 4).

**Pathogenicity and transmission in chickens.** To evaluate the virulence of the H6 isolates in chickens, groups of six 4-week-old SPF chickens were inoculated intranasally with $10^{6.0}$ EID$_{50}$ of each H6 isolate. No clinical sign was observed in the inoculated chickens. We further examined the replication of the H6 viruses by detecting the viruses in different tissue and swabs of inoculated chickens. The GD/1268/H6N2 and GD/1127/H6N2 viruses were detected in the trachea of one inoculated chicken, respectively. FJ/D3480/H6N6 was detected in the duodenum of one inoculated chicken, while HN/2282/H6N6 and ZJ/B2028/H6N6 were not detected in any tested tissues of 3 inoculated chickens at 3dpi. Only GD/E3503/H6N2 was detected in the oropharyngeal swab from one chicken at 4 dpi. GD/E3503/H6N2 and HN/A729/H6N6 were detected in the cloacal swab from one chicken at 2 dpi and 6 dpi, respectively. None of 3 chickens inoculated with HN/A729/H6N6 showed sero-positive to specific H6 antibody, and only some chickens inoculated with the other four viruses were seroconverted. One contact chicken in the GD/E3503/H6N2 group was seroconverted at 14 dpi, while no specific H6 antibody was detected in other contact chickens (Table 5). The results suggested the replication and transmission of H6 viruses with different genotypes were limited in chickens.

**Discussion**

Since the late 1990s, H6 AIVs have been circulating in Southern China. The H6 viruses have been become more prevalent over time with an increasing isolation rate of H6N1, H6N2 and H6N6 subtypes.\(^5,15,42\) In this study, we analyzed the molecular evolution and pathogenicity in mice and chickens of H6 AIVs isolated from the LPMs in Southern China from 2011 to 2017, and provided a glimpse of the genetic diversity and pathogenicity of H6 AIVs in mammals and chickens.

Multiple H6 virus genotypes emerged from 2011 to 2017 resulted from the internal gene reassortment between H6 and other subtype viruses. It has been noted that most of the H6N1/H6N2 viruses isolated from Southern China from 2000 to 2005 were clustered with the G1-like, W312-like or H9N2 Ck/Bei-like lineage based on the PB2 and PB1 genes, and the PA and NP genes of most H6 viruses clustered with H5 and H9 viruses, M and NS genes with G1-like or H9N2 Ck/Bei-like lineage viruses.\(^4,15,42\) Previous studies showed that the H6 isolate from ducks replicated poorly in chicken trachea and that the viruses recovered were W312-like viruses.\(^4,15,42\) However, in our study, none of the internal genes were related closely to G1-like or W312-like viruses. These results suggested that the H6 subtype viruses in Southern China were genetically diverse, which might increase the potential for H6 viruses to transmit from ducks to other animals.

| Virus  | Genotype | Subtype | Virus titer (lgEID$_{50}$/ml) |
|--------|----------|---------|-------------------------------|
| GD/1268 | A201     | H6N2    | 3.42 ± 0.14                   |
| GD/1127 | A201     | H6N2    | 4.25 ± 0.66                   |
| GD/E3503 | A202    | H6N2    | 6.00 ± 0.43                   |
| GD/E3742 | A202    | H6N2    | 5.08 ± 0.52                   |
| GD/E3780 | A202    | H6N2    | 4.75 ± 0.43                   |
| GD/F3891 | A202    | H6N2    | 3.75 ± 0.43                   |
| GD/F4173 | B201    | H6N2    | 4.33 ± 0.14                   |
| HN/A729 | B601     | H6N6    | 2.08 ± 0.72                   |
| HN/2282 | B601     | H6N6    | 5.50 ± 0.00                   |
| GD/D1501 | B601    | H6N6    | 4.42 ± 0.76                   |
| ZJ/B2039 | B602    | H6N6    | /                             |
| ZJ/B2044 | B602    | H6N6    | 3.58 ± 2.08                   |
| ZJ/B2028 | B602    | H6N6    | 5.08 ± 1.89                   |
| GD/E3415 | B602    | H6N6    | 4.58 ± 0.88                   |
| GD/E3724 | B602    | H6N6    | 3.25 ± 0.87                   |
| GD/E3798 | B602    | H6N6    | 1.25(0.98/2.5)                |
| JS/G93  | B602     | H6N6    | 5.33 ± 0.14                   |
| ZJ/B1994 | B603     | H6N6    | 5.00 ± 0.60                   |
| FJ/D3480 | B604     | H6N6    | 6.25 ± 0.00                   |
| JS/F336  | B604     | H6N6    | 4.00 ± 1.39                   |
| JS/E1201 | B605     | H6N6    | 2.57 ± 2.75                   |
| JS/G91  | B606     | H6N6    | /                             |

H6N6 and JS/G91/H6N6. Lower viral titers of some H6N6 viruses were detected in livers, spleens and brains in some inoculated mice, suggesting that those viruses replicate in mice (Table 4).

Table 4. Replication of H6 viruses in mice. a Virus was detected in all of three mice, the virus titers were shown as mean ± SEM. b Virus was detected in part of three mice, the virus titers were shown respectively. c Virus was not detected in any of three mice.

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Molecular analyses suggested that all of the 22 H6 isolates were low pathogenic AIVs. The receptor-binding sites in the viral HA proteins possess the residues Q226 and G228, similar to those H6 isolates reported previously, which preferentially bind to the α-2,3-linked sialic acid receptors predominant in avian species. However, the E190V and N192D substitutions of H6 HA have been associated with interspecies transmission of AIVs from ducks to chickens, but those amino acids were not found in the five H6 AIVs (GD/1127/H6N2, GD/E3503/H6N2, HN/A729/H6N6, ZJ/B2028/H6N6 and FJ/D3480/H6N6) in this study, which would be the most likely reason that the replication of the H6 isolates in chickens was surprisingly limited. The residues 228S, 137N, 186L, A13S, and A193N at HA associated with human receptor-binding preference were not found in H6 isolates in this study. Additionally, almost all H6N6 isolates have the substitution of HA at V187D, the binding affinity might be altered to adapt to mammalian receptors.

None of E191V, H275Y, R293K and N295S substitution in the NA were found, which suggested those H6 isolates are sensitive to neuraminidase inhibitors such as oseltamivir. The deletion of 11 amino acids in the stalk region of the NA of FJ/D3480/H6N6, ZJ/B1994/H6N6and JS/F336/H6N6, which was also found in a swine H6N6 virus, might be associated with the infectivity of H6N6 viruses in mammals through affecting NA activity and the balance between HA and NA. It is notable that the S31N substitution in the M2 protein, which is associated with amantadine resistance of influenza virus, was found in GD/E3503/H6N2 and GD/F3891/H6N2.

Some of the H6 viruses replicated efficiently on MDCK and A549 cells and in the lungs of mice. The direct contact transmission of H6 viruses in guinea pigs was confirmed previously, which suggested the H6 viruses pose a clear threat to mammals. We found that all of the H6H2 isolates were able to replicate efficiently in lung and nasal turbinate without prior adaptation in mice, but the replication ability of H6N6 varies. Even with the same genotype, different H6N6 viruses showed distinct replication abilities in mice, suggesting some amino acid mutations affected the replication of the viruses. Lower titers of some H6 viruses were detected in livers, spleens and brains in a part of inoculated mice, which suggested those viruses posed a potential to adapt and caused a systemic infection in mammals.

Five H6 AIVs selected from different genotypes caused no clinical signs in any of the inoculated chickens. The replication and transmission of H6N2 and H6N6 viruses were limited in SFP chicken in this study, although the pathological changes caused by H6N2 in chickens were reported in California. Previous study showed that H6N2 isolates caused seroconversion in infected chickens, but no virus was recovered from the tissue samples, which suggested that the H6N2 strains replicated poorly and were nonpathogenic to chickens. Our findings showed that the five H6 strains selected from different genotypes caused no clinical signs in any of the inoculated chickens, but the serological test results suggested that chickens in four groups have been infected despite limited recovery of inoculated viruses from the tissue samples. The replication and transmission of H6N2 and H6N6 viruses were limited in SFP chicken in this study.

Overall, our study suggested that the H6 AIVs circulating in South China are genetically diverse and pose potential threat to mammals, and continual surveillance of H6 viruses is necessary in China.

Table 5. Replication of the H6 viruses in chickens. *The number of virus positive samples/total. bThe number of antibody positive samples/total, the HI titers ≥ 4 were considered as antibody positive samples.

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References
1. Wu, H. et al. Isolation and characterization of novel reassortant H6N1 avian influenza viruses from chickens in Eastern China. Virol. J. 15, 164. https://doi.org/10.1186/s12985-018-1063-y (2018).
2. Cox, N. J. & Subbarao, K. Influenza. The Lancet 354, 1277–1282. https://doi.org/10.1016/s0140-6736(99)01241-6 (1999).
3. Tong, S. et al. New world bats harbor diverse influenza A viruses. PLoS Pathog. 9, e1003657. https://doi.org/10.1371/journal.ppat.1003657 (2013).
4. Wu, Y., Wu, Y., Tefsen, B., Shi, Y. & Gao, G. F. Bat-derived influenza-like viruses H17N10 and H18N11. Sci. Rep. 7, 13150. https://doi.org/10.1038/s41598-017-13150-7 (2017).
5. Luo, P. et al. Complete genomic sequence of a novel natural recombinant H6N2 influenza virus from chickens in Guangdong, Southern China. J. Virol. 86, 7717–7718. https://doi.org/10.1128/jvi.00963-12 (2012).
6. Peng, Y. et al. Epidemiological surveillance of low pathogenic avian influenza virus (LPAIV) from poultry in Guangxi Province, Southern China. PLoS ONE 8, e77132. https://doi.org/10.1371/journal.pone.0077132 (2013).
7. Luo, S. et al. Surveillance of live poultry markets for low pathogenic avian influenza viruses in Guangxi Province, Southern China, from 2012–2015. Sci. Rep. 7, 17577. https://doi.org/10.1038/s41598-017-17740-0 (2017).
46. Ni, F., Kondrashkina, E. & Wang, Q. Structural and functional studies of influenza virus A/H6 hemagglutinin. PLoS ONE 10, e0134576. https://doi.org/10.1371/journal.pone.0134576 (2015).
47. Abolnik, C., Strydom, C., Rauff, D. L., Wandrag, D. B. R. & Petty, D. Continuing evolution of H6N2 influenza a virus in South African chickens and the implications for diagnosis and control. BMC Vet. Res. 15, 455. https://doi.org/10.1186/s12917-019-2210-4 (2019).
48. Castrucci, M. R. & Kawaoka, Y. Biologic importance of neuraminidase stalk length in influenza A virus. J. Virol. 67, 759–764 (1993).
49. Mitnaul, L. J. et al. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J. Virol. 74, 6015–6020. https://doi.org/10.1128/jvi.74.13.6015-6020.2000 (2000).
50. Radosevic, D. et al. Virtual screen for repurposing of drugs for candidate influenza a M2 ion-channel inhibitors. Front. Cell. Infect. Microbiol. 9, 67. https://doi.org/10.3389/fcimb.2019.00067 (2019).
51. Gillim-Ross, L. et al. Avian influenza h6 viruses productively infect and cause illness in mice and ferrets. J. Virol. 82, 10854–10863. https://doi.org/10.1128/jvi.01206-08 (2008).
52. Simulundu, E. et al. The zoonotic potential of avian influenza viruses isolated from wild waterfowl in Zambia. Arch. Virol. 159, 2633–2640. https://doi.org/10.1007/s00705-014-2124-1 (2014).

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Author contributions
Z.L. designed research; H.C. and W.L. performed research with the help of L.L., Y.S., X.L.; Q.T., J.Y., Q.L. and J.D. analyzed data; and Z.L., Q.L., W.L., H.C. wrote the main manuscript text. All authors reviewed and revised the drafts of this manuscript.

Competing interests
The authors declare no competing interests.

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