Infection, Genetics and Evolution 74 (2019) 103918

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Adaptive amino acid substitutions enhance the virulence of an avian-origin H6N1 influenza virus in mice☆

Haibo Wu, Fan Yang, Yixin Xiao, Fumin Liu, Hangping Yao, Nanping Wu*

State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, 310003 Hangzhou, China

ARTICLE INFO

Keywords:
Avian influenza virus
H6N1
Mouse-adapted
Substitutions
Virulence

ABSTRACT

The H6N1 subtype avian influenza virus (AIV) is a zoonotic infectious disease pathogen, which poses a threat to human health. In order to study the possible substitution of H6N1 AIV for mammals, an avian-origin H6N1 virus was successively passaged in mice. The results showed that PB2 (L193H and E627K), PA (S709F) and HA (V127I) proteins had multiple amino acid substitutions. The virulence of the mouse-adapted virus was stronger than that of the wild virus, and it was highly pathogenic to mice. Therefore, continued surveillance of these substitutions in poultry H6N1 viruses is required.

Influenza virus is one of the most common zoonotic infectious diseases, and avian influenza virus (AIV) can continuously infects humans and cause deaths. Since 1997, the H6 subtype AIV has been infected in poultry and caused widespread dissemination in live poultry markets (LPMs) in Asian (Chin et al., 2002; Huang et al., 2012; Wang et al., 2014; Wu et al., 2016a; Wu et al., 2015b; Zhao et al., 2011). In 2013, Taiwan reported the first case of human infection with H6N1 AIVs (Shi et al., 2013; Wei et al., 2013), followed by the isolation of H6N1 virus from dogs in Taiwan, which has high homology with human H6N1 virus, and harbored the substitution of E627K in polymerase basic protein 2 (PB2)(Lin et al., 2015). In addition, human-derived H6N1 virus has evolved a preference for human receptors (Wang et al., 2015), and has the ability to infect mammals across species barriers (Cheng et al., 2014; Lin et al., 2015). These results show that H6 AIV is a potential threat to human health, and suggest that people need to continue to monitor the circulating of H6 AIV in poultry.

The aim of this study was to identify possible alternatives to the novel reassortant Chinese H6N1 AIV for mammalian adaptation, and to determine the pathogenicity of the mouse adaptive H6N1 virus in vivo. The adaptation process of an avian-origin H6N1 virus in mouse lungs is carried out through successive lung-lung passage, according to the methods described elsewhere (Chen et al., 2015; Wu et al., 2016b; Yao et al., 2013). The successive lung-lung passage steps of mice were as follows: 6–8 weeks old female BALB/c mice (n = 6) were inoculated intranasally with $10^{6.0}$ 50% embryo infectious dose (EID_{50}) of the H6N1 virus, A/chicken/Zhejiang/1664/2017(H6N1) (ZJ1664, accession nos. KJ933371-8), in 0.05 mL phosphate buffered saline (PBS). The H6N1 virus originated from chickens in the live poultry market in eastern China's Zhejiang Province in 2017, showed low pathogenicity in mice (Wu et al., 2018). The mice were killed three days after inoculation, and lungs of the virus-inoculated mice were harvested and put into 1 mL PBS to crush. After centrifuging lung tissue, 0.05 mL supernatant was inoculated into a next normal mouse. The ZJ1664 virus (wild-type virus, WT-ZJ1664) was passaged serially (mouse-to-mouse) nine times to obtain the mouse-adapted virus [A/chicken/Zhejiang/1664-mouse-adapted/2017(H6N1), MA-ZJ1664].

Fifteen mice were inoculated with WT-ZJ1664 and MA-ZJ1664 $10^{6.0}$EID50 in the nasal cavity at a dose of 0.05 mL. Three mice were sacrificed at 3, 6 and 9 dpi, respectively. Viruses in lung, brain, heart, liver, kidney and spleen tissues were titrated in 9-day chicken embryos by the Reed and Muench method (Reed and Muench, 1938). Survival and weight-loss were monitored in the remaining six mice in each group. The animal experiments conducted in this study were approved by the First Affiliated Hospital, School of Medicine, Zhejiang University (No. 2015-15).

In order to evaluate the pathological changes of mouse-adapted virus MA-ZJ1664, the lung tissue of mice infected with virus was immobilized in 10% formalin. The tissues were embedded in paraffin wax

☆ All the correspondence regarding the manuscript can address to Haibo Wu, email: wuhaibo2014@163.com. (or) wuhaibo@zju.edu.cn. In the printed version, Haibo Wu needn't listed as the corresponding author.

* Corresponding author at: State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, China.

E-mail address: flwnp@zju.edu.cn (N. Wu).

https://doi.org/10.1016/j.meegid.2019.103918
Received 2 April 2019; Received in revised form 23 May 2019; Accepted 11 June 2019
Available online 12 June 2019

using standard tissue processing procedures, and slices were prepared and fixed on glass slides. Standard hematoxylin and eosin (H&E) staining were performed. Finally, sections were examined under light microscopy, as described elsewhere (Wu et al., 2016b; Wu et al., 2015a).

To elucidate the molecular mechanism of virulence enhancement of mouse adaptive viruses, we sequenced the whole genome of MA-ZJ1664 and compared it with that of WT-ZJ1664 to analyze the differences between nucleotides and amino acids. According to the manufacturer's instructions, RNA was extracted from supernatant of ruptured lung tissue using TRIzol (Life Technologies). RT-PCR was performed using one-step RNA PCR kit (TaKaRa), and all fragments of WT-ZJ1664 and MA-ZJ1664 genomes were amplified with primers described previously (Hoffmann et al., 2001; Wu et al., 2014). BioEdit DNA analysis software was used to compare and analyze the obtained sequences.

The in vitro growth properties of the WT-ZJ1664 and MA-ZJ1664 viruses were characterized in MDCK and A549 cells. Confluent MDCK or A549 cells were infected with WT-ZJ1664 and MA-ZJ1664 viruses at a multiplicity of infection (MOI) of 0.1, overlaid with serum-free DMEM containing TPCK-trypsin as described previously (Tan et al., 2014; Wang et al., 2012). Cell supernatants were harvested every 12 h until 72 h postinoculation and titrated in embryonated chicken eggs by the Reed and Muench method (Reed and Muench, 1938).

On the 4th day of infection with MA-ZJ1664, mice began to lose weight rapidly, while mice infected with WT-ZJ1664 only showed slight weight loss (Fig. 1). The survival rates were 66% (4/6) and 100% (6/6), respectively, suggesting that MA-ZJ1664 was more virulent than WT-ZJ1664 in mice. The WT-ZJ1664 virus was only detected in the lungs of mice, while MA-ZJ1664 virus could be detected in the lungs, hearts, spleens, kidneys, brain and liver of mice (Table S1). Pathological analysis of lung tissue showed that on the 8th day after virus infection, severe interstitial inflammatory hyperemia and exudative lesions were found in the lung tissue of MA-ZJ1664 mice. The lesions of lung tissue were larger and the lesions were fused with multiple patches (Fig. 2).

Previous studies have shown that amino acid substitution occurs in H6N1 (or H6N6) subtypes of AIV after multiple passages in each series of mice and increased the virulence of mouse-adapted virus in mice (Tan et al., 2014; Yao et al., 2013; Yu et al., 2014). In this study, the virulence of MA-ZJ1664 increased after nine passages, and these adaptive mutations were detected; a total of four amino acid substitutions were identified through adaptive lung-to-lung passage in mice, including PB2 (L193H and E627K), PA (S709F), and hemagglutinin (HA) (V127I) substitutions (Table 1 and Table S2). The E627K substitution in the PB2 protein reportedly influences the host range and confers increased virulence in many subtype viruses in animal models, including H6N1 and H6N6 (Tan et al., 2014; Yao et al., 2013; Yu et al., 2014). The L193H substitution was involved in the PB1 and PB2 interacted regions (Ohtsu et al., 2002), and the S709F substitution of the PA protein was located at the C-terminal region binds to the N-terminal region of PB1 (Guu et al., 2008). The V127I substitution of the HA protein occurs at the receptor pocket (Gamblin et al., 2004) and, given its location (Fig. S1). In this study, the mouse-adapted virus displayed expanded tissue tropism and increased replication kinetics in vivo when compared to the wild-type virus.

The growth curves in MDCK and A549 cells showed that WT-ZJ1664 and MA-ZJ1664 viruses reached a maximum at 36 postinoculation. Comparison of the growth curves obtained with these viruses indicated that the MA-ZJ1664 virus replicated faster than the WT-ZJ1664 virus in MDCK and A549 cells between 24 h and 48 h postinoculation ($P < .05$), suggesting that the amino acid substitutions enhanced the in vitro growth properties of the virus (Fig. S2).

Many previous studies have revealed a number of amino acid substitutions have been implicated in the adaptation of H6 AIVs in mice, and these mouse-adapted AIVs displayed increasing virulence and enhanced replication kinetics in mice and cell lines, and these mouse-adapted viruses contain genetic markers for mammalian adaptation and virulence, such as the HA (L111F, H156N, S263R and N394 T), PB2 (V308 M and E627K) and polymerase acidic protein (PA) (I38M, T97I, M155I and G622R) (Tan et al., 2014; Yao et al., 2013; Yu et al., 2014). In this study, the PB2 (L193H), PA (S709F), and HA (V127I) substitutions were first reported in the H6 AIVs.

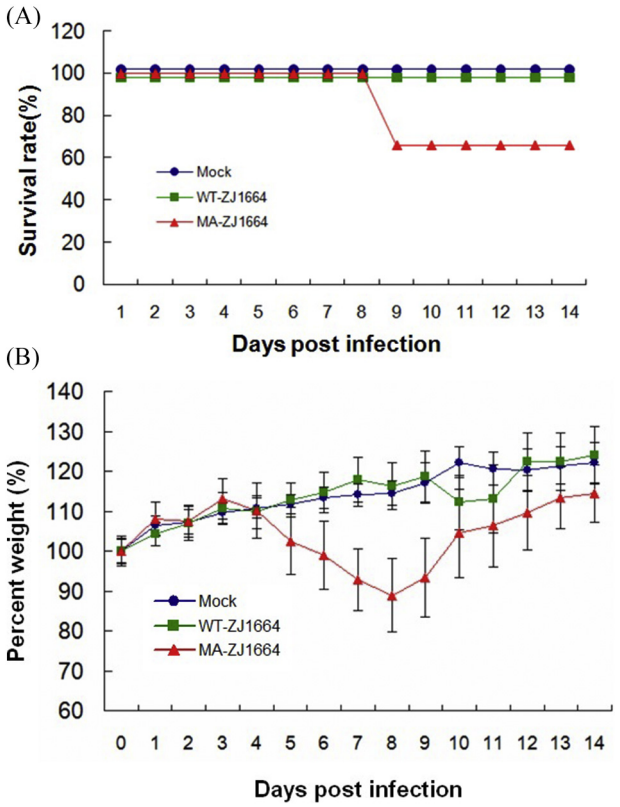

Fig. 1. Survival and body weight were measured in mice infected with the H6N1 avian influenza viruses. Survival (A) and body weight (B) were measured in BABL/c mice infected with the wild-type (WT-ZJ1664) or mouse-adapted (MA-ZJ1664) strains of an H6N1 avian influenza virus (n = 6/group). Each mouse was infected intranasally with $10^{6.0}$ EID_{50} of virus in a 50 μL volume. The number of surviving mice and their body weights were measured daily from the date of challenge to 14 days post inoculation.

The H6 influenza virus has been widely prevalent in poultry all over China, and domestic ducks are considered as the natural reservoir of avian influenza virus.Waterfowls infected with AIV generally do not show obvious clinical symptoms, so they can be used as intermediate hosts between migratory birds and terrestrial poultry in China's avian influenza ecosystem (Cheung et al., 2007; Huang et al., 2012; Zhao et al., 2011). Terrestrial poultry (such as chickens) have the molecular characteristics suitable for being the intermediate host of avian influenza virus to human transmission, which may produce new influenza viruses with pandemic potential (Gambaryan et al., 2008; Guo et al., 2000; Xu et al., 2007).

In this study, amino acid substitutions, in the PB2 (L193H and E627K), PA (S709F), and HA (V127I) proteins were identified in multiple genes of a mouse-adapted H6N1 AIV. These changes were associated with increased virulence compared with the wild-type virus. Furthermore, the adapted virus can cause death in mice, and displayed expanded tissue tropism and increased replication kinetics in vivo. These results suggest that the continuation of H6N1 molecular epidemiological studies and the examination of biological characteristics are essential to elucidate the mutation and evolutionary mechanism of AIV. Therefore, it is necessary to monitor these substitutions of influenza viruses in poultry.

Supplementary data to this article can be found online at https://

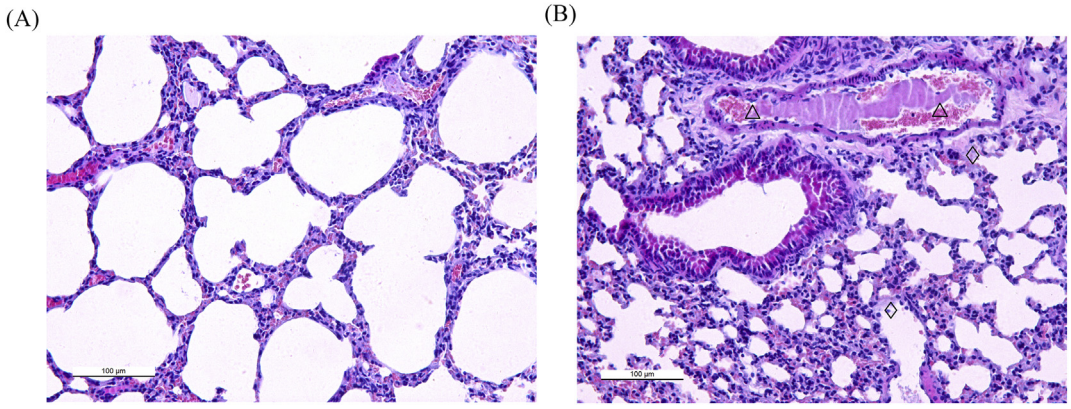

Fig. 2. Histology of mice infected with the H6N1 avian influenza viruses. Lung pathology was determined in mice infected with the wild-type (A) or mouse-adapted (B) strains of an H6N1 avian influenza virus at 6 days post inoculation (dpi). Hematoxylin and eosin staining was used to examine the histology of the lung tissue. Mice infected with the mouse-adapted virus displayed severe interstitial pneumonia in lung tissues, shown by the alveolar lumen flooded with dropout from alveolar cells, inflammatory cells (diamond) and erythrocytes (triangle).

Table 1
Nucleotide and amino acid substitutions identified in a mouse-adapted H6N1 avian influenza virus.

Segment	Nucleotide position	Nucleotide substitution		Amino acid position	Amino acid substitution
PB2	578 (T → A)	Passage0 (P0)	T	193 (L → H)	L
		P2	T		L
		P3	T/A		L/H
		P4	T/A		L/H
		P5	A		H
		P9	A		H
	1879 (G → A)	P0	G	627 (E → K)	E
		P1	A		K
		P9	A		K
PA	2126 (C → T)	P0	C	709 (S → F)	S
					S
					S/F
					F
		P7	C		
		P8	C/T		
		P9	T		
HA	379 (G → A)	P0	G	127 (V → I)	V
		P1	G/A		V/I
		P2	A		I
		P9	A		I

doi.org/10.1016/j.meegid.2019.103918.

Acknowledgements

This work was supported by the National Science Foundation of China (81502852), the Zhejiang Provincial Natural Science Foundation of China (LY19H260006), and the Independent Task of State Key Laboratory for Diagnosis and Treatment of Infectious Diseases (2019ZZ17).

References

Chen, Q., Yu, Z., Sun, W., Li, X., Chai, H., Gao, X., Guo, J., Zhang, K., Feng, N., Zheng, X., Wang, H., Zhao, Y., Qin, C., Huang, G., Yang, S., Qian, J., Gao, Y., Xia, X., Wang, T., Hua, Y., 2015. Adaptive amino acid substitutions enhance the virulence of an H7N7 avian influenza virus isolated from wild waterfowl in mice. Vet. Microbiol. 177, 18–24.

Cheng, K., Yu, Z., Gao, Y., Xia, X., He, H., Hua, Y., Chai, H., 2014. Experimental infection of dogs with H6N1 avian influenza A virus. Arch. Virol. 159, 2275–2282.

Cheung, C.L., Vijaykrishna, D., Smith, G.J., Fan, X.H., Zhang, J.X., Bahl, J., Duan, L., Huang, K., Tai, H., Wang, J., Poon, L.L., Peiris, J.S., Chen, H., Guan, Y., 2007. Establishment of influenza A virus (H6N1) in minor poultry species in southern China. J. Virol. 81, 10402–10412.

Chin, P.S., Hoffmann, E., Webby, R., Webster, R.G., Guan, Y., Peiris, M., Shortridge, K.F., 2002. Molecular evolution of H6 influenza viruses from poultry in southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. J. Virol. 76, 507–516.

Gambaryan, A.S., Tuzikov, A.B., Pazynina, G.V., Desheva, J.A., Bovin, N.V., Matrosovich, M.N., Klimov, A.I., 2008. 6-sulfo sialyl Lewis X is the common receptor determinant recognized by H5, H6, H7 and H9 influenza viruses of terrestrial poultry. Virol. J. 5, 85.

Gamblin, S.J., Haire, L.F., Russell, R.J., Stevens, D.J., Xiao, B., Ha, Y., Vasisht, N., Steinhauer, D.A., Daniels, R.S., Elliot, A., Wiley, D.C., Skehel, J.J., 2004. The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science 303, 1838–1842.

Guo, Y.J., Krauss, S., Senne, D.A., Mo, I.P., Lo, K.S., Xiong, X.P., Norwood, M., Shortridge, K.F., Webster, R.G., Guan, Y., 2000. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 267, 279–288.

Guu, T.S., Dong, L., Wittung-Stafshede, P., Tao, Y.J., 2008. Mapping the domain structure of the influenza a virus polymerase acidic protein (PA) and its interaction with the basic protein 1 (PB1) subunit. Virology 379, 135–142.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.

Huang, K., Zhu, H., Fan, X., Wang, J., Cheung, C.L., Duan, L., Hong, W., Liu, Y., Li, L., Smith, D.K., Chen, H., Webster, R.G., Webby, R.J., Peiris, M., Guan, Y., 2012. Establishment and lineage replacement of H6 influenza viruses in domestic ducks in southern China. J. Virol. 86, 6075–6083.

Lin, H.T., Wang, C.H., Chueh, L.L., Su, B.L., Wang, L.C., 2015. Influenza A(H6N1) virus in dogs, Taiwan. Emerg. Infect. Dis. 21, 2154–2157.

Ohtsu, Y., Honda, Y., Sakata, Y., Kato, H., Toyoda, T., 2002. Fine mapping of the subunit binding sites of influenza virus RNA polymerase. Microbiol. Immunol. 46, 167–175.

Reed, L., Muench, H., 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Shi, W., Shi, Y., Wu, Y., Liu, D., Gao, G.F., 2013. Origin and molecular characterization of the human-infecting H6N1 influenza virus in Taiwan. Protein Cell 4, 846–853.

Tan, L., Su, S., Smith, D.K., He, S., Zheng, Y., Shao, Z., Ma, J., Zhu, H., Zhang, G., 2014. A combination of HA and PA mutations enhances virulence in a mouse-adapted H6N6 influenza A virus. J. Virol. 88, 14116–14125.

Wang, G., Deng, G., Shi, J., Luo, W., Zhang, G., Zhang, Q., Liu, L., Jiang, Y., Li, C., Sriwilaijaroen, N., Hiramatsu, H., Suzuki, Y., Kawaoka, Y., Chen, H., 2014. H6 influenza viruses pose a potential threat to human health. J. Virol. 88, 3953–3964.

Wang, F., Qi, J., Bi, Y., Zhang, W., Wang, M., Zhang, B., Liu, J., Yan, J., Shi, Y., Gao, G.F., 2015. Adaptation of avian influenza A (H6N1) virus from avian to human receptor-binding preference. EMBO J. 34, 1661–1673.

Wang, J., Sun, Y., Xu, Q., Tan, Y., Pu, J., Yang, H., Brown, E.G., Liu, J., 2012. Mouse-adapted H9N2 influenza A virus PB2 protein M147L and E627K mutations are critical for high virulence. PLoS One 7, e40752.

Wei, S.H., Yang, J.R., Wu, H.S., Chang, M.C., Lin, J.S., Lin, C.Y., Liu, Y.L., Lo, Y.C., Yang, C.H., Chuang, J.H., Lin, M.C., Chung, W.C., Liao, C.H., Lee, M.S., Huang, W.T., Chen, P.J., Liu, M.T., Chang, F.Y., 2013. Human infection with avian influenza A H6N1 virus: an epidemiological analysis. Lancet Respir. Med. 1, 771–778.

Wu, H., Peng, X., Xu, L., Jin, C., Cheng, L., Lu, X., Xie, T., Yao, H., Wu, N., 2014. Novel reassortant influenza A(H5N8) viruses in domestic ducks, eastern China. Emerg. Infect. Dis. 20, 1315–1318.

Wu, H., Peng, X., Cheng, L., Lu, X., Jin, C., Xie, T., Yao, H., Wu, N., 2015a. Genetic and molecular characterization of H9N2 and H5 avian influenza viruses from live poultry markets in Zhejiang Province, eastern China. Sci. Rep. 5, 17508.

Wu, H., Peng, X., Cheng, L., Wu, N., 2015b. Molecular characterization of novel reassortant H6N2 subtype avian influenza viruses isolated from poultry in eastern China, in 2014. Infect. Genet. Evol. 36, 41–45.

Wu, H., Lu, R., Peng, X., Cheng, L., Jin, C., Lu, X., Xie, T., Yao, H., Wu, N., 2016a. Isolation and genetic characterization of novel reassortant H6N6 subtype avian influenza viruses isolated from chickens in eastern China. Arch. Virol. 161, 1859–1872.

Wu, H., Peng, X., Cheng, L., Jin, C., Lu, X., Xie, T., Yao, H., Wu, N., 2016b. Multiple amino acid substitutions involved in the adaptation of avian-origin influenza A (H10N7) virus in mice. Arch. Virol. 161, 977–980.

Wu, H., Yang, F., Liu, F., Lu, R., Peng, X., Chen, B., Yao, H., Wu, N., 2018. Isolation and characterization of novel reassortant H6N1 avian influenza viruses from chickens in eastern China. Virol. J. 15, 164.

Xu, K.M., Li, K.S., Smith, G.J., Li, J.W., Tai, H., Zhang, J.X., Webster, R.G., Peiris, J.S., Chen, H., Guan, Y., 2007. Evolution and molecular epidemiology of H9N2 influenza a viruses from quail in southern China, 2000 to 2005. J. Virol. 81, 2635–2645.

Yao, Y., Wang, H., Chen, Q., Zhang, H., Zhang, T., Chen, J., Xu, B., Sun, B., Chen, Z., 2013. Characterization of low-pathogenic H6N6 avian influenza viruses in Central China. Arch. Virol. 158, 367–377.

Yu, Z., Cheng, K., Xin, Y., Sun, W., Li, X., Huang, J., Zhang, K., Yang, S., Wang, T., Zheng, X., Wang, H., Hua, Y., Chai, H., Qin, C., Qian, J., Gao, Y., Xia, X., 2014. Multiple amino acid substitutions involved in the adaptation of H6N1 avian influenza virus in mice. Vet. Microbiol. 174, 316–321.

Zhao, G., Lu, X., Gu, X., Zhao, K., Song, Q., Pan, J., Xu, Q., Duan, Z., Peng, D., Hu, S., Wang, X., Liu, X., 2011. Molecular evolution of the H6 subtype influenza a viruses from poultry in eastern China from 2002 to 2010. Virol. J. 8, 470.