Title	Potency of an inactivated influenza vaccine prepared from A/duck/Hong Kong/960/1980 (H6N2) against a challenge with A/duck/Vietnam/OIE-0033/2012 (H6N2) in mice
Author(s)	Nishi, Tatsuya; Sakoda, Yoshihiro; Okamatsu, Masatoshi; Kida, Hiroshi
Citation	Archives of Virology, 159(10), 2567-2574 https://doi.org/10.1007/s00705-014-2107-2
Issue Date	2014-10
Doc URL	http://hdl.handle.net/2115/59860
Rights	The final publication is available at link.springer.com
Type	article (author version)
File Information	Arch Virol.159(10)p.2567.pdf

[Instructions for use](#)

**Potency of an inactivated influenza vaccine prepared from A/duck/Hong Kong/960/1980
(H6N2) against a challenge with A/duck/Vietnam/OIE-0033/2012 (H6N2) in mice**

Tatsuya Nishi • Yoshihiro Sakoda • Masatoshi Okamatsu • Hiroshi Kida*

T. Nishi • Y. Sakoda • M. Okamatsu • H. Kida

Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary
Medicine, Hokkaido University, Sapporo 060-0818, Japan

H. Kida

Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan

*Corresponding author: Laboratory of Microbiology, Department of Disease Control, Graduate
School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060-0818,
Japan

Tel.: +81-11-706-5207; Fax: +81-11-706-5273

E-mail: kida@vetmed.hokudai.ac.jp

Abstract

H6 influenza viruses are prevalent in domestic and wild birds in Eurasian countries and have been isolated from pigs and a human. To prepare for influenza pandemic, we have established an influenza virus library consisting of more than 1,300 influenza virus strains, including 144 combinations of 16 hemagglutinin and 9 neuraminidase subtypes. H6 viruses in the library were classified into Early, Group II, Group III, and W312 sublineages and the North America lineage on the basis of their phylogenetic features. Chicken antisera to A/duck/Hong Kong/960/1980 (H6N2) of the Early sublineage broadly reacted with viruses of different sublineages in hemagglutinin inhibition test. A whole inactivated virus particle vaccine was prepared from A/duck/Hong Kong/960/1980 (H6N2) which was stocked in the influenza virus library. The potency of this vaccine against A/duck/Vietnam/OIE-0033/2012 (H6N2), which belongs to a different sublineage was evaluated in mice. The test vaccine was sufficiently potent to induce an immune response that reduced the impact of disease caused by a challenge with A/duck/Vietnam/OIE-0033/2012 (H6N2) in mice. The present results indicate that the whole inactivated virus particle vaccine prepared from a virus strain in the influenza virus library is useful as a vaccine against pandemic influenza.

Introduction

Influenza A viruses infect a large variety of birds and mammals including humans. In humans, H1 and H3 viruses are causing seasonal influenza. Since neutralizing (NT) antibodies to the hemagglutinin (HA) or neuraminidase (NA) are subtype-specific, human population is immunologically naïve to infection with viruses of novel subtypes. Influenza viruses of each of the HA subtypes (H1–H13) replicate in the respiratory tract of pigs [13]. In addition, avian influenza viruses acquire the potential to infect humans after multiple infections in pigs [25]. Case of human infection with H1, H2, H3, H5, H6, H7, H9, and H10 influenza viruses have been reported [1, 3, 4, 21, 24, 30]. Therefore, it is recommended to prepare vaccines against viruses of each of the HA subtype for the preparedness for future pandemics.

To provide information on the precursor genes of future pandemic influenza viruses, global surveillance of avian influenza has been conducted since 1977 in Japan, Alaska, Siberia, Mongolia, and Australia [9, 15, 20, 23, 29]. Virus isolates in the surveillance have been stored in the influenza virus library in our laboratory for vaccine and diagnostic use. Their biological characteristics have been analyzed, and the data are available at <http://virusdb.czc.hokudai.ac.jp/>. In our previous study, vaccines against infections of H1, H5, H7, and H9 influenza viruses were prepared from viruses in the library, and they induced effective immunity against challenge viruses in mice and cynomolgus macaques [10, 11, 17, 19, 27]. It was also revealed that inactivated whole particle virus vaccines

were more effective than ether-split vaccines [6, 19].

H6 influenza viruses are prevalent in poultry populations in Eurasian countries [8, 14]. The HA genes of Eurasian H6 viruses have been phylogenetically divided into Early, Europe, Group I, Group II, Group III, and W312 sublineages [8]. H6 viruses were also isolated from domestic pigs in southern China [31]. In May 2013, an H6N1 virus was isolated from a 20-year-old woman with symptoms including fever, cough, headache, and muscle ache in Taiwan [30]. In the present study, thus, to prepare for a potential influenza pandemic, H6 viruses from the influenza virus library were analyzed genetically and antigenically to select a vaccine strain. The efficacy of an inactivated whole virus particle vaccine prepared from the strain was evaluated in mice.

Materials and methods

Viruses and cells

A/duck/Hong Kong/960/1980 (H6N2) (Dk/Hong Kong/960) and A/teal/Hong Kong/W312/1997 (H6N1) were kindly provided by Dr. Shortridge K.F., and Dr. Guan Y., University of Hong Kong, Hong Kong SAR, China, respectively. A/duck/Vietnam/OIE-0033/2012 (H6N2) (Dk/Vietnam/2012) was isolated from a domestic duck in our surveillance study in Vietnam [18]. The HA sequence data of the representative viruses of each sublineage, Dk/Hong Kong/960, Dk/Vietnam/2012, A/teal/Hong Kong/W312/1997 (H6N1), A/shearwater/South Australia/1/1972

(H6N5), and A/turkey/Massachusetts/3740/1965 (H6N2) have been deposited at GenBank/EMBL/DDBJ (accession no. AB294213, AB727927, AF250479, AB278600, and AB296072). All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h, and infectious allantoic fluids were stored at –80 °C until use.

Madin–Darby canine kidney (MDCK) cells were grown in MEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with calf serum and used to titrate virus infectivity by plaque assay [25].

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluids of embryonated chicken eggs using the TRIzol LS Reagent (Invitrogen, Carlsbad, California, U.S.A.) and reverse-transcribed with the Uni12 primer (5'-AGCAAAAGCAGG-3') [26] and M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, California, U.S.A.). The full-length cDNAs of the eight gene segments were amplified by polymerase chain reaction with Ex-Taq (TaKaRa, Shiga, Japan) and gene-specific primer sets [7]. Direct sequencing of each gene segment was performed using the 3500 Genetic Analyzer (Life Technologies). For phylogenetic analysis, sequence data of the genes together with those from the public database were analyzed by the maximum-likelihood method using MEGA 5.0 software (<http://www.megasoftware.net/>).

89

90 Serological tests

91 Hemagglutinin inhibition (HI) and NT tests were performed basically according to Sakabe *et al.*
92 [22]. The HI titer was expressed as the reciprocal of the highest serum dilution showing complete
93 inhibition of the hemagglutination of 8 HA units of the virus. In NT tests, titers were determined as
94 the reciprocals of serum dilution of the complete inhibition of the cytopathic effect of 100 50%
95 tissue culture infectious dose (TCID₅₀) of viruses using MDCK cells.

96

97 Virus growth in embryonated chicken eggs

98 One hundred 50% egg infectious dose (EID₅₀) of viruses were inoculated into 10-day-old
99 embryonated chicken eggs and incubated at 35 °C. Allantoic fluids were collected at 12, 24, 36, 48,
100 60, and 72 h after inoculation to determine virus titers by plaque assay in MDCK cells.

101

102 Vaccine preparation

103 Dk/Hong Kong/960 and Dk/Vietnam/2012 were inoculated into the allantoic cavities of
104 10-day-old embryonated chicken eggs and propagated at 35 °C for 48 h. The viruses in the
105 allantoic fluids were purified by differential centrifugation and sedimentation through a sucrose
106 gradient [12]. The purified virus was inactivated with 0.2% formalin at 4 °C for 7 days. The

protein concentration was measured using the BCA Protein Assay Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

Potency test of vaccines in mice

Each of the whole inactivated vaccines of Dk/Hong Kong/960 (2, 10, or 50 µg of protein) or Dk/Vietnam/2012 (2 or 10 µg of protein) was injected once intraperitoneally into ten 4-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan). PBS was injected into the control mice. Three weeks later, serum samples were collected and mice were challenged with 10^6 PFU/40 µl of Dk/Vietnam/2012 intranasally under anesthesia. At 3 days post-challenge, five mice of each group were sacrificed, and their lungs were collected. Virus titers in the lung homogenates were quantified by plaque assay in MDCK cells. Five other mice of each group were observed for weight loss for 14 days. Each of the inactivated vaccines of Dk/Hong Kong/960 (2, 10 or 50 µg of protein) was also injected twice into mice with a 2-week interval. Two weeks after the second vaccination, serum samples were collected, and mice were challenged with 10^6 PFU/40 µl of Dk/Vietnam/2012. Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approved numbers: 13-0104), and all experiments were performed according to the guidelines of this committee.

Results

Genetic analysis of H6 influenza viruses

Nucleotide sequences of the HA genes of the 94 H6 viruses in the influenza virus library were phylogenetically analyzed by the maximum-likelihood method with other representative H6 strains of each lineage. On the basis of the results of phylogenetic analysis, H6 HA genes were classified into the Early, Group II, Group III, and W312 sublineages and the North America lineage (Fig. 1). The H6 viruses isolated from poultry in Hong Kong in 1980 were classified into the Early sublineage. The H6 viruses isolated from poultry in Asia in the past decade were classified into Group II, and the isolates from wild birds in the East Asia and poultry in Taiwan were classified into Group III.

Antigenic analysis of H6 influenza viruses

Sixteen H6 influenza virus strains were selected from 94 strains in the library and antigenically analyzed using the HI test (Table 1). H6 viruses of the Group II displayed low reactivity with the antisera of H6 viruses of other sublineages. Antiserum against Dk/Hong Kong/960 of the Early sublineage reacted with H6 viruses of all sublineages. These results indicate that the antigenicity of H6 viruses of Group II is different from the other H6 viruses and the vaccine strain for H6 virus had better to be selected from the viruses of the Early sublineage.

143

144 Selection of a vaccine strain from H6 influenza viruses in the influenza virus library

145 To select a vaccine strain, the growth in embryonated chicken eggs of six H6 viruses of the
146 Early sublineage, A/duck/Hong Kong/834/1980 (H6N1), A/duck/Hong Kong/882/1980 (H6N2)
147 (Dk/Hong Kong/882), A/duck/Hong Kong/926/1980 (H6N2), A/duck/Hong Kong/941/1980 (H6N1),
148 A/duck/Hong Kong/943/1980 (H6N5), and Dk/Hong Kong/960 were assessed. Dk/Hong
149 Kong/882 and Dk/Hong Kong/960 replicated most effectively in chicken embryos. The highest
150 virus titers for 72 h were $10^{7.4}$ and $10^{7.1}$ PFU/ml, respectively. Their replications had reached a
151 plateau by 36 and 24 h, respectively (data not shown). Following this, protein yields after virus
152 purification were assessed. Dk/Hong Kong/882 was purified from 30 embryonated chicken eggs,
153 and 650 µg of purified virus were obtained. Dk/Hong Kong/960 was purified from the same
154 number of embryonated chicken eggs, and 990 µg of purified virus were obtained, suggesting that
155 Dk/Hong Kong/960 exhibited a higher protein yield after virus purification than Dk/Hong Kong/882.
156 Therefore, Dk/Hong Kong/960 was selected as a vaccine strain.

157

158 Potency test of the vaccine against an H6 influenza virus strain in mice

159 The inactivated vaccine of Dk/Hong Kong/960 or Dk/Vietnam/2012 was intraperitoneally
160 injected once into mice. The serum antibody titers of mice against the vaccine and the challenge

strain were examined (Table 2). The NT antibodies to the homologous vaccine strain were detected in the sera of all mice vaccinated with 10 or 50 µg of protein of the Dk/Hong Kong/960 vaccine and 2 or 10 µg of protein of the Dk/Vietnam/2012 vaccine. The NT antibodies to Dk/Vietnam/2012 were also detected in the sera of more than half of the mice vaccinated with 10 or 50 µg of protein of the Dk/Hong Kong/960 vaccine.

Further, to assess the potency of the vaccine against a challenge with Dk/Vietnam/2012, 10^{6.0} PFU of the virus were intranasally inoculated into mice vaccinated once. The virus titers in the lungs after the challenge were measured to assess the protective immunity induced by the vaccines (Table 2). The virus titers in the lungs were <10^{1.0-3.8} PFU/g in mice injected with 2 or 10 µg of protein of the Dk/Vietnam/2012 vaccine. The virus titers in the lungs of the mice injected with the Dk/Hong Kong/960 vaccine containing 10 or 50 µg of protein were 10^{4.2-5.3} PFU/g, and those of mice injected with 2 µg of protein were not significantly different from those of control mice. These results indicate that the Dk/Vietnam/2012 and Dk/Hong Kong/960 vaccines induced immunity in mice to suppress virus replication in the lungs. The potency of the vaccine was also evaluated by the weight loss of mice after the challenge (Fig. 2). The mice injected with 2 or 10 µg of the Dk/Vietnam/2012 vaccine or 50 µg of the Dk/Hong Kong/960 vaccine did not display remarkable weight loss. The body weight of mice injected with 2 or 10 µg of the Dk/Hong Kong/960 vaccine recovered faster than the control group mice. These results indicate that the Dk/Vietnam/2012 and

Dk/Hong Kong/960 vaccines induced immunity in mice and suppressed the impact of disease caused by the challenge strain.

To improve the efficacy of the Dk/Hong Kong/960 vaccine, the vaccine was intraperitoneally injected twice into mice. At 2 weeks post-injection, the serum NT antibody titers against Dk/Hong Kong/960 were higher than those of mice injected once (Table 3). NT antibodies against Dk/Vietnam/2012 were also detected in the sera of all vaccinated mice (Table 3). The virus replication in the lungs of all the vaccinated mice was significantly suppressed compared with that of control group (Table 3). The mice injected twice with 50 µg of the Dk/Hong Kong/960 vaccine did not display remarkable weight loss, and those injected with 2 or 10 µg of the Dk/Hong Kong/960 vaccine recovered their weight faster than the control group mice (Fig. 3). These results indicate that the potency of the Dk/Hong Kong/960 vaccine against the challenge strain was enhanced by two intraperitoneal injections in mice.

Discussion

The HA genes of H6 virus strains in the influenza virus library were phylogenetically divided into five groups: the Early, Group II, Group III, and W312 sublineages and the North America lineage (Fig. 1). Antigenic analysis revealed that the antigenicity of the viruses of each sublineage differed from each other (Table 1). Since it is not possible to predict which virus of any

sublineage would cause pandemic influenza in humans, it is important to prepare a vaccine that broadly cross-reacts with H6 viruses of all sublineages. It was reported that the live attenuated A/teal/Hong Kong/W312/1997 (H6N1) vaccine induced cross protective immunity against the viruses of the Early, W312 sublineages and the North America lineage in mice and ferrets [2, 28]. In the present study, the antiserum to an Early sublineage virus broadly reacted with viruses of all sublineages, including Group II and Group III (Table 1); therefore, Dk/Hong Kong/960 of the Early sublineage was selected as a vaccine strain. To evaluate the potency of this vaccine, Dk/Vietnam/2012 from the H6 viruses of Group II, which are antigenically different from other H6 viruses, was selected as a challenge strain.

It was reported that whole virus particle vaccines induced strong immune responses, and an H5N1 whole particle vaccine induced protective immunity against a challenge with antigenically distinct virus [6, 10, 16]. In the present study, an inactivated whole particle vaccine was prepared from an H6 avian influenza virus, Dk/Hong Kong/960, from the influenza virus library. The potency of this vaccine against Dk/Vietnam/2012 was evaluated in mice. The Dk/Hong Kong/960 vaccine conferred immunity in mice to reduce the impact of disease caused by a challenge with Dk/Vietnam/2012 (Fig. 2 and 3, Table 2 and 3). The present results indicate that the whole particle vaccine has potency even against the antigenically distinct H6 virus in mice.

Vaccination is one of the important control measures for human influenza; however,

approximately 6 months are required to produce vaccines [5]. To prepare for future influenza pandemics, avian influenza viruses of 144 combinations of 16 HA and 9 NA subtypes have been stocked in the influenza virus library. Since the pathogenicity, antigenicity, genetic information, and yield in embryonated chicken eggs of the viruses in the influenza virus library were previously assessed, we can provide vaccine strains immediately. The present study indicates that the whole virus particle vaccine prepared from a virus strain from the influenza virus library is useful as a vaccine against pandemic influenza.

Acknowledgements

We thank Dr. Shortridge K. F. and Dr. Guan Y. for providing viruses. This study was supported by the Strategic Funds for the Promotion of Science and Technology (2011–2013), Japan (JST), the Global Center of Excellence (GCOE) Program of Hokkaido University, and the Japan Initiative for Global Research Network on Infectious Disease (J-GRID).

References

1. Arzey GG, Kirkland PD, Arzey KE, Frost M, Maywood P, Conaty S, Hurt AC, Deng YM, Iannello P, Barr I, Dwyer DE, Ratnamohan M, McPhie K, Selleck P (2012) Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia. *Emerg Infect Dis* 18:814-816
2. Chen Z, Santos C, Aspelund A, Gillim-Ross L, Jin H, Kemble G, Subbarao K (2009) Evaluation of live attenuated influenza a virus h6 vaccines in mice and ferrets. *J Virol* 83:65-72
3. Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM, Team NS-OIAHNVI (2009) Emergence of a novel swine-origin influenza A

237 (H1N1) virus in humans. *N Engl J Med* 360:2605-2615

238 4. DeLay PD, Casey HL, Tubiash HS (1967) Comparative study of fowl plague virus and a virus
239 isolated from man. *Public Health Rep* 82:615-620

240 5. Gerdil C (2003) The annual production cycle for influenza vaccine. *Vaccine* 21:1776-1779

241 6. Hagenslaars N, Mastrobattista E, Glansbeek H, Heldens J, van den Bosch H, Schijns V, Betbeder
242 D, Vromans H, Jiskoot W (2008) Head-to-head comparison of four nonadjuvanted inactivated
243 cell culture-derived influenza vaccines: effect of composition, spatial organization and
244 immunization route on the immunogenicity in a murine challenge model. *Vaccine* 26:6555-6563

245 7. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the
246 full-length amplification of all influenza A viruses. *Arch Virol* 146:2275-2289

247 8. Huang K, Bahl J, Fan XH, Vijaykrishna D, Cheung CL, Webby RJ, Webster RG, Chen H, Smith
248 GJ, Peiris JS, Guan Y (2010) Establishment of an H6N2 influenza virus lineage in domestic
249 ducks in southern China. *J Virol* 84:6978-6986

250 9. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H (1995) Perpetuation of influenza A
251 viruses in Alaskan waterfowl reservoirs. *Arch Virol* 140:1163-1172

252 10. Itoh Y, Ozaki H, Tsuchiya H, Okamoto K, Torii R, Sakoda Y, Kawaoka Y, Ogasawara K, Kida H
253 (2008) A vaccine prepared from a non-pathogenic H5N1 avian influenza virus strain confers
254 protective immunity against highly pathogenic avian influenza virus infection in cynomolgus
255 macaques. *Vaccine* 26:562-572

256 11. Itoh Y, Ozaki H, Ishigaki H, Sakoda Y, Nagata T, Soda K, Isoda N, Miyake T, Ishida H, Okamoto
257 K, Nakayama M, Tsuchiya H, Torii R, Kida H, Ogasawara K (2010) Subcutaneous inoculation of
258 a whole virus particle vaccine prepared from a non-pathogenic virus library induces protective
259 immunity against H7N7 highly pathogenic avian influenza virus in cynomolgus macaques.
260 *Vaccine* 28:780-789

261 12. Kida H, Yanagawa R (1979) Isolation and characterization of influenza A viruses from wild
262 free-flying ducks in Hokkaido, Japan. *Zentralbl Bakteriell Orig A* 244:135-143

263 13. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG (1994)
264 Potential for transmission of avian influenza viruses to pigs. *J Gen Virol* 75 (Pt 9):2183-2188

265 14. Kim HR, Lee YJ, Lee KK, Oem JK, Kim SH, Lee MH, Lee OS, Park CK (2010) Genetic
266 relatedness of H6 subtype avian influenza viruses isolated from wild birds and domestic ducks in
267 Korea and their pathogenicity in animals. *J Gen Virol* 91:208-219

268 15. Kishida N, Sakoda Y, Shiromoto M, Bai GR, Isoda N, Takada A, Laver G, Kida H (2008) H2N5
269 influenza virus isolates from terns in Australia: genetic reassortants between those of the
270 Eurasian and American lineages. *Virus Genes* 37:16-21

271 16. Lu X, Edwards LE, Desheva JA, Nguyen DC, Rekstin A, Stephenson I, Szretter K, Cox NJ,
272 Rudenko LG, Klimov A, Katz JM (2006) Cross-protective immunity in mice induced by

- live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine 24:6588-6593
17. Nomura N, Sakoda Y, Soda K, Okamatsu M, Kida H (2012) An H9N2 influenza virus vaccine prepared from a non-pathogenic isolate from a migratory duck confers protective immunity in mice against challenge with an H9N2 virus isolated from a girl in Hong Kong. J Vet Med Sci 74:441-447
18. Okamatsu M, Nishi T, Nomura N, Yamamoto N, Sakoda Y, Sakurai K, Chu HD, Thanh LP, Van Nguyen L, Van Hoang N, Tien TN, Yoshida R, Takada A, Kida H (2013) The genetic and antigenic diversity of avian influenza viruses isolated from domestic ducks, muscovy ducks, and chickens in northern and southern Vietnam, 2010-2012. Virus Genes 47:317-329
19. Okamatsu M, Sakoda Y, Hiono T, Yamamoto N, Kida H (2013) Potency of a vaccine prepared from A/swine/Hokkaido/2/1981 (H1N1) against A/Narita/1/2009 (H1N1) pandemic influenza virus strain. Virol J 10:47
20. Okazaki K, Takada A, Ito T, Imai M, Takakuwa H, Hatta M, Ozaki H, Tanizaki T, Nagano T, Ninomiya A, Demenev VA, Tyaptirganov MM, Karatayeva TD, Yamnikova SS, Lvov DK, Kida H (2000) Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch Virol 145:885-893
21. Peiris M, Yam WC, Chan KH, Ghose P, Shortridge KF (1999) Influenza A H9N2: aspects of laboratory diagnosis. J Clin Microbiol 37:3426-3427
22. Sakabe S, Sakoda Y, Haraguchi Y, Isoda N, Soda K, Takakuwa H, Saijo K, Sawata A, Kume K, Hagiwara J, Tuchiya K, Lin Z, Sakamoto R, Imamura T, Sasaki T, Kokumai N, Kawaoka Y, Kida H (2008) A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus in chickens. Vaccine 26:2127-2134
23. Sakoda Y, Sugar S, Batchluun D, Erdene-Ochir TO, Okamatsu M, Isoda N, Soda K, Takakuwa H, Tsuda Y, Yamamoto N, Kishida N, Matsuno K, Nakayama E, Kajihara M, Yokoyama A, Takada A, Sodnomdarjaa R, Kida H (2010) Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory. Virology 406:88-94
24. Schäfer JR, Kawaoka Y, Bean WJ, Süss J, Senne D, Webster RG (1993) Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology 194:781-788
25. Shichinohe S, Okamatsu M, Sakoda Y, Kida H (2013) Selection of H3 avian influenza viruses with SA α 2,6Gal receptor specificity in pigs. Virology 444:404-408
26. Skehel JJ, Hay AJ (1978) Nucleotide sequences at the 5' termini of influenza virus RNAs and their transcripts. Nucleic Acids Res 5:1207-1219

- 309 27. Soda K, Sakoda Y, Isoda N, Kajihara M, Haraguchi Y, Shibuya H, Yoshida H, Sasaki T,
310 Sakamoto R, Saijo K, Hagiwara J, Kida H (2008) Development of vaccine strains of H5 and H7
311 influenza viruses. *Jpn J Vet Res* 55:93-98
- 312 28. Talaat KR, Karron RA, Luke CJ, Thumar B, McMahon BA, Chen GL, Lamirande EW, Jin H,
313 Coelingh KL, Kemble G, Subbarao K (2011) An open label Phase I trial of a live attenuated
314 H6N1 influenza virus vaccine in healthy adults. *Vaccine* 29:3144-3148
- 315 29. Yamamoto N, Sakoda Y, Motoshima M, Yoshino F, Soda K, Okamatsu M, Kida H (2011)
316 Characterization of a non-pathogenic H5N1 influenza virus isolated from a migratory duck
317 flying from Siberia in Hokkaido, Japan, in October 2009. *Virology* 418:65
- 318 30. Yuan J, Zhang L, Kan X, Jiang L, Yang J, Guo Z, Ren Q (2013) Origin and Molecular
319 Characteristics of a Novel 2013 Avian Influenza A(H6N1) Virus Causing Human Infection in
320 Taiwan. *Clin Infect Dis* 57:1367-8
- 321 31. Zhang G, Kong W, Qi W, Long LP, Cao Z, Huang L, Qi H, Cao N, Wang W, Zhao F, Ning Z,
322 Liao M, Wan XF (2011) Identification of an H6N6 swine influenza virus in southern China.
323 *Infect Genet Evol* 11:1174-1177
- 324

Figure captions

Fig. 1 Phylogenetic tree of H6 HA genes of influenza viruses. Nucleotides 123–857 (735 bp) of the HA gene were used for maximum-likelihood phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node indicate the confidence level in bootstrap analysis with 1,000 replications. The representative viruses of each sublineage are underlined. The viruses in the influenza virus library are presented in bold. The vaccine and challenge strains are highlighted

Fig. 2 Changes in the body weight of mice vaccinated intraperitoneally once with the Dk/Hong Kong/960 (A) or Dk/Vietnam/2012 (B) vaccine after a challenge with Dk/Vietnam/2012. Data are shown as the mean body weight $\pm$ standard error. *, $P < 0.05$ versus the group of mice injected with PBS

Fig. 3 Changes in the body weight of mice vaccinated intraperitoneally twice with the Dk/Hong Kong/960 vaccine after a challenge with Dk/Vietnam/2012. Data are shown as the mean body weight $\pm$ standard error. *, $P < 0.05$ versus the group of mice injected with PBS

Fig. 1 Nishi *et al.*

Fig. 2 Nishi *et al.*

Fig. 3 Nishi *et al.*

Table 1 The cross-reactivity of H6 viruses with chicken antisera to representative strains of each lineage in HI test

Lineage	Sublineage ^a	Virus	Antiserum to ^b					North America
			Eurasia					
			—	Group II	Group III	W312	Early	
			S.Aus/72	VN4429/10	Hok/04	W312	HK/960	
Eurasia	—	Shearwater/S. Australia/1/1972 (H6N5)	<u>2,560</u>	40	640	640	2,560	640
	Group II	Duck/Vietnam/OIE-4429/2010 (H6N2)	160	<u>5,120</u>	160	40	640	2,560
		Duck/Vietnam/OIE-0033/2012 (H6N2)	40	2,560	160	40	320	640
		Duck/Vietnam/OIE-2562/2011 (H6N6)	80	5,120	160	40	320	160
		Duck/Vietnam/OIE-1213/2012 (H6N6)	160	2,560	160	20	320	40
		Duck/Fujian/1695/2005 (H6N2)	40	2,560	1,280	40	2,560	1,280
	Group III	Duck/Hokkaido/262/2004 (H6N1)	1,280	160	<u>2,560</u>	320	2,560	160
		Duck/Hokkaido/31/2010 (H6N2)	640	320	2,560	320	2,560	2,560
		Duck/Hokkaido/144/2012 (H6N1)	640	160	1,280	160	1,280	640
		Chicken/Tainan/V156/1999 (H6N1)	320	320	640	320	1,280	320
	W312	Teal/Hong Kong/W312/1997 (H6N1)	320	40	320	<u>1,280</u>	640	160
	Early	Duck/Hong Kong/960/1980 (H6N2)	640	1,280	2,560	320	<u>2,560</u>	2,560
		Duck/Hong Kong/882/1980 (H6N2)	1280	1280	2,560	320	2,560	1,280
		Duck/Hong Kong/834/1980 (H6N1)	1280	160	2,560	320	2,560	1,280
		Duck/Hong Kong/943/1980 (H6N5)	640	320	1,280	80	1,280	320
North America		Turkey/Massachusetts/3740/1965 (H6N2)	2,560	1,280	1,280	640	2,560	<u>2,560</u>

^a "—" indicates not belong to any sublineage.^b Homologous titers were underlined.

Abbreviations: S.Aus/72, Shearwater/S. Australia/1/1972 (H6N5); VN4429/10, Duck/Vietnam/OIE-4429/2010 (H6N2); Hok/04, Duck/Hokkaido/262/2004 (H6N1); W312, Teal/Hong Kong/W312/1997 (H6N1); HK/960, Duck/Hong Kong/960/1980 (H6N2); Mass/65, Turkey/Massachusetts/3740/1965 (H6N2).

Table 2 NT antibody titers of mice injected once with the vaccine and virus titers in the lungs after challenge

Vaccine	Dose of vaccine	NT titer to		Virus titer ^a (log PFU/g)
		Dk/Hong Kong/960	Dk/Vietnam/2012	
PBS	—	<40, <40, <40, <40, <40	<40, <40, <40, <40, <40	5.8, 5.4, 5.0, 5.4, 5.3
Dk/Hong Kong/960	50 µg	80, 80, 40, 40, 40	40, 40, 40, <40, <40	4.3, 4.4, 5.4, 4.2, 4.6 *
	10 µg	40, 40, 40, 80, 40	40, 40, 40, 40, <40	5.0, 5.0, 4.7, 5.3, 4.9 *
	2 µg	<40, <40, 40, 40, 40	<40, <40, <40, 40, 40	4.8, 5.3, 5.2, 5.5, 5.4
Dk/Vietnam/2012	10 µg	ND ^b	320, 320, 320, 320, 160	<1.0, <1.0, <1.0, 1.4, <1.0 *
	2 µg	ND	40, 160, 80, 40, 80	3.2, 2.9, 1.5, 3.8, 2.2 *

Each of vaccine was injected intraperitoneally into 10 mice. Serum samples were collected 3 weeks after the vaccination. Mice were challenged with 10^{6.0} PFU of Dk/Vietnam/2012 intranasally.

^a The lung samples were collected at 3 dpc and virus titers were measured.

^b "ND" indicates not determined.

*, $P < 0.05$ versus the group of mice injected with PBS

Table 3 NT antibody titers of mice injected twice with the vaccine and virus titers in the lungs after challenge

Vaccine	Dose of vaccine	NT titer to		Virus titer ^a (log PFU/g)
		Dk/Hong Kong/960	Dk/Vietnam/2012	
PBS	—	<40, <40, <40, <40	<40, <40, <40, <40	5.2, 5.5, 5.5, 5.2, 5.2
Dk/Hong Kong/960	50 µg	80, 80, 160, 80, 160	80, 160, 80, 80, 80	4.0, 4.7, 4.4, 3.5, 4.4 *
	10 µg	80, 40, 80, 80, 40	80, 40, 40, 40, 40	4.0, 5.0, 5.1, 4.6, 5.2 *
	2 µg	80, 80, 40, 80, 80	40, 40, 40, 40, 40	4.5, 4.6, 5.6, 4.4, 4.6 *

Each of vaccine was injected intraperitoneally with a 2-week interval. Serum samples were collected 2 weeks after the final vaccination. Mice were challenged with 10^{6.0} PFU of Dk/Vietnam/2012 intranasally.

^a The lung samples were collected at 3 dpc and virus titers were measured.

*, $P < 0.05$ versus the group of mice injected with PBS