HPAIV strains have persisted throughout the world for more than 15 years, and antigenic variants have been selected because some countries use vaccines for the control of HPAIV infection. In the chickens vaccinated against HPAIV, it is hard to find infected birds because they do not show clinical signs, despite shedding of the virus. As a result, HPAIV has returned to migratory water birds from domestic poultry, and many feral water birds have died on the way back to their northern territory in Siberia in spring. Some migratory water birds infected with the virus must have returned to their nesting lakes in Siberia and then disseminated the virus to other birds though water-borne transmission at their nesting lakes. To prevent the perpetuation of HPAIVs among migratory water birds at their nesting lakes in Siberia, HPAIVs should be contained within poultry in Asia. Thus, we strongly recommend that a stamping-out strategy is the only way to achieve prompt eradication of H5N1 HPAIV and that vaccination may be an optional tool for the control of HPAI in addition to the stamping-out policy. Otherwise, disasters will occur every year throughout Asian countries.

METHODS

Viruses. The H5N1 viruses isolated in the present study and the reference H5 viruses shown in Table 2 were propagated in 10-day-old embryonated chicken eggs. As reference strains, H5 NPAIVs isolated from the faecal material of migratory ducks (Yamamoto *et al.*, 2011) and the H5N1 HPAIVs shown in Table 2 (Kajihara *et al.*, 2011; Mase *et al.*, 2005; Muramoto *et al.*, 2006; Okamatsu *et al.*, 2010; Sakoda *et al.*, 2010; Suarez *et al.*, 1998) were used for antigenic analyses.

Isolation and identification of viruses. Virus isolation was carried out from faecal samples, tracheal and cloacal swabs, or homogenates of the tissues of wild birds and chickens throughout the year. Faecal samples were mixed with transport medium containing minimum essential medium (Nissui), 10 000 U penicillin G (Meiji Seika) ml^{-1}, 10 mg streptomycin (Meiji Seika) ml^{-1}, 0.3 mg gentamicin (Merck) ml^{-1}, 250 U nystatin (Sigma) ml^{-1} and 0.5 % BSA fraction V (Roche) to yield a 10–20 % suspension. Tracheal and cloacal swabs were mixed with 2 ml transport medium. Organ tissue was homogenized with transport medium to yield a 10 % suspension. Samples from wild birds and chickens were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs, and the subtypes of the HA and NA of influenza virus isolates were identified by haemagglutination inhibition (HI) and neuraminidase inhibition tests, respectively, according to a standard protocol (OIE, 2011).

H5N1 HPAIVs were isolated from 17 species of dead or diseased wild birds found at the waterside of their resting areas and in the gardens of private houses from November 2010 to March 2011 (Table 1): whooper swan (*Cygnus cygnus*), greater scaup (*Aythya marila*), pintail (*Anas acuta*), peregrine falcon (*Falco peregrinus*), tufted duck (*Aythya fuligula*), mute swan (*Cygnus olor*), common pochard (*Aythya ferina*), little grebe (*Tachybaptus ruficollis*), great crested grebe (*Podiceps cristatus*), tundra swan (*Cygnus columbianus*), black-headed gull (*Larus ridibundus*), black swan (*Cygnus atratus*), ural owl (*Strix uralensis*), mandarin duck (*Aix galericulata*), grey heron (*Ardea cinerea*), hooded crane (*Grus monacha*) and goshawk (*Accipiter gentilis*).

Experimental infection of chickens with H5N1 isolates. To assess the pathogenicity of the representative H5N1 virus isolates, A/duck/Fukushima/2/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Tochigi/15/2011 (H5N1) and A/peregrine falcon/Aomori/7/2011 (H5N1) were inoculated intravenously into 4–6-week-old chickens (*Gallus gallus*) for an IVPI test according to a standard protocol (OIE, 2011). Each bird was housed in a self-contained isolator unit (Tokiwa Kagaku) at a Biosafety Level 3 facility at Hokkaido University, Japan.

Sequencing and phylogenetic analysis. For the genetic analysis, 30 isolates were selected from 63 isolates of wild birds, and three isolates were also selected from 24 isolates of chickens. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs using TRIzol LS reagent (Invitrogen) and reverse transcribed with the Uni12 primer (Hoffmann *et al.*, 2001) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The full-length or partial sequence of each gene segment was amplified by PCR with gene-specific primer sets reported previously (Hoffmann *et al.*, 2001) or designed exclusively in the present study. The sequences of primers designed in the present study were: PB2-826F: 5′-GTTAGGAGAGCAACAGTATCAG-3′, PB2-2135R: 5′-TCATTGATGCTCAATGCCGG-3′, PB1-547F: 5′-ACACATTTCCAGAGAAAGAG-3′, PB1-2128R: 5′-TCCACCATGCTAGAAATCCC-3′, PA-38F: 5′-GTGCGACAATGCTTCAATCC-3′, PA-1372R: 5′-CCTGCAATGGGATACTTCCGC-3′, NP-57F: 5′-TGGAAACTGGTGGAGAACGC-3′, NP-1456R: 5′-TTGTCTCCGAAGAAATAAGA-3′, M-19F: 5′-GTCGAAACGTACGTTCTCTC-3′, M-853R: 5′-GAATCCACAATATCAAGTGCAAG-3′ and NS-848R: 5′-TCATTAAATAAGCTGGAACG-3′. Direct sequencing of each gene segment was performed using a 3130 or 3500 Genetic Analyzer (Applied Biosystems). To assess the genetic relationship among influenza virus isolates, nt 34–1019 (986 bp) of HA, nt 197–1206 (1010 bp) of NA, nt 1017–1929 (913 bp) of PB2, nt 1064–1657 (594 bp) of PB1, nt 269–1218 (950 bp) of PA, nt 760–1329 (570 bp) of NP, nt 97–771 (675 bp) of M and nt 73–750 (678 bp) of NS of isolates in the present study were compared with those of other recent H5N1 isolates in Asia. For the NA and internal genes, reference strains of each genotype according to a previous report (Duan *et al.*, 2008) were included. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using MEGA 5 software (http://www.megasoftware.net/).

Antigenic analysis. The antigenic properties of the representative H5 viruses [A/duck/Hokkaido/WZ83/2010 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1) and A/peregrine falcon/Aomori/7/2011 (H5N1)] were compared with those of the reference H5 viruses by a fluorescent antibody method using mAbs against H5 HA (Soda *et al.*, 2008). Madin–Darby canine kidney cells infected with H5 influenza viruses were fixed with cold 100 % acetone at 8 h post-inoculation. The reactivity patterns of the H5 viruses with mAbs were investigated with an FITC-conjugated goat anti-mouse IgG (MP Biomedicals) using a fluorescence microscope (Axiovert 200; Carl Zeiss).

The antigenic properties of the representative H5 viruses were also assessed using hyperimmunized chicken antisera against A/mallard/Hokkaido/24/2009 (H5N1) and A/whooper swan/Hokkaido/1/2008 (H5N1) by an HI test according to a standard protocol (OIE, 2011). HI titres were expressed as the reciprocals of the highest serum dilutions that showed complete HI.

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1 *Virology*

2 *Full paper*

3 **An H9N2 influenza virus vaccine prepared from a non-pathogenic isolate from a migratory**

4 **duck confers protective immunity in mice against challenge with an H9N2 virus isolated from**

5 **a girl in Hong Kong**

6

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19 *Running head:* A VACCINE AGAINST H9N2 INFLUENZA VIRUS

20

21 **ABSTRACT.** H9N2 influenza viruses circulate in wild birds and poultry in Eurasian countries,
22 and have been isolated from pigs and humans in China. H9N2 viruses isolated from birds, pigs and
23 humans have been classified into three sublineages based on antigenic and genetic features.
24 Chicken antisera to H9N2 viruses of the Korean sublineage reacted with viruses of different
25 sublineages by the hemagglutination-inhibition test. A test vaccine prepared from a non-pathogenic
26 A/duck/Hokkaido/49/1998 (H9N2) strain of the Korean sublineage, obtained from our influenza
27 virus library, induced immunity in mice to reduce the impact of disease caused by the challenge with
28 A/Hong Kong/1073/1999 (H9N2), which is of a different sublineage. The present results indicate
29 that an inactivated whole virus vaccine prepared from a non-pathogenic influenza virus from the
30 library could be used as an emergency vaccine during the early stage of a pandemic caused by H9N2
31 infection.

32

33 KEY WORDS: H9N2 influenza virus, antigenicity, vaccine

34

35 **INTRODUCTION**

36 Avian influenza viruses of various subtypes are circulating in poultry worldwide [1, 18-19, 21,
37 29, 38]. In particular, H9N2 influenza virus is prevailing poultry populations in Eurasian countries
38 [9-11, 24]. Since H9N2 viruses were isolated from quails in Hong Kong in 1988, they have
39 become prevalent in live bird markets and poultry farms in Asia [8, 32]. The wide spread of H9N2
40 virus have been greatly concerned not only in the poultry industry but also for public health [8, 38].
41 The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into
42 G1, Y280, and Korean sublineages [10]. H9N2 viruses do not substantially cause severe disease in
43 poultry, but co-infection with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*,
44 or attenuated coronavirus vaccine exacerbates the disease [13, 22]. H9N2 viruses were also
45 isolated from domestic pigs in China [38] and Korea, and from humans with febrile respiratory
46 illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4-5, 23, 31]. It has therefore been
47 postulated that H9N2 virus has the potential to cause pandemic influenza in humans.

48 In the present study, as the preparedness for pandemic influenza, H9 virus strains from the
49 influenza virus library in our laboratory [19] were analyzed antigenically and phylogenetically to
50 select a strain suitable for a vaccine. A/duck/Hokkaido/49/1998 (H9N2) was selected and an
51 inactivated whole virus vaccine was prepared. The efficacy of the vaccine against challenge with
52 A/Hong Kong/1073/1999 (H9N2) was assessed in mice.

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56 **MATERIALS AND METHODS**

57 *Viruses:* A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2),
58 A/quail/Hong Kong/G1/1997 (H9N2), A/chicken/Hong Kong/FY20/1999 (H9N2), A/silkie

59 chicken/Hong Kong/SF43/1999 (H9N2), and A/quail/Hong Kong/A17/1999 (H9N2) were provided
60 by Dr. K. F. Shortridge (The University of Hong Kong, China). A/ostrich/South
61 Africa/9508103/1995 (H9N2) and A/chicken/Pakistan/2/1999 (H9N2) were provided by Dr. I. H.
62 Brown (Animal Health and Veterinary Laboratories Agency, Weybridge, U. K.). A/Hong
63 Kong/1073/1999 (H9N2) (HK/1073/99), which was isolated from a 4-year-old girl in Hong Kong in
64 1999 [23], was provided by Dr. A. J. Hay (MRC National Institute for Medical Research, U. K.).
65 H9N2 influenza virus strains isolated from birds and mammals, and A/duck/Hokkaido/49/1998
66 (H9N2) (Dk/Hok/49/98) [25] are listed in Table 1. The viruses were grown in 10-day-old
67 embryonated chicken eggs and infectious allantoic fluids were stored at -80°C until use.

68 *Phylogenetic analysis:* Viral RNAs were extracted from the allantoic fluids of chicken embryos
69 infected with viruses using TRIzol LS Reagent (Invitrogen, CA, U.S.A.) and reverse-transcribed
70 using the Uni12 primer [14] and M-MLV reverse transcriptase (Invitrogen). The cDNA was
71 amplified by using the Takara Ex Taq (Takara Bio, Inc., Shiga, Japan). The first cycle of the
72 amplification program consisted of a 5 min period at 94 °C and was followed by 30 cycles with the
73 following conditions; 98 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 1 min. The last cycle was
74 done at 72 °C for 10 min. Polymerase chain reaction amplification of the viral genes was
75 performed using a PTC-200 thermal cycler (BIO-RAD, CA, U.S.A.). The primers used for HA
76 gene amplification were H9-101F (5'-GGCCACCAGTCAACAAACTC-3') [24] and H9-1341R
77 (5'-GTTTACATTCGCATCATGCTC-3'). Direct sequencing of the HA gene was performed using
78 a CEQ 2000XL autosequencer (Beckman Coulter, CA, U.S.A.). For phylogenetic analysis,
79 sequence data obtained for the genes together with those from public databases were analyzed using
80 the neighbor-joining method [34] using MEGA 5.0 software (http://www.megasoftware.net/).

81 *Antigenic analysis:* Antigenic characterization of H9N2 influenza viruses was done by
82 hemagglutination-inhibition (HI) test [35]. Hyperimmunized chicken antisera against seven H9N2

83 viruses were prepared [20]. Briefly, the sera were serially two-fold diluted with phosphate buffered
84 saline (PBS) in 96-well microplates. The diluted sera were mixed with 8 hemagglutinin units of
85 virus antigen and incubated at room temperature for 30 min. Chicken red blood cells (0.5%) were
86 added to the antigen-serum dilution mixtures and incubated at room temperature for 30 min. HI
87 titers were expressed as reciprocals of the highest serum dilutions that showed complete HI.

88 *Virus replication and pathogenicity in embryonated chicken eggs:* Viruses were inoculated into
89 10-day-old embryonated chicken eggs and incubated for 48 h at 35°C. HA titers and 50% egg
90 infectious dose (EID_{50}) were measured every 12 h post-inoculation. Pathogenicity of
91 Dk/Hok/49/98 against embryonated chicken eggs was evaluated by the mean death time as described
92 Abenes *et al.* [2].

93 *Vaccine preparation:* Dk/Hok/49/98 and HK/1073/99 were injected into the allantoic cavities of
94 10-day-old embryonated chicken eggs and propagated at 35°C for 48 h. The viruses in the allantoic
95 fluids (512 HA for Dk/Hok/49/98 and 1,024 HA for HK/1073/99) were purified by differential
96 centrifugation and sedimentation through a sucrose gradient [14]. The protein concentration was
97 measured using the BCA Protein Assay Reagent (Thermo Fisher Scientific K. K., MA, U.S.A.).
98 The purified virus was inactivated with 0.1% formalin at 4°C for 7 days. The HA content was
99 standardized as described [28]. Proteins of purified viruses were separated by sodium dodecyl
100 sulfate-polyacrylamide gel electrophoresis on a 15% gel (BIO-RAD) and stained with Coomassie
101 brilliant blue. The gel image was captured and analyzed by LumiVisionPRO (AISIN, Aichi, Japan),
102 and the ratio of HA protein to total protein was calculated. On the basis of this method,
103 concentration of HA protein was 14.7 μg in 50 μg of vaccine.

104 *Challenge with HK/1073/99 into mice vaccinated once or twice:* Inactivated Dk/Hok/49/98 or
105 HK/1073/99 vaccines were injected once intraperitoneally into 4-week-old female BALB/c mice
106 (Japan SLC, Inc., Shizuoka, Japan). PBS was injected into control mice. Three weeks later, 10

107 mice in each group were challenged intranasally with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under
108 anesthesia. Mixture of tiletamine hydrochloride (20 mg/kg) (United States Pharmacopeia,
109 Maryland, U.S.A.), zolazepam hydrochloride (20 mg/kg) (United States Pharmacopeia), and
110 xylazine (20 mg/kg) (Bayer HelthCare, Osaka, Japan) was injected intraperitoneally into mice for
111 anesthesia.

112 Inactivated Dk/Hok/49/98 vaccines were also injected twice intraperitoneally into 4-week-old
113 female BALB/c mice. Two weeks later, the vaccine was again intraperitoneally injected into the
114 mice. One week after the second vaccination, 10 mice in each group were challenged intranasally
115 with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under anesthesia.

116 On day 3 post-infection, five mice in each group were sacrificed and the lungs were
117 homogenized to make a 10% (w/v) suspension with minimal essential medium (Nissui, Tokyo,
118 Japan) with antibiotics (penicillin G potassium, streptomycin sulfate, gentamicin sulfate, and
119 nystatin) and 0.5% Bovine Serum Albumin Fraction V (Roche, Basel, Switzerland). The virus
120 titers of the supernatants of the lung tissue homogenates were calculated in 10-day-old embryonated
121 chicken eggs and expressed as the EID_{50}/g of tissue.

122 In neutralization (NT) tests, titers were determined as the reciprocal of that maximum antibody
123 dilution that completely prevented cytopathic effect caused by 100 plaque forming units of virus
124 using MDCK cells.

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128 **RESULTS**

129 *Phylogenetic analysis of the HA genes of H9N2 influenza viruses:* The HA genes of 22 H9N2
130 viruses were sequenced and phylogenetically analyzed by the neighbor-joining method. All of the

131 HA genes were classified into the Eurasian lineage, and further classified into the Korean (n=11),
132 Y280 (n=7), and G1 (n=4) sublineages (Fig. 1). The H9 viruses of the Korean and Y280
133 sublineages were isolated from water birds, poultry, pigs, and humans in east Asian countries, and
134 those of the G1 sublineage were isolated from poultry in west Asian countries (Fig. 1).

135 *Antigenicity of the H9N2 influenza viruses:* H9N2 influenza viruses were antigenically analyzed
136 by HI test (Table 2). Antisera against H9N2 viruses of the Y280 sublineage reacted slightly with
137 H9N2 viruses of the G1 and Korean sublineages. Antisera against H9N2 viruses of the G1
138 sublineage reacted more with H9N2 viruses of the Y280 sublineages than those of the Korean
139 sublineage. On the other hand, antisera against H9N2 viruses of the Korean sublineage reacted
140 with H9N2 viruses of all sublineages. This result suggested that the H9N2 vaccine strain should be
141 selected from the viruses of the Korean sublineage.

142 *Selection of H9N2 vaccine strain:* To select an H9N2 vaccine strain, four H9N2 viruses,
143 Dk/Hok/49/98, A/duck/Hokkaido/13/2000 (H9N2) (Dk/Hok/13/00), A/duck/Hokkaido/9/1999
144 (H9N2) (Dk/Hok/9/99), and A/duck/Hokkaido/26/1999 (H9N2) (Dk/Hok/26/99), were selected from
145 11 isolates of the Korean sublineage, and their replication and pathogenicity in embryonated chicken
146 eggs were assessed. HA titers of Dk/Hok/49/98, Dk/Hok/13/00, Dk/Hok/9/99, and Dk/Hok/26/99
147 were 512, 512, 256, and 128, respectively. Virus titers were $10^{9.7}$, $10^{8.3}$, $10^{8.3}$, and $10^{7.3}$ EID_{50}/ml,
148 respectively, indicating that Dk/Hok/49/98 replicated efficiently in 10-day-old embryonated chicken
149 eggs. Pathogenicity of Dk/Hok/49/98 in the embryonated chicken eggs was determined by mean
150 death time and that of Dk/Hok/49/98 was 91.8 h, indicating that Dk/Hok/49/98 had low
151 pathogenicity in chicken embryos. This virus was selected as a candidate H9N2 vaccine strain.

152 *Protective efficacy of the test vaccine in mice against H9N2 virus challenge:* To assess the
153 efficacy of the vaccine against H9N2 virus infection, HK/1073/99 was intranasally inoculated into
154 mice that had previously been vaccinated once with inactivated HK/1073/99 or Dk/Hok/49/98.

155 Immunogenicity of the inactivated vaccine was assessed by NT test, and virus titers in the lungs
156 were measured to assess protective immunity induced by the vaccine (Table 3). Serum antibodies
157 were detected in mice injected with 50, 10, and 2 μg protein of HK/1073/99 vaccine. The virus
158 titers in the lungs were $<10^{1.5}$-$10^{3.7}$ EID_{50}/g in mice injected with 50 or 10 μg protein of HK/1073/99
159 vaccine, and $10^{4.7}$-$10^{6.8}$ EID_{50}/g in the 2 and 0.4 μg vaccine groups, and in the PBS control group
160 (Table 3). A reduction in body weight was observed in mice injected with 10, 2, and 0.4 μg protein,
161 and in the control group from day 2 post-infection, reaching up to 10% body weight loss at days 3-4
162 post-infection, compared with in the mice that received 50 μg of protein (Fig. 2A).
163 We also tested the efficacy of vaccination with Dk/Hok/49/98 on protection against subsequent
164 intranasal infection with HK/1073/99. Serum antibodies were slightly detected in mice injected
165 with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein (Table 3). The virus titers in the lungs
166 of mice injected with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein were $10^{4.3}$-$10^{5.3}$
167 EID_{50}/g. In the mice injected with 2 and 0.4 μg protein, the virus titers in the lungs of mice were
168 similar to those of non-vaccinated control mice (Table 3). Although, a reduction in body weight
169 was observed in mice at all doses of the Dk/Hok/49/98 vaccine, slight significant difference was
170 observed in mice injecteded with 50 μg protein, compared with in mice injected with PBS (Fig. 2B).
171 In the mice injected twice with Dk/Hok/49/98 vaccine on days 0 and 14, serum antibodies
172 were detected in mice in the 50, 10, and 2 μg groups at one week after the second injection (Table 4).
173 The virus titers in the lungs were $<10^{1.5}$-$10^{3.8}$ EID_{50}/g in mice injected with 50, 10, and 2 μg protein,
174 and $10^{5.3}$-$10^{6.5}$ EID_{50}/g in the other vaccinated mice (Table 4). A reduction in body weight was
175 observed in mice injected with 2 and 0.4 μg protein, and in control group, reaching up to 10%
176 weight loss from days 4-6 post-infection, compared with in mice injected with 50 and 10 μg protein
177 (Fig. 3). These results suggest that the repeat administration of the test vaccine confers immunity,
178 and prevents body weight loss and decreases virus replication, after infection of mice with H9

179 influenza virus.

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183 **DISCUSSION**

184 H9N2 viruses of each of the three sublineages, G1, Y280, and Korean, were recently isolated
185 from wildbirds and poultry worldwide [3, 8, 27]. H9N2 viruses were also isolated from pigs and
186 humans in China [4-5, 37] and Korea, suggesting that these viruses have the potential to cause
187 pandemic influenza in humans. H9N2 viruses isolated from pigs in China and Korea were
188 classified into the Y280 and Korean sublineages, while H9N2 viruses isolated from humans in China
189 was classified into the G1 and Y280 sublineages [4-5, 23, 31]. It is suggested that H9N2 viruses
190 isolated from pigs and humans are antigenically distinct among viruses of the Korean, Y280, and G1
191 sublineages [5, 23, 31, 37]. Therefore, it is important that any H9N2 influenza virus vaccine to be
192 used for pandemic influenza can broadly cross-react with antisera against all sublineage viruses. In
193 the present study, Dk/Hok/49/98 was selected from the Korean sublineage, since antisera to the virus
194 cross-reacted with all sublineages virus. Furthermore, Dk/Hok/49/98 replicated efficiently in
195 embryonated chicken eggs and was non-pathogenic in chicken embryos. Recently, H9N2 viruses
196 were isolated from pigs and humans in China [4-5, 23, 31], it is necessary to analyze the antigenicity
197 of these H9 isolates and evaluate the efficacy of test vaccine against them. Taken together, it is
198 important to carry out surveillance of avian influenza consecutively and to analyze the isolates
199 antigenically and phylogenetically.

200 In the present study, it was suggested that the test whole particle vaccine has the potency
201 against challenge with H9N2 virus of different sublineage in mice. It was already reported that
202 whole particle vaccine induced strong immune responses and H5N1 whole particle vaccine induced

203 protective immunity against antigenically distinct challenge virus [12, 26]. Although the efficacy
204 of the test vaccine observed slightly in mice vaccinated once due to the antigenic difference between
205 Dk/Hok/49/98 and HK/1073/99, it was clear that the vaccine induced protective immunity in mice
206 injected twice, indicating the usefulness for the preparedness of the pandemic.

207 The current cycle of seasonal influenza vaccine production requires detailed planning up to 6
208 months before vaccine manufacture [7]. In the case of influenza pandemic in 2009, it also took 5
209 months to have an H1N1 vaccine available [6, 36]. To prepare for the emergence of pandemic
210 influenza in birds and mammals including humans, we have carried out global surveillance of avian
211 influenza [15, 30, 33, 39]. Avian influenza viruses of 144 combinations of HA and NA subtypes
212 have been stocked for use in vaccine and diagnosis. Since the viruses stocked in our influenza
213 virus library were already assessed the pathogenicity and replication in embryonated chicken eggs,
214 we can exclude those tests to select a vaccine strain and prepare a vaccine rapidly [16-17, 19]. The
215 present results indicate that the inactivated whole virus vaccine prepared from an influenza virus
216 from the library could be used as an emergency vaccine during the early stage of a pandemic caused
217 by H9N2 influenza virus infection.

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361 **FIGURE LEGENDS**
362 Fig. 1. Phylogenetic tree of the HA genes of H9N2 influenza viruses. Nucleotides 163-1,048
363 (886 bases) of the HA genes were used for the analysis. Horizontal distances are proportional to
364 the minimum number of nucleotide differences required to join nodes and sequences. Numbers at
365 the nodes indicate confidence levels in a bootstrap analysis with 1,000 replicates. Viruses stocked
366 in our laboratory are highlighted in gray. Representative viruses in each sublineage are underlined.
367 Abbreviations: Sw, swine; Ck, chicken; SCk, silky chicken; Dk, duck; Osr, ostrich; Qa, quail; Ty,
368 turkey; HK, Hong Kong; Hb, Hebei; S.Af, South Africa; Hok, Hokkaido; Pak, Pakistan; and Wis,
369 Wisconsin.
370