Potency of an inactivated influenza vaccine prepared from A/duck/Mongolia/119/2008 (H7N9) against the challenge with A/Anhui/1/2013 (H7N9)

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Abstract

H7N9 influenza virus infection in humans was reported in China on March 31, 2013. Humans are immunologically naïve to the H7N9 subtype, for which the seasonal influenza vaccine is not effective. Thus, the development of an H7N9 influenza virus vaccine is an urgent issue. To prepare for the emergence of an influenza pandemic, we have established a library comprising more than 1,300 influenza virus strains with 144 different combinations of 16 HA and 9 NA subtypes. An H7N9 virus strain isolated from a 35-year-old woman, A/Anhui/1/2013 (H7N9), was found to be antigenically similar to H7N9 influenza viruses isolated from migratory ducks. In the present study, the potency of an inactivated whole virus particle vaccine prepared from an H7N9 low pathogenic avian influenza virus, A/duck/Mongolia/119/2008 (H7N9), selected from the library, was assessed by a challenge with A/Anhui/1/2013 (H7N9). The results indicate that the test vaccine was potent enough to induce sufficient immunity to reduce the impact of disease caused by the challenge with A/Anhui/1/2013 (H7N9) in mice. The present results indicate that an inactivated whole virus particle vaccine prepared from an influenza virus strain stored in the library could be useful as a vaccine strain in case of an influenza pandemic.

1. Introduction

In March 2013, the first identified cases of human infection with an H7N9 influenza A virus occurred in China. As of February 20, 2014, 355 cases of human infection and 112 deaths have been reported, and the number of cases continues to increase in China [1]. The hemagglutinin (HA) and the neuraminidase (NA) genes of H7N9 viruses isolated from humans are derived from avian H7N3 and H7N9 viruses, respectively, and the internal genes are derived from H9N2 viruses circulating in poultry in China [2,3]. Characterization of the H7N9 influenza virus indicated that it has the potential to infect humans [4,5]. Currently, no vaccine is available for the prevention of H7N9 influenza virus infection. The urgent need for the development of such a vaccine has been acknowledged by the World Health Organization [6].

Each of the known subtypes of influenza A virus perpetuates among migratory ducks and their nesting lake water in nature. We have conducted global surveillance studies of influenza in birds and mammals since 1977 and have established a library of virus strains comprising 144 combinations of 16 HA and 9 NA subtypes for vaccine and diagnostic use [7-9]. The biological characters of these strains have been analyzed and the data are available at http://virusdb.czc.hokudai.ac.jp/. Several inactivated whole virion influenza vaccines were prepared from the virus strains in the library and were found to be effective against a challenge with influenza viruses A(H1N1)pdm09, H5N1,

H7N7, and H9N2 using cynomolgous macaques and mouse models [10-13]. In addition, whole virion influenza vaccines are shown to be more effective than ether-split vaccines [13,14].

In the present study, H7N9 influenza virus strains from the library in our laboratory were analyzed antigenically and genetically to select a suitable strain for the vaccine. A selected influenza virus strain that was an isolate from a fecal sample of a migratory duck in Mongolia in 2008, A/duck/Mongolia/119/2008 (H7N9) (Dk/Mon/119/08), was used to prepare an inactivated whole virus particle vaccine. The potency of this test vaccine was evaluated by a challenge with A/Anhui/1/2013 (H7N9) (Anhui/1/13).

2. Materials and methods

2.1. Viruses and cells

Anhui/1/13 was provided by Dr. M. Tashiro (National Institute of Infectious Diseases, Japan). A/turkey/Italy/4580/1999 (H7N1) was provided by Dr. I. Capua (Istituto Zooprofilattico Sperimentale delle Venezie, Italy). A/duck/Hong Kong/301/1978 (H7N2) was provided by Dr. K. F. Shortridge (University of Hong Kong, Hong Kong SAR), and A/seal/Massachusetts/1/80 (H7N7) (Seal/Mass/1/80) was provided by Dr. R. G. Webster (St. Jude Children's Research Hospital, USA). Dk/Mon/119/08 was isolated from fecal samples of migratory ducks [15]. Dk/Mon/119/08 was applied to plaque purification

79 twice in Madin–Darby canine kidney (MDCK) cells to improve the propagation efficiency.
80 All viruses used in the present study were propagated in 10-day-old embryonated
81 chicken eggs at 35°C for 48 h, and infectious allantoic fluids were stored at −80°C until
82 use.
83 MDCK cells were maintained in minimum essential medium (Nissui, Tokyo, Japan)
84 supplemented with 10% calf serum and used for titration of viral infectivity.
85
86 *2.2. Sequencing and phylogenetic analysis*
87 Viral RNAs were extracted from the allantoic fluids of chicken embryos infected with
88 viruses using TRIzol LS Reagent (Life Technologies, Foster City, CA, USA) and
89 reverse-transcribed using the Uni12 primer [16] and M-MLV reverse transcriptase (Life
90 Technologies). The cDNA was amplified by polymerase chain reaction (PCR) with
91 TaKaRa Ex Taq (Takara Bio, Inc., Shiga, Japan). Primers used in this study are as
92 follows; BmHA-1F, 5′-primer TATTCGTCTCAGGGAGCAAAAGCAGGGG-3′; NS-890R,
93 5′-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3′; H7-368F, 5′-primer
94 CAGGCGGAATTGACAAGGAG-3′; and H7-1141R, 5′-primer
95 TGCAGCAGTTCCCTCTCCTTGTGC-3′ [16]. PCR products were reacted with
96 gene-specific primers and using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life
97 Technologies). Sequences of the DNA templates were determined using the

98 3500/3500xL genetic analyzer (Life Technologies). Sequencing data was analyzed using
99 GENETYX version 11 (Genetyx Corporation, Tokyo, Japan). For phylogenetic analysis,
100 sequence data obtained for the genes together with those from GenBank/EMBL/DDBJ
101 and GISAID database (Table 1) were analyzed using the neighbor-joining method [17]
102 with MEGA 5.0 software (http://www.megasoftware.net/).
103
104 *2.3. Antigenic analysis*
105 The antigenic properties of Dk/Mon/119/08 and Anhui/1/13 were assessed using
106 hyper-immunized chicken antisera against five H7 viruses by hemagglutinin inhibition
107 (HI) test according to a standard protocol [18]. HI titers were expressed as the
108 reciprocals of the highest serum dilutions that showed complete HI.
109 Anhui/1/13, Dk/Mon/119/08, and other H7 influenza viruses were antigenically
110 compared by the fluorescent antibody (FA) method using monoclonal antibodies (MAbs)
111 to the H7 HA according to the method of Sakabe *et al.* [19]. In brief, MDCK cells
112 infected with H7 influenza viruses were fixed with cold 100% acetone at 8 h after
113 inoculation. The reactivity patterns of the H7 viruses with MAbs were investigated
114 with an FITC-conjugated goat anti-mouse IgG (MP Biomedicals, Santa Ana, CA, USA)
115 using a fluorescence microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany).
116

117 *2.4. Vaccine preparation*

118 Dk/Mon/119/08 and Anhui/1/13 were inoculated into the allantoic cavities of
119 10-day-old embryonated chicken eggs and propagated at 35°C for 48 hours. The viruses
120 in the allantoic fluids were purified by differential centrifugation and sedimentation
121 through a sucrose gradient [20]. The protein concentration was measured using the
122 BCA Protein Assay Reagent (Thermo Fisher Scientific K. K., Waltham, MA, USA). The
123 purified virus was inactivated with 0.1% formalin at 4°C for 7 days. The HA content
124 was standardized according to the method of Ninomiya *et al.* [21]. On the basis of this
125 method, dose of HA concentration was estimated 14.7 μg in 50 μg of vaccine.

126

127 *2.5. Potency test of vaccine against Anhui/1/13 in mice*

128 Dk/Mon/119/08 and Anhui/1/13 vaccines with 2, 10, and 50 μg protein were injected
129 subcutaneously into groups of 10 4-week-old female BALB/c mice (Japan SLC, Inc.,
130 Shizuoka, Japan), respectively. PBS was injected into control mice. Three weeks later,
131 serum samples were collected and 30 μl of $10^{4.0}$ PFU of Anhui/1/13 was intranasally
132 inoculated into the mice under anesthesia. Three days after the challenge, five mice
133 from each group were sacrificed and the lungs were collected. Virus titers in the lung
134 homogenates were quantified by plaque assay in MDCK cells. Five other mice from
135 each group were observed for 14 days for clinical signs and weight loss. These vaccines

were also injected into mice twice with a 2-week interval. Two weeks after the final injection, the serum samples were collected and Anhui/1/13 was inoculated into mice. Data were statistically analysed by using T-test.

Animal experiments were authorized by the Committee of Institutional Animal Care and Use at the Graduate School of Veterinary Medicine, Hokkaido University (approved numbers: 13-0104); all experiments were performed according to the guidelines of this committee.

3. Results

3.1. Antigenic analysis of H7 influenza viruses

To prepare H7N9 influenza virus vaccine, four H7N9 influenza virus strains isolated from fecal samples of ducks, Dk/Mon/119/08, A/duck/Mongolia/147/2008, A/duck/Mongolia/128/2008, and A/duck/Mongolia/129/2010 were selected from the library. Dk/Mon/119/08 was selected as a vaccine strain, showing the highest growth potential in embryonated chicken eggs (data not shown). Dk/Mon/119/08 and Anhui/1/13 were antigenically analyzed by the neutralization tests (Table 2). The infectivity of Anhui/1/13 was neutralized by all the antisera to H7 avian influenza viruses, particularly with the antiserum to Dk/Mon/119/08 at a titer of 1:640 as the homologous titer was 1:1280.

To clarify more precise antigenic relationship between Anhui/1/13 and Dk/Mon/119/08, we compared reactivity patterns of H7 viruses with a panel of monoclonal antibodies recognizing 5 different epitopes on the H7 HA by FA test (Table 3). The results revealed that HA of Dk/Mon/119/08 is antigenically related closely to that of Anhui/1/13.

3.2. Phylogenetic analysis of the H7 HA of avian, equine, and human influenza viruses

Nucleotide sequences of the HA genes of the 39 H7 viruses, including the H7N9 viruses isolated from humans (Table 1), were phylogenetically analyzed using the neighbor-joining method (Fig. 1). The H7N9 viruses isolated from humans were closely related to H7 low pathogenic avian influenza viruses. Based on the results of phylogenetic analysis, the identity of amino acid sequences of HA between Dk/Mon/119/08 and Anhui/1/13 was 96.6%. Genetic analysis revealed that the strain selected for vaccine preparation, Dk/Mon/119/08, is closely related to Anhui/1/13.

3.3. Potency of the test vaccines in mice against the challenge with H7N9 virus isolated from humans

To assess the immunogenicity of inactivated whole virus particle vaccines derived from the Dk/Mon/119/08 and Anhui/1/13, serum neutralizing antibody titers of mice

against Anhui/1/13 were measured (Table 4). Neutralizing antibodies were induced by each vaccine in a dose-dependent manner. These results indicate that the Dk/Mon/119/08 vaccine was as effective as the Anhui/1/13 vaccine in conferring antibody responses against the Anhui/1/13 virus strain.

To assess the potency of the vaccines against the challenge with Anhui/1/13, 10^4 PFU of Anhui/1/13 were intranasally inoculated into mice that had been previously injected once subcutaneously with inactivated Dk/Mon/119/08 or Anhui/1/13. The potency of the test vaccines was evaluated by virus titration of the lungs of the mice (Table 4). The virus titers in the lungs were $10^{2.0}$ -$10^{6.2}$ PFU/g in mice injected with 50, 10, and 2 μg protein of Anhui/1/13 vaccine. The virus titers in the lungs of mice injected with Dk/Mon/119/08 vaccine containing 50, 10, and 2 μg protein were $10^{2.9}$ -$10^{6.0}$ PFU/g. These results indicate that the test vaccine prepared from Anhui/1/13 or Dk/Mon/119/08 induced immunity to reduce virus replication in the lungs of vaccinated mice compared with those injected with PBS. The rates of weight loss in the mice after virus challenge are shown in Fig. 2. The mice injected with the test vaccine survived for 14 days, although they showed some weight loss, whereas the non-vaccinated control mice showed significant weight loss after the challenge. The fluctuations in body weight did not differ significantly between mice vaccinated with Dk/Mon/119/08 (50 μg or 10 μg) and those vaccinated with Anhui/1/13 (50 μg or 10 μg). In addition, the rate of weight loss in

mice injected with 10 μg of whole virus particle Dk/Mon/119/08 vaccine soon returned to normal (5 days post challenge), compared with those injected with 10 μg of whole virus particle Anhui/1/13 vaccine.

To examine further the efficacy of two shots of these vaccines, an additional experiment was performed (Table 5 and Fig. 3). The results showed that antibody titers were higher than that of mice injected once with each vaccine of 50, 10 and 2 μg protein (Table 5). The body weight loss of the mice vaccinated twice with Anhui/1/13 or Dk/Mon/119/08 vaccine was less than those vaccinated once (Fig. 3). The virus titers in the lungs of the mice vaccinated twice were lower than those of them vaccinated once with either Dk/Mon/119/08 or Anhui/1/13 vaccines. These results indicate that Dk/Mon/119/08 vaccine reduced the impact of disease caused by infection with A/Anhui/1/13 which was isolated from humans in mice.

4. Discussion

Before 2013, several H7 subtype infections were reported in humans, including A/Netherlands/33/03 (H7N7), A/New York/107/2003 (H7N2), and Seal/Mass/1/80, as a result of direct transmission from animal to human or laboratory accidents [22-25]. H7N9 influenza virus infections in humans have been reported in China since March, 2013. The H7N9 influenza virus isolated from humans is a reassortant virus, with all

212 the genes of avian origin [2]. Some isolates of the H7N9 influenza viruses bind equally
213 well to human- and avian-type receptors [4]. Although there is no evidence of efficient
214 human-to-human transmission of H7N9 influenza viruses, these viruses may have the
215 potential to cause pandemic influenza in humans. Consequently, vaccines are required
216 for H7N9 influenza virus infection in humans.

217 In the early stage of influenza pandemic, the antigenicity, pathogenicity and growth
218 ability of novel virus may not be known. In this study, we compared antigenically and
219 genetically H7 avian influenza viruses in the library with Anhui/1/13 virus.
220 Anhui/1/13 is antigenically similar to H7 avian influenza viruses isolated from ducks
221 (Table 2). Kida *et al.* showed that antigenic drift extensively occurs in human strains,
222 whereas the hemagglutinins of duck viruses were antigenically highly conserved [26].
223 Therefore, the pandemic virus strains emerging in humans are considered to be
224 antigenically similar to that of avian influenza virus strains in the library. The H7N9
225 virus strains in the library are certainly low pathogenic in both humans and poultry,
226 since the viruses does not have a pair of dibasic amino acid residues at the cleavage site
227 of the HA. In addition, the amino acid sequence at the receptor binding site of the HAs
228 indicates that the viruses specifically recognize α2,3 sialic acid receptor.
229 Dk/Mon/119/08 replicated efficiently in embryonated chicken eggs and was low
230 pathogenic in chicken embryos. The HA of Dk/Mon/119/08 was antigenically similar to

231 that of A/Anhui/1/13. Therefore, Dk/Mon/119/08 should be an ideal vaccine strain for
232 H7N9 virus infection.

233 Whole virus particle vaccines have been reported to induce protective immunity
234 more effectively than ether-split vaccines [13,14], and the influenza H7N9 virus-like
235 particle vaccine was effective in mice against a challenge with H7N9 influenza virus
236 isolated from humans [27]. In the present study, an inactivated whole particle H7N9
237 influenza vaccine was prepared from an H7N9 avian influenza virus, Dk/Mon/119/08,
238 present in the influenza virus library [15]. In the mice injected once with Anhui/1/13
239 vaccine or Dk/Mon/119/08 vaccine, virus titers in the lungs of mice were lower compared
240 with those of control animals after the challenge with A/Anhui/1/13 virus. One shot of
241 Dk/Mon/119/08 vaccine conferred protective immunity in mice against the challenge
242 with Anhui/1/13. Two shots of the vaccines induced stronger immunity to prevent the
243 body weight loss and to reduce virus replication in the lungs of mice than those of one
244 shot of vaccines (Table 5 and Fig. 3). These results indicate that Dk/Mon/119/08
245 vaccine induced enough immunity to prevent the impact of the disease.

246 Vaccination is one of the important control measures against influenza.
247 Approximately 6 months is required to produce vaccines [28,29]. To prepare for future
248 influenza pandemics, we have conducted surveillance of avian influenza since 1977.
249 The pathogenicity, antigenicity, genetic information, and yield in embryonated chicken

250 eggs of the virus strains in the library have been assessed. Avian influenza viruses of
251 144 combinations of 16 HA and 9 NA subtypes are stocked in our influenza virus library.
252 The present whole virus vaccine prepared from an influenza virus from the library
253 should be useful as a vaccine strain in the case of the emergence of influenza pandemic.
254
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261

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Conflict of interest statement

The authors declare that they have no conflict of interests.

357 **Figure captions**

358 Fig. 1. Phylogenetic tree of H7 HA of influenza viruses. Full-length nucleotide
359 sequences of the HA genes were used for the analysis. Horizontal distances are
360 proportional to the minimum number of nucleotide differences required to join nodes and
361 sequences. Numbers at the nodes indicate confidence levels in a bootstrap analysis with
362 1,000 replicates. Viruses were used to generate vaccines are highlighted. H7N9
363 influenza viruses in China are indicated by black circles and those stocked in the virus
364 library are indicated by white circles. H7 viruses used for neutralization tests are
365 underlined.

366

367 Fig. 2. Changes in body weight of mice vaccinated once following challenge with
368 Anhui/1/13. Five mice from each group injected with Dk/Mon/119/08 vaccine (a) or
369 Anhui/1/13 vaccine (b) were inoculated intranasally with Anhui/1/13. Body weight was
370 monitored for 14 days. Data are shown as the mean body weight change in each group
371 with the corresponding standard error. Asterisks indicate that body weights of the
372 vaccinated groups recovered significantly more than the PBS-injected group ($P < 0.05$).

373

374 Fig. 3. Changes in body weight of mice vaccinated twice following challenge with
375 Anhui/1/13. Five mice from each group injected with Dk/Mon/119/08 vaccine (a) or

376 Anhui/1/13 vaccine (b) were inoculated intranasally with Anhui/1/13. Body weight was
377 monitored for 14 days. Data are shown as the mean body weight change in each group
378 with the corresponding standard error. Asterisks indicate that body weights of the
379 vaccinated groups recovered significantly more than the PBS-injected group ($P < 0.05$).
380

Tables

Table 1 H7 viruses used in this study

Pathogenicity	Virus	Accession number[a]
HPAIV	A/chicken/Germany/1934 (H7N1)	GU052946
	A/FPV/Weybridge/1934 (H7N7)	L37794
	A/equine/Prague/1/1956 (H7N7)	CY096907
	A/turkey/England/1963 (H7N3)	CY015065
	A/chicken/Victoria/1976 (H7N7)	CY024786
	A/turkey/England/647/77 (H7N7)	AF202247
	A/duck/Taiwan/Ya103/1993 (H7N7)	AB297925*
	A/chicken/Queensland/1994 (H7N3)	CY022685
	A/turkey/Italy/4603/1999 (H7N1)	AF364147
	A/turkey/Italy/4580/1999 (H7N1)	GU052930
LPAIV	A/duck/Victoria/1976 (H7N7)	CY061602
	A/turkey/Oregon/1971 (H7N3)	AB269693*
	A/turkey/Tennessee/1/1979 (H7N3)	AB269692*
	A/duck/Hong Kong/293/1978 (H7N2)	CY006029
	A/duck/Hong Kong/301/1978 (H7N2)	AB302789*
	A/seal/Massachusetts/1/1980 (H7N7)	AB269696*
	A/swan/Shimane/42/1999 (H7N8)	AB269872*
	A/duck/Taiwan/4201/1999 (H7N7)	AB269695*
	A/mallard/Netherlands/12/2000 (H7N3)	KF695239
	A/duck/Mongolia/867/2002 (H7N1)	AB473543*
	A/duck/Hokkaido/Vac-2/2004 (H7N7)	AB243420*
	A/duck/Mongolia/720/2007 (H7N6)	AB450448*
	Dk/Mon/119/08 (H7N9)	AB481212*
	A/duck/Mongolia/147/2008 (H7N9)	AB828685*
	A/duck/Mongolia/128/2008 (H7N9)	AB829332*
	A/quail/Aichi/1/2009 (H7N6)	AB538456
	A/duck/Mongolia/129/2010 (H7N9)	AB828686*
	A/duck/Korea/A79/2010 (H7N7)	JN244243
	A/duck/Hokkaido/1/2010 (H7N7)	AB622425*
	A/duck/Zhejiang/12/2011 (H7N3)	JQ906576
	A/duck/Iwate/301012/2012 (H7N1)	AB698075*
	A/duck/Mongolia/47/2012 (H7N7)	AB755793*
	A/chicken/Shanghai/S1053/2013 (H7N9)	CY146956
	A/environment/Shanghai/S1088/2013 (H7N9)	CY147124
	Anhui/1/13 (H7N9)	EPI_ISL_138739[b]
	A/Hangzhou/1/2013 (H7N9)	KC853766
	A/pigeon/Shanghai/S1069/2013 (H7N9)	CY147172
	A/Shanghai/2/2013 (H7N9)	EPI_ISL_138738[b]
	A/Shanghai/1/2013 (H7N9)	EPI_ISL_138737[b]

[a] GenBank/EMBL/DDBJ Accession number.
[b] GISAID Accession number.
* The HA gene sequence was submitted to the GenBank/EMBL/DDBJ databases in this study.
LPAIV, Low pathogenic avian influenza virus; HPAIV, Highly pathogenic avian influenza virus

Table 2 Cross-reactivity of H7N9 viruses with antisera by neutralization test

Lineage	Viruses	Antiserum to*				
		Anhui/1/13	Mon/119	HK/301	Vac-2/04	Mas/1
Eurasia	Anhui/1/13 (H7N9)	**640**	640	1,280	1,280	80
	Dk/Mon/119/08 (H7N9)	320	**1,280**	5,120	1,280	160
	Duck/Hong Kong/301/78 (H7N2)	320	1,280	**5,120**	320	320
	Duck/Hokkaido/Vac-2/04 (H7N7)	640	320	5,120	**10,240**	2,560
	Turkey/Italy/4580/99 (H7N1)	160	320	640	160	20
North America	Seal/Massachusetts/1/80 (H7N7)	320	1,280	5,120	5,120	**2,560**

* The titers for a homologous combination are underlined.
Mon/119, Dk/Mon/119/08 (H7N9); HK/301, Duck/Hong Kong/301/78 (H7N2); Vac-2/04, Duck/Hokkaido/Vac-2/04 (H7N7); Mas/1, Seal/Massachusetts/1/80 (H7N7).

Table 3 Antigenic analyses of H7 influenza viruses using monoclonal antibodies

Viruses[a]	Monoclonal antibodies				
	55/2[b]	129/3[c]	8/4[b]	81/6[b]	187/1[c]
Human					
Anhui/1/13 (H7N9)	+	+	−	−	+
LPAIV					
Dk/Mon/119/08 (H7N9)	+	+	−	−	+
Duck/Hokkaido/Vac-2/04 (H7N7)	+	+	−	−	+
Duck/Mongolia/147/01 (H7N1)	+	+	+	+	+
Duck/Mongolia/555/02 (H7N7)	+	+	−	−	+
Duck/Hokkaido/114/03 (H7N7)	+	+	+	−	+
Duck/Hokkaido/W34/04 (H7N7)	+	+	+	−	+
Duck/Hong Kong/301/78 (H7N2)	+	+	+	−	+
Swan/Tottori/42/80 (H7N7)	+	+	−	+	+
Gull/Shimane/91/88 (H7N8)	+	+	−	+	+
Duck/Taiwan/4201/99 (H7N7)	+	+	+	+	+
HPAIV					
Turkey/England/63 (H7N3)	+	+	+	+	+
Chicken/Pakistan/447/95 (H7N3)	+	+	+	+	+
Chicken/Netherlands/2586/03 (H7N7)	+	+	+	+	+
Turkey/Itary/4580/99 (H7N1)	+	+	+	+	+
North American					
Seal/Massachusetts/1/80 (H7N7)	+	+	+	+	+
Equine					
Equine/Prague/1/56 (H7N7)	+	−	−	−	−

[a] Vaccine strains used in this study are underlined.
[b] MAbs to the HA of Seal/ Massachusetts/1/80 (H7N7) (Kida *et al*.,[30]).
[c] MAbs to the HA of Duck/Hokkaido/Vac-2/04 (H7N7) (Sakabe *et al*.,[19]).
LPAIV, Low pathogenic avian influenza virus; HPAIV, Highly pathogenic avian influenza virus

Table 4 Neutralizing antibody titers before challenge and virus titers of the lungs after challenge in mice vaccinated once

Vaccine	Dose of vaccine	Neutralizing antibody titer to		Virus titer[a] Mean log_{10} PFU/g ± Se[b]
		Anhui/1/13	Dk/Mon/119/08	
Anhui/1/13	50 μg	320, 320, 160, 320, 320	ND[c]	4.8 ± 0.28*
	10 μg	40, 40, 40, 20, 40	ND	5.2 ± 0.37*
	2 μg	20, 20, 20, 20, 40	ND	4.5 ± 0.30**
Dk/Mon/119/08	50 μg	80, 160, 80, 80, 80	160, 320, 160, 160, 160	2.3 ± 0.60*
	10 μg	40, 80, 80, 160, 40	80, 40, 40, 80, 40	5.4 ± 0.23*
	2 μg	20, 40, <20, <20, <20	<20, <20, <20, <20, <20	6.0 ± 0.16*
PBS	-	<20, <20, <20, <20, <20	<20, <20, <20, <20, <20	6.6 ± 0.05

Each vaccine was injected subcutaneously in 10 mice. Serum samples were collected 3 weeks after the vaccination. Mice were challenged with $10^{4.0}$ PFU of Anhui/1/13 intranasally.

[a] The lung samples were collected at 3 days post challenge and virus titers were measured.

[b] Data for 5 mice.

[c] "ND" indicates not determined.

*: P<0.05, vs. virus titers in PBS group.

**: P<0.01, vs. virus titers in PBS group.

Table 5 Neutralizing antibody titers before challenge and virus titers of the lungs after challenge in mice vaccinated twice

Vaccine	Dose of vaccine	Neutralizing antibody titer to		Virus titer[a] Mean log_{10} PFU/g ± Se[b]
		Anhui/1/13	Dk/Mon/119/08	
Anhui/1/13	50 μg	640, 320, 640, 640, 1280	ND[c]	-
	10 μg	640, 640, 640, 320, 160	ND	0.4 ±0.40**
	2 μg	80, 20, 40, 40, 40	ND	4.6 ±0.39**
Dk/Mon/119/08	50 μg	80, 40, 40, 80, 80	80, 160, 160, 320, 160	0.6±0.38**
	10 μg	40, 80, 40, 40, 40	40, 40, 40, 40, 40	2.7 ±1.12**
	2 μg	20, 40, 40, <20, 20	20, 40, 40, 40, 40	5.3 ±0.39**
PBS	-	<20, <20, <20, <20, <20	<20, <20, <20, <20, <20	6.5 ±0.08

Each vaccine was injected subcutaneously in 10 mice. Serum samples were collected 3 weeks after the vaccination. Mice were challenged with $10^{4.0}$ PFU of Anhui/1/13 intranasally.

[a] The lung samples were collected at 3 days post challenge and virus titers were measured; - : indicates viruses could not be detected in all mice.

[b] Data for 5 mice.

[c] "ND" indicates not determined.

*: P<0.05, vs. virus titers in PBS group.

**: P<0.01, vs. virus titers in PBS group.

Figures

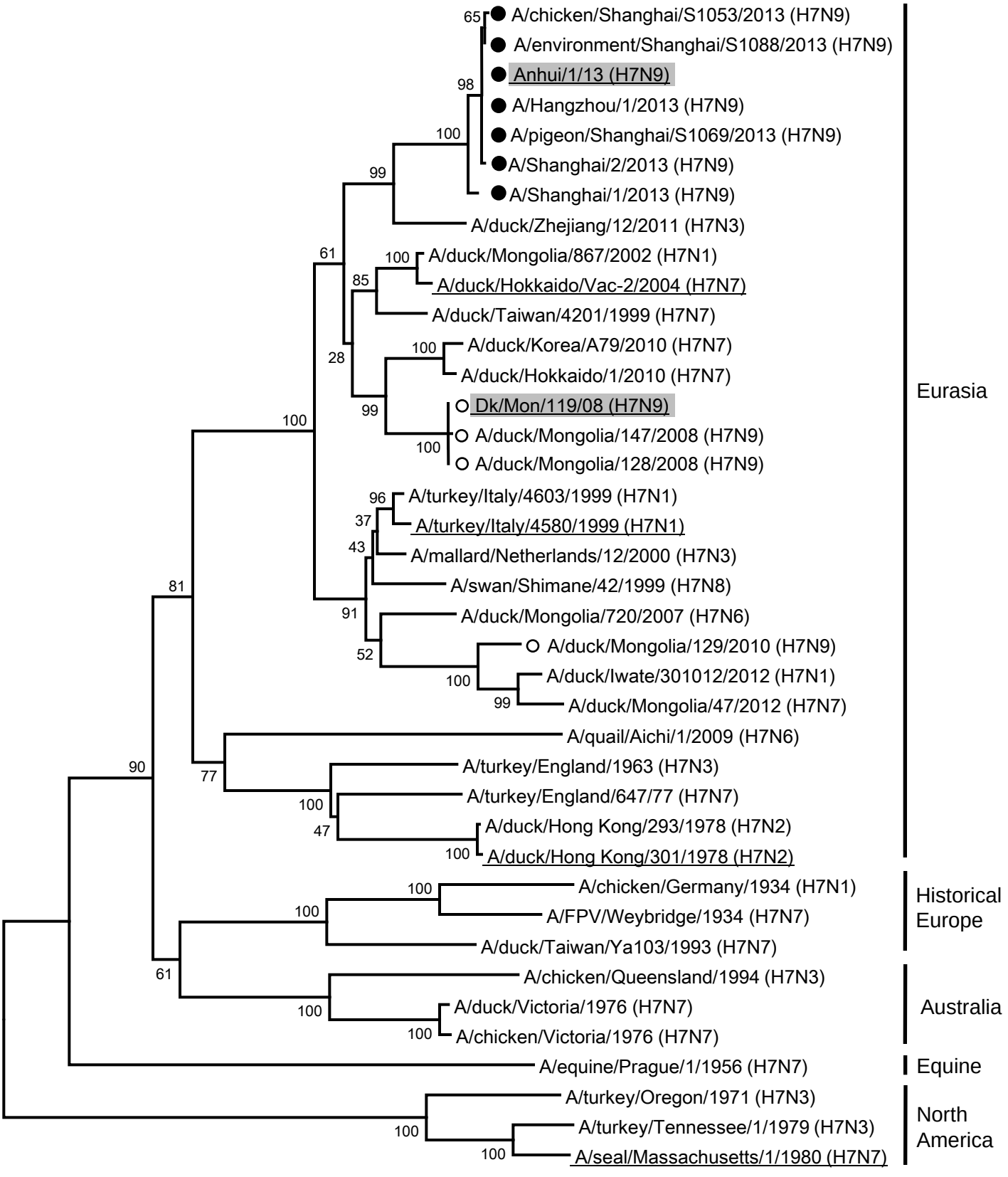
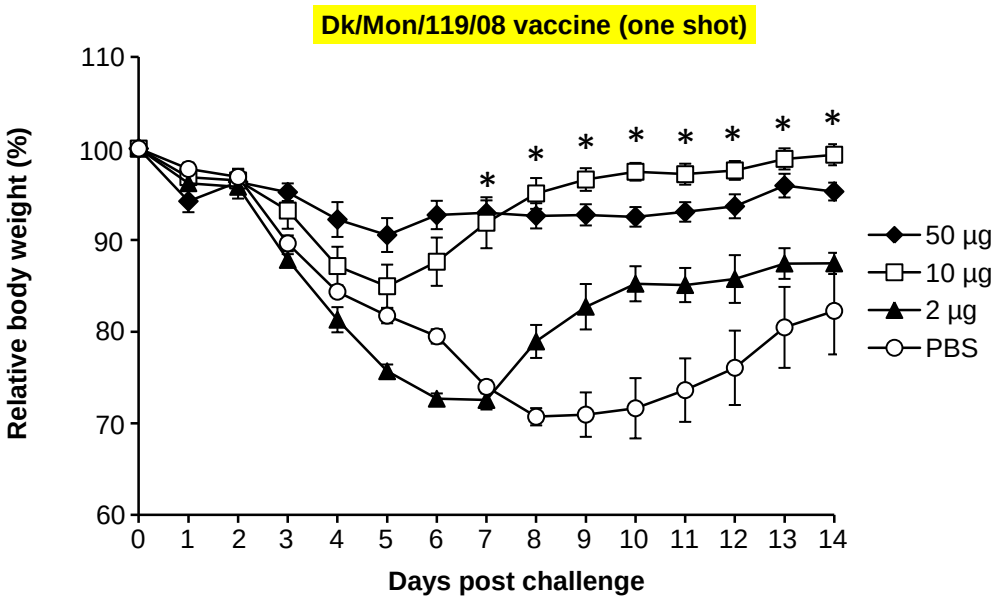


Fig. 1 Huy *et al.*

(a)



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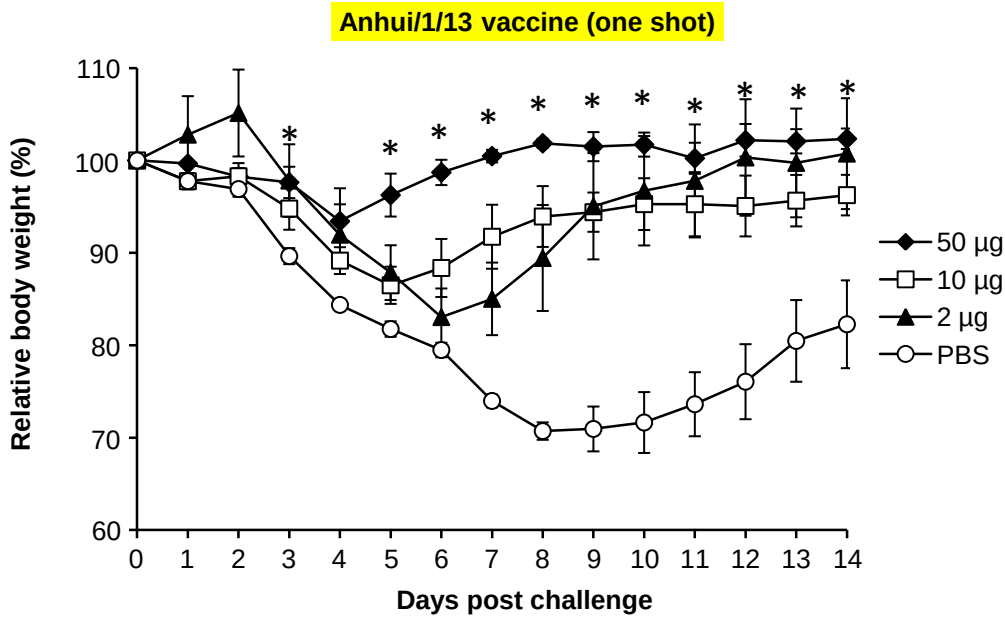

Fig. 2 Huy *et al.*

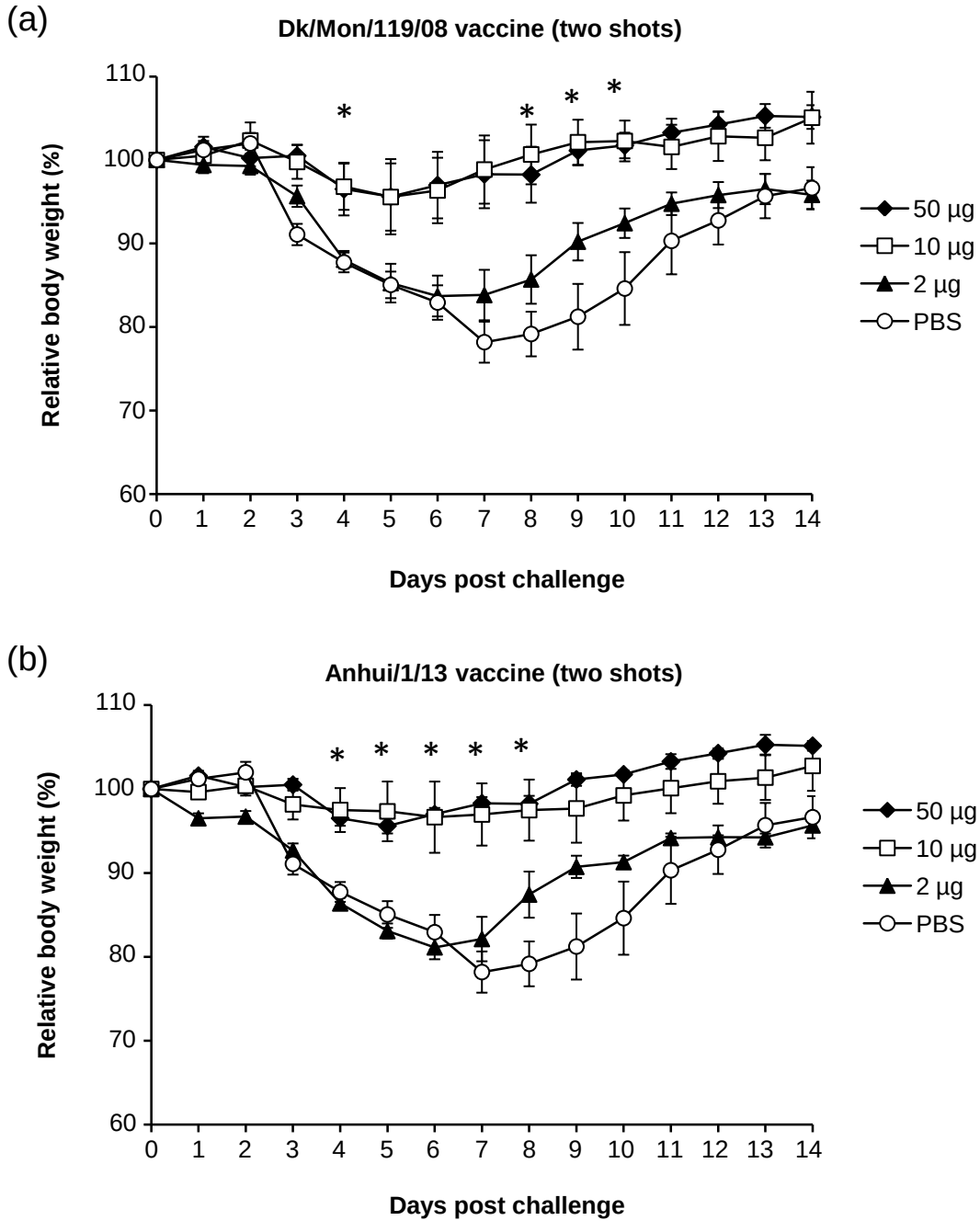

Fig. 3 Huy *et al.*