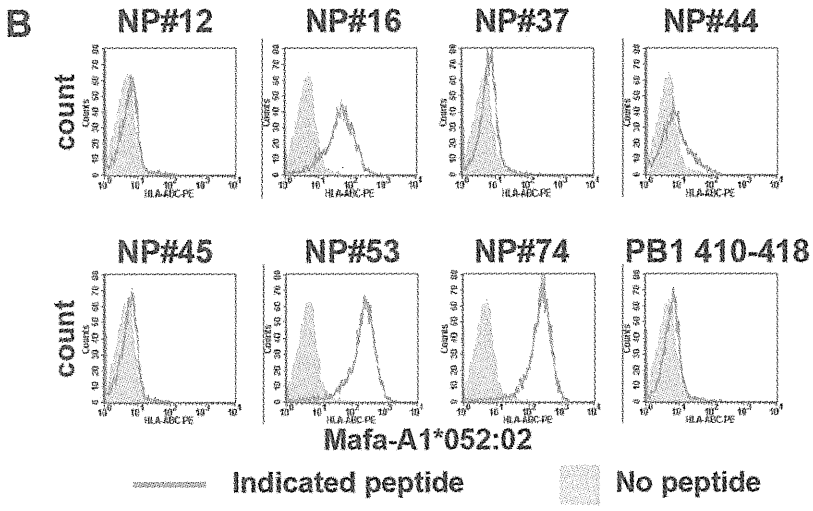
Figure 7. NP peptides bound to Mafa-A1*052:02.

A

Peptide	Amino acid sequence
NP#12(56-65)	LIQNSITIER
NP#16(76-85)	NKYLEEHPSA
NP#37(181-190)	AAVKGVGTIA
NP#44(216-225)	RVAYERMCNI
NP#45(221-230)	RMCNILKGKF
NP#53(261-270)	RSALILRGSV
NP#74(366-375)	ASNENVETMD
PB1 410-418	GMFNMLSTV



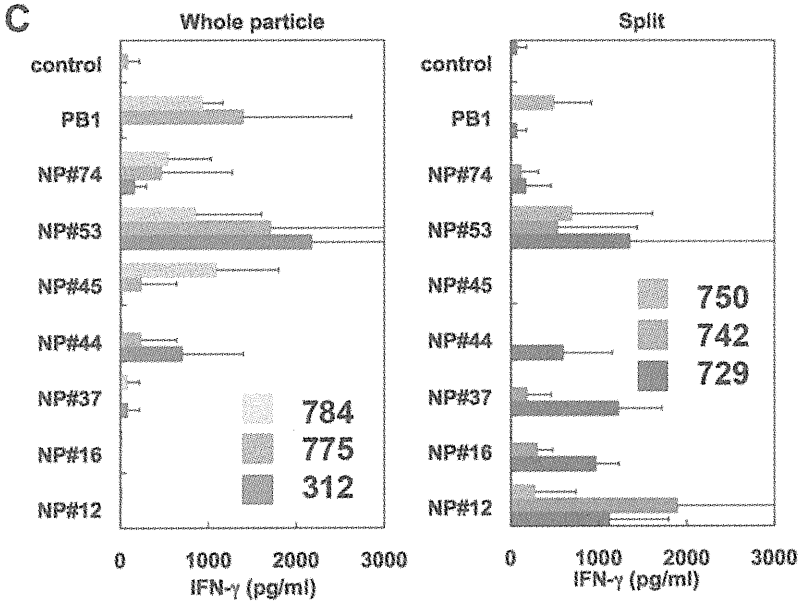


Figure 7. NP peptides bound to Mafa-A1*052:02. (A) Amino acid sequences of 10-mer peptides derived from Narita1 nucleoprotein are shown as one-letter code. The sequence of influenza A virus PB1 peptide was used as a representative of non-binding peptides to Mafa-A1*052:02. The numbers in parentheses indicate residues from the N-terminus of NP. (B) RMA-S expressing Mafa-A1*052:02 and human β2-microglobulin was

cultured with NP peptides at 27°C for 1 h and then at 37°C for 4 h. Stable expression of Mafa-A1*052:02 on the cell surface was examined by staining with W6/32 antibody. Orange lines: cells cultured with indicated peptides. Blue shade: cells cultured without peptide. (C) $CD8^+$ cells were isolated from spleens of indicated macaques and cultured with APC and indicated peptides. IFN-γ production was determined using ELISA. Averages and SD of triplicate culture are shown. Representative results of three independent experiments are shown.
doi:10.1371/journal.pone.0037220.g007

Discussion

We examined antibody and T cell responses induced by subcutaneous vaccination with two forms of preparation, whole particle vaccine and split vaccine, using immunologically naïve cynomolgus macaques for influenza virus infection. The macaque model enabled evaluation of vaccine efficacy without consideration of pre-existing immunity, which has been difficult to exclude in human studies [2,13,14]. Since most inactivated vaccines are inoculated subcutaneously or intramuscularly in humans [14,24], we subcutaneously inoculated vaccines into macaques to reflect results in the present study to human vaccination usage. As a result, the whole particle vaccine derived from our virus library was more effective for conferring memory immune responses and prohibiting virus replication than was the split vaccine.

We previously revealed that pandemic (H1N1) 2009 influenza virus caused viral pneumonia in cynomolgus macaques, which resembled pneumonia in human patients during the pandemic [2–6]. Furthermore, we were able to analyze immune responses in macaques using antibodies against human molecules [25], whereas reagents for immunological analyses were not sufficient in ferrets that showed human-like clinical symptoms by human influenza viruses. Therefore, we considered the macaque models to be suitable for extrapolation of responses and efficacy of vaccines against pandemic influenza virus infection in humans.

As shown in Fig. 7C, $CD8^+$ T cells of individual vaccinated macaques responded to different sets of NP peptides since the macaques used in the present study were not inbred. To compare peptide-specific T cell responses between two vaccinations, we required a parameter shared by all studied macaques. Therefore, we selected macaques carrying Mafa-A1*052:02 and identified NP262-270 as a peptide that induced $CD8^+$ T cell responses in all vaccinated macaques. The variety of stimulatory peptides except NP262-270 among macaques might be due to antigen presentation on other Mafa class I molecules, i.e., the second allelic product of Mafa-A1 other than Mafa-A1*052:02 and Mafa-B (Table 1). Therefore, we examined $CD8^+$ T cell responses specific for NP262-270 plus Mafa-A1*052:02 to compare memory T cell responses in macaques vaccinated with the whole particle vaccine with those in macaques vaccinated with the split vaccine.

Table 1. Mafa-A1 alleles of macaques used in the present study.

	Mafa-A1	
Animal ID	**Allele 1**	**Allele 2**
785	052:02	089:03
848	052:02	089:03
994	052:02	008:02
312	052:02	004:01
775	052:02	093:01
784	052:02	089:03
729	052:02	094:01
742	052:02	093:02
750	052:02	094:01

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One of the peptides that bound to Mafa-A1*052:02, NP#74, contained a known CTL epitope (ASNENVETM, NP366-374) bound to mouse MHC class I $H\text{-}2D^b$, in which N at position 5 and M at position 9 are known as main anchor residues and N at position 3 is a minor anchor [26]. The results presented in Fig. 8 showed that anchor positions of NP262-270 were aligned with the same intervals as those seen in NP366-374 bound to $H\text{-}2D^b$. Therefore, it is possible that the Mafa-A1*052:02 binding motif is similar to that of $H\text{-}2D^b$, i.e., hydrophobic or non-polar residues at positions 3, 5 and 9. This was supported by analyses of peptides eluted from Mafa-A1*052:02 (Table 2). Based on these results, the Mafa-A1*052:02 binding motif appeared to be hydrophobic residues (L, I or V) at position 3, hydrophobic residues (L or I) at position 5 and hydrophobic or aromatic residues (L, V, M, F or Y) at position 9 as anchors.

Using macaques carrying Mafa-A1*052:02, we showed advantages of the whole particle vaccine in three aspects, immunological memory, immunogenicity and cross-reactivity. Firstly, we proved the advantage of the whole particle vaccine in memory CTL responses. A higher percentage of IFN-γ-positive $CD8^+$ cells specific for the NP262-270 peptide was observed in macaques vaccinated with the whole particle vaccine than in macaques without vaccination and macaques vaccinated with the split vaccine (Fig. 9). Therefore, it is thought that memory T cells were maintained in macaques inoculated with the whole particle vaccine and expanded promptly after challenge infection.

The whole particle vaccine also induced immunological memory in antibody responses more effectively than did the split vaccine. Neutralizing activity of sera against the challenge strain increased 2 days after the challenge infection in macaques vaccinated with the whole particle vaccine, whereas increase of neutralization activity against the challenge strain in sera from macaques vaccinated with the split vaccine was detected on days 8 to 10 after challenge infection (Fig. 6). This was only 2 days earlier than that in macaques without vaccination. Therefore, the whole particle vaccine generated immunological memory in both cellular and humoral responses more effectively than did the split vaccine. Since recall memory B cell responses were impaired in MyD88-deficient mice, Toll-like receptors (TLR) stimulated by whole particle vaccines would play a critical role in generation of memory B cells [27].

Secondly, as previously shown in mouse and human studies [28–30], the whole particle vaccine induced higher titers of neutralizing antibody in macaques than did the split vaccine (Fig. 2). This indicates that inactivated whole viral particles were more immunogenic than soluble split antigens in macaques. Several studies have indicated that a virus-like particle (VLP) antigen was superior to a soluble protein in inducing Th1 and CTL responses with effective antigen cross-presentation by dendritic cells [31–33]. We also showed that one immunization with the whole particle vaccine induced neutralizing antibody at a level similar to that induced by two immunizations with the split vaccine. This result suggests that we could reduce the dose of vaccination by using whole particle vaccines to save vaccine amounts and distribute vaccines to more people than by using split vaccines.Not only formulation of antigens but also additional

Figure 8. Determination of a core sequence and anchor residues of NP#53 peptides bound to Mafa-A1*052:02.

A

Peptide	Amino acid sequence
NP#53(261-270)	RSALILRGSV
NP261-269	RSALILRGS
NP261-268	RSALILRG
NP261-267	RSALILR
NP262-270	SALILRGSV
NP263-270	ALILRGSV
NP264-270	LILRGSV

C

Peptide	Amino acid sequence
NP262-270	SALILRGSV
262A	AALILRGSV
263G	SGLILRGSV
264A	SAAILRGSV
265A	SALALRGSV
266A	SALIARGSV
267A	SALILAGSV
268A	SALILRASV
269A	SALILRGAV
270A	SALILRGSA

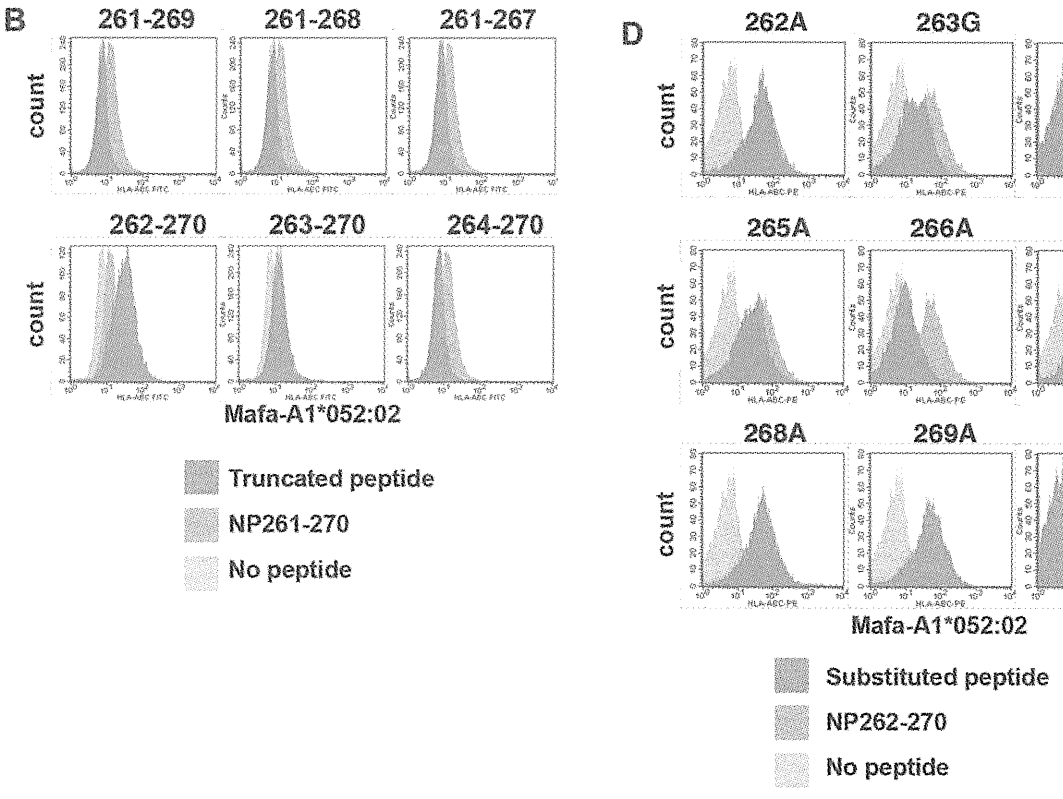

Figure 8. A core sequence and anchor residues of NP#53 peptides bound to Mafa-A1*052:02. (A) Amino acid sequences of truncated NP#53 peptides. (B) Truncated NP#53 peptides were cultured with the Mafa-A1*052:02 transfectant as described in the legend to Fig. 7. Orange: cells cultured with truncated NP#53 indicated in each histogram. Green: cells cultured with untruncated NP#53 (261–270). Blue: cells cultured without peptide. (C) Amino acid sequences of substituted NP262-270 peptides. Amino acids of NP262-270 were substituted to alanine except residue 263 that was substituted to glycine. (D) Substituted NP262-270 peptides were cultured with the Mafa-A*052:02 transfectant. Orange: cells cultured with substituted NP262-270 indicated in each histogram. Green: cells cultured with unsubstituted NP262-270. Blue: cells cultured without peptide. Representative results of three independent experiments are shown.
doi:10.1371/journal.pone.0037220.g008

molecules included in the whole particle vaccines were thought to affect the immunogenicity. IL-12 and tumor necrosis factor (TNF)-α production by human dendritic cells was enhanced by whole particle vaccines but not by subunit vaccines [34]. This response might be partly mediated by TLR signals [35]. Inactivated whole virus particles showed adjuvant effects by activating the TLR7-MyD88 pathway in plasmacytoid dendritic cells [36]. Type I interferon production by plasmacytoid dendritic cells was crucial for induction of primary B cell and $CD4^+$ T cell responses and cross-presentation of antigen by dendritic cells, resulting in

Table 2. Amino acid sequences of peptides eluted from Mafa-A1*052:02.

sources	NCBI accession	start position	MW[1]	amino acid sequences[2]										
	GI number						1	2	3	4	5	6	7	8	9[3]
Guanylate cyclase soluble subunit α3 isoform A	67763816	127	1112.66			V	P	V	E	V	I[4]	K	E	S	L
S-adenosylmethionine synthase isoform type 2	5174529	150	1235.75		M	P	L	T	I	V	L	A	H	K	L
proliferation-associated protein 2G4	124494254	180	1225.62		T	P	I	E	G	M	L	S	H	Q	L
tudor domain containing 7	112293287	975	1296.84	R	V	L	L	K	G	I	L	T	N	G	L
FLJ00043 protein/EH domain-binding protein 1-like protein-1	150378549	904	980.59				A	P	V	T	Q	P	R	V	L
CAP-Gly domain-containing linker protein1/2	4506751	273/280	1250.77		A	P	I	H	K	V	I	R	I	G	F
SEC14-like protein 1	221316682	206	1238.74	V	V	I	P	E	A	A	L	K	E	G	L
60 S ribosomal protein L15	15431293	83	1403.79	K	P	V	H	H	G	V	N	Q	L	K	F
structural maintenance of chromosomes flexible hinge domain-containing protein 1	148839305	807	1156.72			R	P	L	P	S	K	A	I	K	F
mitotic checkpoint protein BUB3	4757880	11	1303.65	Q	P	P	E	D	G	I	S	S	V	K	F
dnaJ homolog subfamily A member 1	4504511	313	1520.86	R	P	Y	E	K	G	R	L	I	I	E	F
FH1/FH2 domain-containing protein 1	118572599	107	1182.76			K	P	T	L	I	L	R	T	Q	L
intraflagellar transport protein 57 homolog	8922256	100	1193.56				R	P	F	E	Q	P	Q	E	Y
ubiquitin-conjugating enzyme E2 D4	8393719	43	1001.47				S	P	Y	Q	G	G	V	F	F
vacuolar protein sorting-associated protein 13A	66346674	2184	1043.42				T	C	V	T	S	I	C	E	M

[1]Molecular weight of eluted peptides.
[2]Amino acid sequences of peptides eluted from Mafa-A1*052:02 are shown as a one-letter code.
[3]C-terminals of peptides are aligned as position 9.
[4]Bold letters are amino acids that are compatible with the predicted Mafa-A1*052:02-binding motif.
doi:10.1371/journal.pone.0037220.t002

polarization to Th1 cells and activation of CTL [37,38]. The TLR signal also stimulated naïve B cells directly and enhanced B cell responses [39,40]. Concordant with the previous reports, we showed that IgG1 responses, one of the hallmarks of Th1 responses in human immune responses, were induced more effectively in cynomolgus macaques after vaccination with the whole particle vaccine than with the split vaccine (Fig. 1), indicating that whole particle vaccines including virus RNA as a natural adjuvant enhanced immunogenicity and induced Th1 responses [28,41,42]. Therefore, whole particle vaccines do not necessarily need inoculation with an additional adjuvant.

Thirdly, sera from macaques inoculated with the whole particle vaccine showed neutralizing activity against not only the vaccine strain but also the challenge strain (Figs. 2 and 6), resulting in lower virus titers after challenge in macaques vaccinated with the whole particle vaccine than in macaques vaccinated with the split vaccine (Fig. 5). In the present study, similarities of HA and NA between the vaccine strain and the challenge virus strain were 89% and 83% at the amino acid level, respectively. These results indicated that whole particle vaccines induced not only a larger amount of specific antibody but also a more broadly cross-reactive antibody than did split vaccines [43]. This might be explained by results of previous studies showing that whole particle vaccines and split vaccines activated different B cell repertoires (clones) [44] and that two formulations of vaccines induced differentiation of distinct T helper cells such as Th1 and Th2 as discussed above, resulting in differences in the number of reacting B cells and affinity maturation [28,45]. Furthermore, since sequences of NP including CTL epitopes were conserved in influenza A viruses compared with HA and NA, CTL specific for NP and other internal proteins might react to heterosubtypic influenza viruses [46,47]. Thus, whole particle vaccines would be effective even if viral antigens were changed by gene mutations within a certain range. Therefore, our virus library containing 144 combinations of 16 HA and 9 NA would be useful when pandemic vaccines are prepared as whole particle vaccines to induce both antibody and CTL responses with broad cross-reactivity [8].

In summary, we showed that inactivated whole virus particles from the virus library were effective against pandemic (H1N1) 2009 influenza virus infection using the cynomolgus macaque model. Whole particle vaccines were superior for induction of memory antibody and CTL responses, which were confirmed at the level of peptide-specific responses. We are examining other Mafa class I and class II types and we are breeding macaques carrying specific MHC haplotypes. These macaques will be useful for evaluation of vaccination and analysis of both cellular and humoral immune responses in future studies.

Materials and Methods

Animals and Ethics Statement

?This study was carried out in strict accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of Research Center for Animal Life Science at Shiga University of Medical Science and STANDARDS RELATING TO THE CARE AND MANAGEMENT, ETC. OF EXPERIMENTAL ANIMALS (Notification No.6, March 27, 1980 of the Prime Minister's Office, Japan). The protocol was approved by the Shiga University of Medical Science Animal Experiment Committee (Permit number: 2009-5-2H) and the Biosafety Committee (Permit number: 2009-2). The animal experiments were conducted in strict compliance with animal husbandry and welfare regulations. All procedures were performed under ketamine and xylazine anesthesia, and all efforts were made to minimize suffering. Food pellets of CMK-2 (CLEA Japan, Inc., Tokyo,

Figure 9. Recall T cell responses after challenge infection with pandemic H1N1 virus.

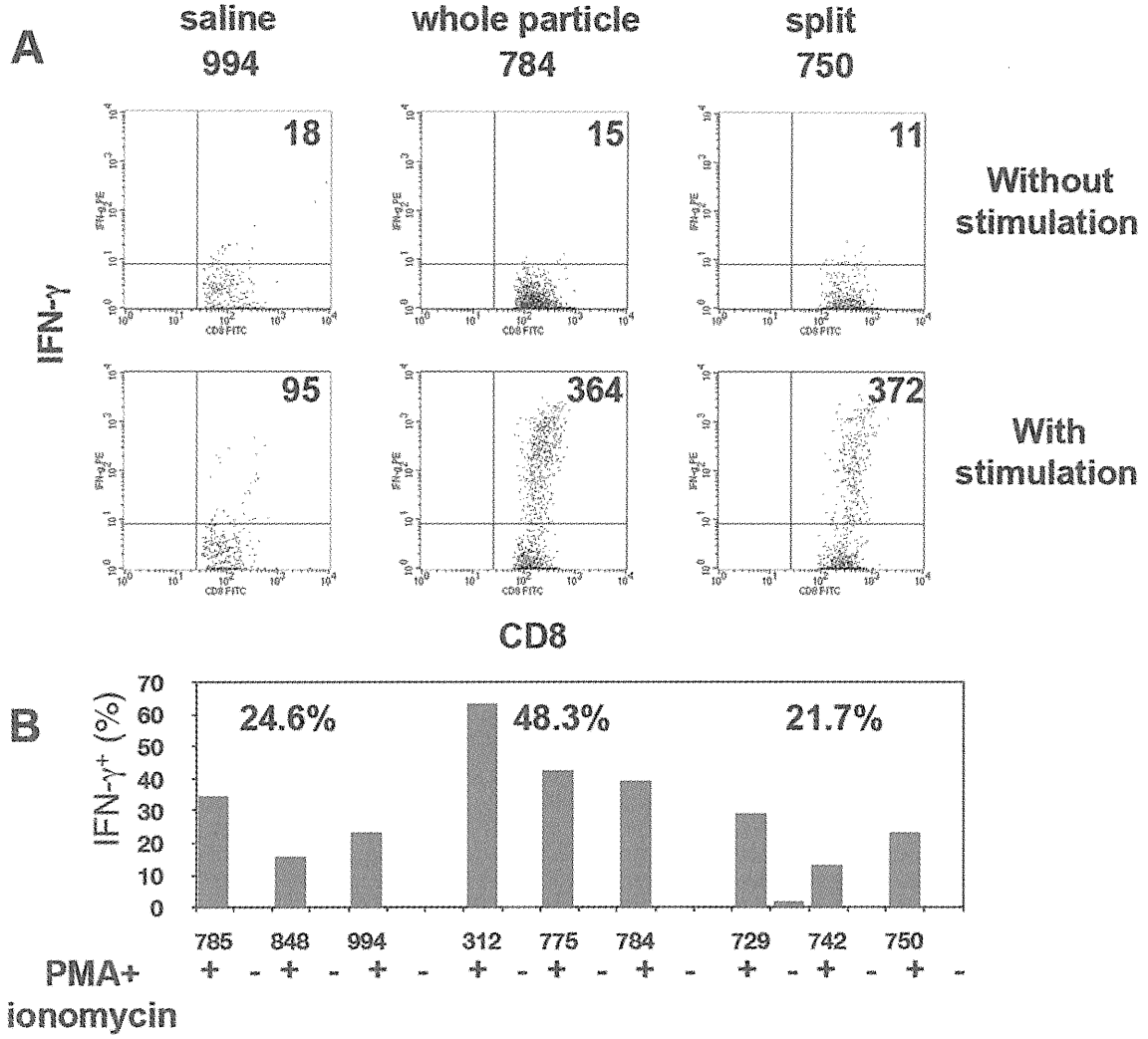

Figure 9. Recall T cell responses after challenge infection with pandemic H1N1 virus. Cervical lymph node cells from the macaques 14 days after challenge with the pandemic H1N1 virus were cultured with NP262-270 peptide for 5 d. The recovered cells were stimulated with (+) or without (-) PMA and ionomycin for 5 h. Thereafter, intracellular IFN-γ and surface CD8 were stained. (A) Representative IFN-γ and CD8 profiles of each group are shown after gating of $CD8^+$ cells. Upper figures: culture without PMA and ionomycin, lower figures: culture with PMA and ionomycin. Mean fluorescence intensity (MFI) of IFN-γ staining is indicated in each figure. The averages of MFI of IFN-γ^+ $CD8^+$ cells stimulated with PMA and ionomycin are 147, 395 and 285 in the saline group, the whole particle vaccine group and the split vaccine group, respectively. (B) The percentages of IFN-γ^+ cells in $CD8^+$ cells are shown. The averages of percentages of three macaques in each group are indicated in the graph. There are significant differences ($P<0.05$) between the saline group and whole particle vaccine group and between the whole particle vaccine group and split vaccine group.

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Japan) fed once a day after recovery from anesthesia and drinking water were available *ad libitum*. Animals were singly housed under controlled conditions of humidity (40±5%), temperature (25±1°C), and light (12 h light/12 h dark cycle, lights on at 8:00 A.M.). Five- to seven-year-old female cynomolgus macaques from the Philippines (Ina Research Inc., Ina, Japan) were used. The cynomolgus macaques used in the present study were healthy young adults. In the text and figures, individual macaques are distinguished by identification numbers. The absence of influenza A virus NP-specific antibodies in their sera was confirmed before experiments using an antigen-specific enzyme-linked immunosorbent assay (ELISA), AniGen AIV Ab ELISA (Animal Genetics Inc., Kyonggi-do, Korea), for currently circulating influenza virus. Two weeks before virus inoculation, a telemetry probe (TA10CTA-D70, Data Sciences International, St. Paul, MN) was implanted in the peritoneal cavity of each macaque under ketamine/xylazine anesthesia followed by isoflurane inhalation to monitor body temperature. The macaques used in this study were

free from B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica*.

Vaccines (1 mg/dose) were inoculated subcutaneously into macaques using syringes twice with a two-week interval between injections under ketamine/xylazine anesthesia. Saline instead of the vaccine was inoculated into macaques as unvaccinated controls. The macaques were challenged with Narita1 (2×10^5 $TCID_{50}$/1 ml) into nasal cavities (0.5 ml for each nostril) with pipettes 7 weeks after the second vaccinations under ketamine/xylazine anesthesia. Experiments using Narita1 were performed in the biosafety level 3 facility of the Research Center for Animal Life Science, Shiga University of Medical Science.

Under ketamine/xylazine anesthesia, 2 cotton sticks (TE8201, Eiken Chemical, Ltd., Tokyo, Japan) were used to collect fluid samples in nasal cavities and tracheas, and the sticks were subsequently immersed in 1 ml of PBS containing 0.1% bovine serum albumin (BSA) and antibiotics.

Viruses and Vaccines

We used influenza virus A/swine/Hokkaido/2/1981 (H1N1) (National Center for Biotechnology Information (NCBI) taxonomy database ID: 387253) as a vaccine strain [48].

The Hokkaido2 virus was propagated in allantoic cavities of 10-day-old embryonated hen's eggs at 35°C for 48 h. To prepare an inactivated whole particle vaccine, the allantoic fluid infected with Hokkaido2 was concentrated and purified by high-speed centrifugation (112,500 g for 90 min) through a 10–50% sucrose density gradient and then treated in 0.1% formalin at 4°C for one week [9]. For preparation of a split vaccine, ether was mixed for 30 min with a suspension of the viral particles purified and inactivated by the same method as that for preparation of the whole particle vaccine. Thereafter, ether was evaporated with bubbing at room temperature without removal of hydrophilic solution. The amount of the whole particle vaccine and that of the split vaccine were indicated as the amount of entire proteins including HA and other viral proteins. With an HA test, we confirmed that the two types of vaccine included the same amounts of HA.

Pandemic influenza virus A/Narita/1/2009 pdm (NCBI taxonomy ID: 645520) was used as a challenge virus (kindly provided by Dr. Takato Odagiri, National Institue of Infectious Disease (NIID), Japan) [22]. In a preliminary study, we confirmed that Narita1 inoculated into nostrils, oral cavities and tracheas caused pneumonia in cynomolgus macaques as severe as that observed in macaques inoculated with A/California/04/2009 (H1N1) (NCBI taxonomy ID: 641501) [2]. Narita1 was propagated in embryonated eggs twice at NIID and once in Madin-Darby canine kidney (MDCK) cells (the American Type Culture Collection, Manassas, VA) at the Shiga University of Medical Science [49]. The amino acid sequence identities between Hokkaido 2 and Narita1 were 89% in HA (GI: 216409430 vs. GI: 237659680) and 83% in NA (GI: 216409434 vs. GI: 23761745).

In order to assess virus replication, serial dilutions of swab samples were inoculated onto confluent MDCK cells as described previously [9]. Cytopathic effects were examined under a microscope 72 h later.

Detection of Antibody Specific for Virus Antigen with ELISA

The antibody titers of serum and swab samples against Hokkaido2 antigens were determined using ELISA [50]. Ninety-six-well plates were coated with 50 µl of purified Hokkaido2 (20 µg/ml) disrupted with 0.05 M Tris-HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl. Serially diluted samples were incubated overnight in the coated plates. After washing five times, horseradish peroxidase (HRP) -conjugated anti-monkey IgG antibodies (MP Biomedicals, Inc./Cappel, Aurora, OH) (1:2000×50 µl), anti-monkey IgA antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) (1:4000×50 µl) or IgM (Rockland Inc., Gilbertsville. PA) (1:5000×50 µl) were added and incubated for 1 h at room temperature. For detection of IgG1, incubation with anti-human IgG1 (clone: HP6069, Calbiochem) (1:2000×50 µl) was followed by incubation with HRP-conjugated anti-mouse Ig (Bio-Rad Laboratories, Inc.) (1:2000×50 µl). HRP activity was assessed using 3, 3′, 5, 5′-tetramethyl benzidine substrate (100 µl). The reaction was stopped by the addition of 1 M hydrogen chloride (100 µl). Optical density was measured at 450 nm.

Cytokine Assay

In the experiment for which results are shown in Fig. 3, lymphocytes were purified from peripheral blood of the macaques using a density gradient (Wako Pure Chemical Industries Ltd., Osaka, Japan). After washing, $CD8^+$ cells were isolated using CD8 microbeads for non-human primates and magnetic cell sorting (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), followed by separation of $CD4^+$ cells using CD4 microbeads. The cells remaining after removal of $CD4^+$ and $CD8^+$ cells were used as antigen-presenting cells (APC) after irradiation at 30 Gy. $CD4^+$ or $CD8^+$ T cells (1×10^5 cells/well) and APC (0.5×10^5 cells/well) were cultured with the inactivated whole particle antigens or the ether split vaccine antigen in the presence of anti-CD28 (clone: CD28.2) and CD49d (clone: 9F10) antibodies (0.5 µg/ml, eBioscience Inc., San Diego, CA) in 96-well U-bottom plates for 48 h and supernatants were collected [10]. The concentrations of IFN-γ and IL-2 in the supernatants were measured using the Milliplex MAP non-human primate cytokine panel and Luminex200 (Millipore Corp., Billerica, MA).

In the experiment for which results are shown in Fig. 7C, spleen cells obtained at autopsy on day 14 after the challenge infection were used after homogenization. After washing, $CD8^+$ cells were isolated using CD8 microbeads as described above. $CD8^+$ T cells (1×10^5 cells/well) and APC (0.5×10^5 cells/well) were cultured with various peptides (10 µM) in the presence of anti-CD28 and CD49d antibodies (0.5 µg/ml) in 96-well U-bottom plates for 48 h and supernatants were collected. The concentrations of IFN-γ in the supernatants were measured by ELISA using purified anti-human IFN-γ (clone: MD-1, eBioscience) and biotinylated anti-human IFN-γ (clone: 4S.B3, BioLegend, Inc., San Diego, CA) followed by HRP-labeled streptavidin (BD Biosciences). HRP activity was assessed using 3, 3′, 5, 5′-tetramethyl benzidine substrate as described above.

In the experiment for which results are shown in Fig. 9, unfractionated cells from cervical lymph nodes obtained at autopsy on day 14 after the challenge infection were incubated with NP262-270 peptide (10 µM, Hokkaido System Sciences, Sapporo, Japan) in the presence of anti-CD28, anti-CD49d (0.5 µg/ml) and human IL-2 (10 ng/ml) for 5 d. The cultured lymph node cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (0.1 µg/ml) and ionomycin (1 µg/ml) for 5 h. Monensin (2 µM) was added for the last 4 h. Thereafter, cells were washed with PBS containing EDTA (0.5 µM). Surface CD8 was stained with fluorescein isothiocyanate (FITC)-conjugated specific antibodies (clone: RPA-T8, eBioscience). Intracellular IFN-γ was stained with phycoerythrin (PE)-conjugated antibody (clone: 4S.B3, BD Biosciences) after fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin.

Virus Neutralization Assay

Serum samples were pretreated with a receptor-destroying enzyme (RDEII, Denka Seiken, Tokyo, Japan) at 37°C overnight and then inactivated at 56°C for 1 h. The diluted samples were mixed with 50 $TCID_{50}$ of the viruses for 1 h. Then the mixture was added onto an MDCK monolayer. After 1-h incubation, the cells were cultured in MEM containing 0.1% BSA and 5 μg/ml trypsin. After incubation at 35°C for 3 days, the number of wells with cytopathic effects was counted in quadruplicate culture. Neutralization titers were expressed as the dilution in which cytopathic effects were observed in 50% of the wells.

Typing of Mafa-A1 and Transfection of the *Mafa-A1*052:02* Gene

Blood was collected from cynomolgus macaques and immediately mixed with Trizol (Invitrogen Corporation, Carlsbad, CA). After extracting total RNA, cDNA was synthesized using oligo dT primer and ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). To amplify Mafa-A genes, PCR was performed using a set of primers (each 0.5 μM), sense 5′-GATGGCTATCATGGCGCC-3′ and anti-sense 5′-TCTTCATGCCTTCTCTTTGTGACT-3′, and KOD FX polymerase (0.4 units, Toyobo) [51]. The cycling parameters were as follows: an initial denaturation at 98°C for 1 min followed by 30 cycles of 98°C for 10 s and 68°C for 1 min. The amplified DNA fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI). Thereafter, nucleotide sequences were determined using an ABI3130 genetic analyzer (Applied Biosystems, Foster City, CA) in accordance with the protocol of the Big Dye terminator method.

The *Mafa-A1*052:02* (GenBank AM943361, nomenclature based on the WHO Nomenclature Committee for Factors of the HLA System) gene was inserted as an EcoRI DNA fragment into the EcoRI site of a pTA2 vector (Toyobo) and then cloned into EcoRI sites of a pcDNA3.1$^+$ vector (Invitrogen). The *Mafa-A1*052:02* gene cloned in pcDNA3.1 was transfected into RMA-S-expressing human *β2-microglobulin* gene (kindly provided by Dr. Masanori Matsui) using Nucleofector Solution T and a Nucleofector I device (Lonza Group Ltd., Basel, Switzerland) in accordance with the manufacturer's instructions. Cells that stably expressed the introduced genes were selected with G418 (0.5 mg/ml) and hygromycin B (0.35 mg/ml).

Peptide Binding Assay

Overlapped peptides based on the amino acid sequence of Narita1 nucleoprotein (GenBank GQ169303, protein ID ACR20063.1) were synthesized by the Sigma Pepscreen system (Sigma-Aldrich Co., St. Louis, MI). Each peptide shared 5 amino acids with consecutive peptides. RMA-S-expressing human *β2-microglobulin* and *Mafa-A1*052:02* genes were cultured overnight at 27°C [52,53]. Then the cells were washed twice with PBS. RMA-S (5×10^5 cells/tube) was incubated with each peptide for 30 min at 27°C and then for 4 h at 37°C. Surface Mafa-A1*052:02 molecules were detected by biotinylated W6/32 and PE-conjugated streptavidin (eBioscience). Dead cells were excluded by using propidium iodide. Expression of Mafa-A1*052:02 on the cell surface was analyzed using a flow cytometer.

Establishment of 721.221 Transfected with *Mafa-A1*052:02*

pcDNA 3.1$^+$ vector containing *Mafa-A1*052:02* gene was introduced into an MHC class I-deficient human B cell line, 721.221 (kindly provided by Dr. Masanori Matsui), by electroporation [54]. Two μg of pcDNA 3.1$^+$- Mafa-A1*052:02 was added to 721.221 (2×10^6 cells) in 100 μl of Nucleofector^{TM} Solution T and electroporated using program A-24 on a Nucleofector I device. G418-resistant cells were isolated.

Collection of Peptides from Mafa-A1*052:02 and LC-MS/MS Analysis

Mafa-A1*052:02-associated peptides were purified using a modification of a previously described protocol [21]. Briefly, one hundred sixty 75-cm^2 flasks (BD Biosciences) of 721.221 transfectants expressing Mafa-A1*052:02 were collected and washed twice with PBS. The cells were resuspended in ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES-NaOH pH 7.5). The cells were homogenized using a tight-fitting glass dounce homogenizer. The homogenate was spun at 1,000 g for 7 min at 4°C and the supernatant was retained. The pellet was further washed twice with homogenization buffer and the supernatants were added to the first harvest. After centrifugation at 12,000 g for 30 min at 4°C, the pellet was resuspended in lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% (v/v) CHAPS solution containing protease inhibitor Cocktail (Roche Ltd.)) for 1 h on ice. After centrifugation at 30,000 g for 1 h at 4°C, the supernatant was immunoaffinity purified with 1.7 mg of purified antibody W6/32 coupled with 1 ml of CNBr-activated Sepharose 4B (GE Healthcare UK Ltd., Buckinghamshire, UK) following the manufacturer's protocol. Bound peptides were eluted from Mafa-A1*052:02 with 0.2 M acetic acid (pH 2.7) and were then filtered through a Centricon 10 kDa YM-10 membrane (Millipore Corp.) at 3,500 g for 5 h at 4°C. The filtered sample was concentrated by a vacuum centrifuge, Savant SpeedVac SC-100A (Thermo Fisher Scientific Inc., Waltham, MA), and resuspended in a solution consisting of 2% acetonitrile, 0.1% trifluoroacetic acid and 98% water. The sample was injected for LC-MS/MS analysis.

A liquid chromatography system, Paradigm MG4 (AMR Inc., Tokyo, Japan), was coupled online to a linear ion trap mass spectrometer (LTQ, Thermo Electron Corp., Waltham, MA) equipped with an electrospray interface operated in positive ion mode. All MS/MS spectra were identified using SEQUEST (v.28 (revision 12), Thermo Electron Corp.).

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Author Contributions

Conceived and designed the experiments: YI YS T. Shiina HK KO. Performed the experiments: MA YI MO TM T. Shiina KT SS MN H. Ishigaki H. Ishida KS VLP. Analyzed the data: MA YI MO TM T. Shiina H. Inoko. Contributed reagents/materials/analysis tools: AT HT SN RT T. Shimizu IO. Wrote the paper: YI KO.

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ORIGINAL ARTICLE

Characterization of avian influenza viruses isolated from domestic ducks in Vietnam in 2009 and 2010

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Abstract In the surveillance of avian influenza in Vietnam, 26 H9N2, 1 H3N2, 1 H3N8, 7 H4N6, 3 H11N3, and 1 H11N9 viruses were isolated from tracheal and cloacal swab samples of 300 domestic ducks in April 2009, and 1 H9N6 virus from 300 bird samples in March 2010. Out of the 27 H9 virus isolates, the hemagglutinins of 18 strains were genetically classified as belonging to the sublineage G1, and the other nine belonged to the Korean sublineage. Phylogenetic analysis revealed that one of the 27 H9 viruses was a reassortant in which the PB2 gene belonged to the Korean sublineage and the other seven genes belonged to the G1 sublineage. Three representative H9N2 viruses were intranasally inoculated into ducks, chickens, pigs, and mice. On the basis of experimental infection studies, it was found that each of the three viruses readily infected pigs and replicated in their upper respiratory tracts, and they infected chickens with slight replication. Viruses were recovered from the lungs of mice inoculated with two of the three isolates. The present results reveal that H9 avian influenza viruses are prevailing and genetic reassortment occurs among domestic ducks in Vietnam. It is recommended that careful surveillance of swine influenza with H9 viruses should be performed to prepare for pandemic influenza.

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Introduction

Avian influenza viruses of various subtypes are circulating in poultry in Asian countries [1, 15, 20, 30, 40]. In particular, H9N2 influenza virus is present in poultry in Eurasian countries [9–11, 25]. Since H9N2 viruses were isolated from quails in Hong Kong in 1988, they have become prevalent in live-bird markets and poultry farms in Asia [8, 34]. H9N2 virus infections have greatly affected not only the poultry industry but also public health [8, 40]. The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into G1, Y280, and Korean sublineages [10]. H9N2 viruses do not usually cause severe disease in poultry, but co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, or attenuated coronavirus vaccine exacerbates the disease [12, 21]. H9N2 viruses were also isolated from domestic pigs in China [39] and Korea, and from humans with febrile respiratory illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4, 7, 23, 33, 43]. Thus, it is postulated that H9N2 virus may cause pandemic influenza in humans.

In our laboratory, avian influenza has been surveyed in Japan, Alaska, Siberia, Mongolia, and Australia since 1977

[14, 22, 27, 31, 36, 41]. The isolates were antigenically and phylogenetically analyzed and assessed for pathogenicity in birds and mammals by experimental infection [22, 27, 36, 41]. In the present study, a surveillance of avian influenza was carried out in Vietnam in domestic ducks and wild birds in 2009 and 2010, and the isolates were antigenically and phylogenetically analyzed and their pathogenicity in birds and mammals was assessed.

Materials and methods

Viruses

A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2), and A/duck/Hong Kong/W213/1998 (H9N2) of the Y280 sublineage and A/quail/Hong Kong/G1/1997 (H9N2) of the G1 sublineage were provided by Dr. K. F. Shortridge, the University of Hong Kong, China. A/turkey/Wisconsin/1/1966 (H9N2) of the North American lineage was provided by Dr. R. G. Webster, St. Jude Children's Research Hospital, United States of America. A/duck/Hokkaido/49/1998 (H9N2) and A/duck/Hokkaido/13/2000 (H9N2) of Korean sublineage were isolated from ducks under surveillance in our laboratory [27, 31]. Viruses isolated from domestic ducks in Vietnam in 2009 and 2010 were grown in 10-day-old embryonated chicken eggs, and infectious allantoic fluids were stored at −80°C until use.

Virus isolation and phylogenetic analysis

One hundred tracheal and cloacal swab samples that were viral gene positive from 600 domestic ducks and 207 wild birds (night heron, *Nycticorax nycticorax*; grey heron, *Ardea cinerea*; purple heron, *Ardea purpurea*; chinese pond heron, *Ardeola bacchus*; chinese egret, *Egretta eulophotes*; little egret, *Egretta garzetta*; intermediate egret, *Egretta intermedia*; cormorant, *Phalacrocorax carbo*; little cormorant, *Microcarbo niger*; Japanese bush warbler, *Cettia diphone*; black-browed reed warbler, *Acrocephalus bistrigiceps*; olive bulbul, *Iole virescens*; black capped kingfisher, *Halcyon pileata*; collared kingfisher, *Halcyon chloris*; racket tailed treepie, *Crypsirina temia*; oriental magpie robin, *Copsychus saularis*; tiger shrike, *Lanius tigrinus*; yellow bittern, *Ixobrychus sinensis*; indian cuckoo, *Cuculus micropterus*; common koel, *Eudynamys scolopacea*; and black collared starling, *Sturnus nigricollis*) in April 2009 and March 2010 in southern Vietnam were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. Viral RNA was detected by the reverse transcription loop-mediated isothermal amplification (RT-LAMP) method described previously [42] as a screening test for virus isolation. Viral RNAs were extracted from the allantoic fluids of chicken embryos infected with viruses by TRIzol LS Reagent (Invitrogen, CA, USA) and reverse-transcribed using the Uni12 primer [13] and M-MLV reverse transcriptase (Invitrogen). Polymerase chain reaction for amplification of the viral genes was performed using a PTC-200 thermal cycler (Bio-Rad, CA, USA). Direct sequencing of the viral genes was performed using an autosequencer CEQ 2000XL (Beckman Coulter, CA, USA). For phylogenetic analysis, sequence data for these genes together with those from public database were analyzed by the neighbor-joining method [35] using MEGA 5.0 software (http://www.megasoftware.net/). Accession numbers of the gene sequences of the isolates in the present study are as follows: AB545593, AB545594, AB639351-AB639356 (OIE-2313), AB621343, AB639024-AB639030 (OIE-2326), AB545591, AB545592, AB571519-AB571524 (OIE-2327), AB571525-AB571532 (OIE-2328), AB638754-AB638761 (OIE-2390), AB638722-AB638729 (OIE-2576), AB638746-AB638753 (OIE-2581), AB638730-AB638737 (OIE-2582), AB571533-AB571539, AB572587 (OIE-2583), AB638738-AB638745 (OIE-2584), AB638603-AB638610 (OIE-2587), AB638320-AB638327 (OIE-2592), AB638312-AB638319 (OIE-2593), and AB636530-AB636537 (OIE-2595). Subtypes of influenza virus isolates were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests using chicken antisera to the reference strains of influenza viruses [17].

Animals

Four-week-old Chelly Valley ducks were purchased from Takikawa Shinseien (Hokkaido, Japan). Four-week-old Boris brown chickens were purchased from Hokuren Co. (Hokkaido, Japan). Three-week-old crossbred (Landrace × Duroc × Yorkshire) specific-pathogen-free pigs were purchased from Yamanaka Chikusan (Hokkaido, Japan). Four-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All procedures were performed according to the animal experiment guidelines of Graduate school of Veterinary Medicine, Hokkaido University.

Experimental infection

A/duck/Vietnam/OIE-2327/2009 (Dk/VN/OIE-2327/2009), A/duck/Vietnam/OIE-2328/2009 (Dk/VN/OIE-2328/2009), and A/duck/Vietnam/OIE-2583/2009 (Dk/VN/OIE-2583/2009) were inoculated intranasally into three ducks (100 μl/duck), six chickens (100 μl/chicken), two pigs (1 ml/pig), and ten mice (30 μl/mouse) at a 50% egg infectious dose (EID_{50}) of $10^{5.8}$ EID_{50}, $10^{5.8}$ EID_{50}, $10^{6.8}$ EID_{50}, and $10^{5.0}$ EID_{50}, respectively.

After the inoculation of each influenza virus into three ducks, laryngopharyngeal and cloacal swabs were collected in minimal essential medium (MEM; Nissui, Tokyo, Japan) with antibiotics (penicillin G potassium, streptomycin sulfate, gentamicin sulfate, and nystatin) daily from 1 to 7 days post-infection (d.p.i.). All ducks were clinically observed for 14 days after inoculation with influenza viruses.

After the inoculation of each influenza virus into six chickens, three chickens were sacrificed at 3 d.p.i., and the brain, trachea, lung, and colon were collected and homogenized to make 10% (w/v) suspensions in MEM. The remaining three chickens were clinically observed for 14 days after inoculation.

After the inoculation of each influenza virus into two pigs, nasal swabs from these pigs were collected in MEM from 1 to 7 d.p.i. daily, and two pigs were clinically observed for 14 days after inoculation.

After the inoculation of each influenza virus into ten mice, five mice were sacrificed at 3 d.p.i., and the lungs were collected and homogenized to make 10% (w/v) suspensions in MEM. The other five mice were clinically observed and their body weight was monitored for 14 days after inoculation.

Virus titers in the supernatants of the swabs and the tissue homogenates were determined in 10-day-old embryonated chicken eggs and expressed as the EID_{50}/ml and g of tissue, respectively. Antibody responses to the inoculated viruses in ducks, chickens, and pigs at 14 d.p.i. were examined by HI test or enzyme-linked immunosorbent assay (ELISA) [18].

Results

Isolation of influenza viruses from domestic ducks and wild birds

In the present study, surveillance of avian influenza was carried out in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Vietnam in April 2009 and March 2010. Twelve strains (1 H3N2, 1 H3N8, 6 H4N6, 2 H9N2, 1 H11N3, and 1 H11N9) were isolated from 34 RT-LAMP-positive tracheal and cloacal swab samples from 240 domestic ducks in Vinh Loi district. Nine strains (7 H9N2 and 2 H11N3) were isolated from 38 RT-LAMP-positive swab samples from 160 domestic ducks in Bac Lieu town. Nineteen strains (1 H4N6, 17 H9N2, and 1 H9N6) were isolated from 28 RT-LAMP-positive swab samples of 200 domestic ducks in Hoa Binh district (Table 1). All of the viruses were isolated from domestic ducks in households, live-bird markets, and slaughterhouses in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Vietnam (Fig. 1). No virus was isolated from 207 wild-bird samples in April 2009 and March 2010.

Genetic characterization of viruses isolated from domestic ducks in southern Vietnam

The sequence data of the HA genes of 27 H9 isolates, including reference strains of three different sublineages, were phylogenetically analyzed by the neighbor-joining method (Fig. 2). All of the H9 HA genes were classified as belonging to the Eurasian lineage, and the HA genes of 19 and 8 isolates were grouped into the G1 and Korean sublineage, respectively.

The partial nucleotide sequence of each gene segment of the isolates was analyzed phylogenetically (Fig. 2). Gene constellations of the H9N2 virus isolates were divided into three patterns. H9N2 viruses belonging to the G1 sublineage were isolated from domestic ducks in households A and E. On the other hand, H9N2 viruses belonging to the Korean sublineage were isolated in live-bird market G. Furthermore, one of these H9N2 viruses of the G1 sublineage of the HA gene was isolated in live-bird market G, and this virus also possessed a PB2 gene of the Korean sublineage. Representative isolates of these three patterns are Dk/VN/OIE-2583/2009, Dk/VN/OIE-2327/2009, and Dk/VN/OIE-2328/2009, respectively.

Table 1 Viruses isolated from domestic ducks in southern Vietnam in 2009 and 2010

Place of sampling	Subtypes of isolates	ID number of samples
Household		
A	H4N6	OIE-2454, OIE-2455
	H9N2	OIE-2448
B	H4N6	OIE-2470, OIE-2471
C	H4N6	OIE-2480, OIE-2481
D	H4N6	OIE-2577
	H9N2	OIE-2574, OIE-2575, OIE-2576
E	H9N2	OIE-2580, OIE-2581, OIE-2582, OIE-2583, OIE-2584, OIE-2585, OIE-2586, OIE-2587, OIE-2590, OIE-2591, OIE-2592, OIE-2593, OIE-2594, OIE-2595
Live-bird market		
F	H3N8	OIE-2403
G	H9N2	OIE-2322, OIE-2323, OIE-2325, OIE-2326, OIE-2327, OIE-2328
	H11N3	OIE-2329, OIE-2336
H	H3N2	OIE-2382
	H9N2	OIE-2390
	H11N3	OIE-2391
	H11N9	OIE-2386
I	H9N6	OIE-2334[a]
Slaughter house		
J	H9N2	OIE-2313

[a] This virus was isolated in 2010

Antigenic analysis of the HAs of H9 influenza viruses

H9 influenza viruses isolated from domestic ducks in Vietnam were analyzed by the HI test (Table 2). All of the H9 isolates tested reacted with antisera against the H9 viruses of the Korean and G1 sublineages. However, the isolates of the Korean sublineage showed low cross-reactivity to antisera against the H9 viruses of the G1 sublineage, and all isolates in this study showed moderate and low cross-reactivity to antisera against the H9 viruses of the Y280 sublineage and North American lineage, respectively. This suggests that the antigenicity of the H9 isolates of the Korean sublineage is different from that of viruses of the G1 sublineage. It was also found that reactivity patterns of H9 isolates belonging to the G1 and Korean sublineage in the present study were the same as those of the reference strains.

Susceptibility of ducks, chickens, pigs, and mice to infection with H9N2 isolates

H9N2 isolates were inoculated intranasally into ducks, chickens, pigs, and mice. Clinical signs were not observed during 14 days in any of the ducks. Viruses were recovered from laryngopharyngeal swabs from duck #1 inoculated with DK/VN/OIE-2327/2009 at 3 and 4 d.p.i. Viruses were also recovered from a laryngopharyngeal swab from duck #5 inoculated with Dk/VN/OIE-2328/2009 at 3 d.p.i. In the experimental infection with Dk/VN/OIE-2583/2009, viruses were recovered from a cloacal swab from duck #7 at 5 d.p.i. and laryngopharyngeal swabs from duck #8 at 1 and 3 d.p.i. Antibodies to H9 HA were detected from the sera of all ducks at 14 d.p.i (Table 3).

Fig. 2 Phylogenetic trees for the eight gene segments of H9 influenza viruses. Nucleotides 70-417 (347 bp) of HA, 67-468 (402 bp) of NA, 1,318-1,902 (585 bp) of PB2, 1,135-1,610 (476 bp) of PB1, 756-1,167 (412 bp) of PA, 1,139-1,434 (296 bp) of NP, 55-893 (839 bp) of M, and 25-790 (766 bp) of NS were used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels in bootstrap analysis with 1,000 replications. Viruses isolated in this study are highlighted in gray. Representative viruses in each sublineage are underlined. Abbreviations: Ck, chicken; Dk, duck; Qa, quail; Ty, turkey; HK, Hong Kong; Hok, Hokkaido; Pak, Pakistan; and Wis, Wisconsin. **a** The nucleotide sequences of the HA genes of 11 isolates were the same as that of Dk/VN/OIE-2587/2009. The ID numbers of these 10 isolates are OIE-2448, OIE-2574, OIE-2575, OIE-2580, OIE-2581, OIE-2585, OIE-2586, OIE-2590, OIE-2591, and OIE-2594. **b** The nucleotide sequences of the HA genes of two isolates were the same as that of Dk/VN/OIE-2390/2009. The ID numbers of these two isolates are OIE-2322 and OIE-2323

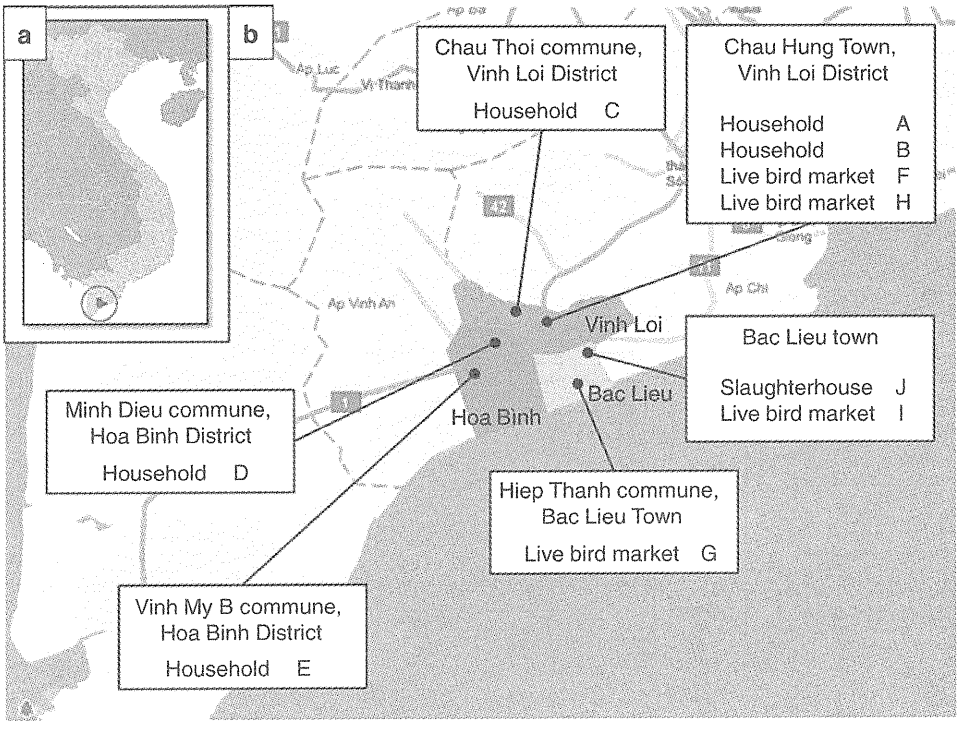
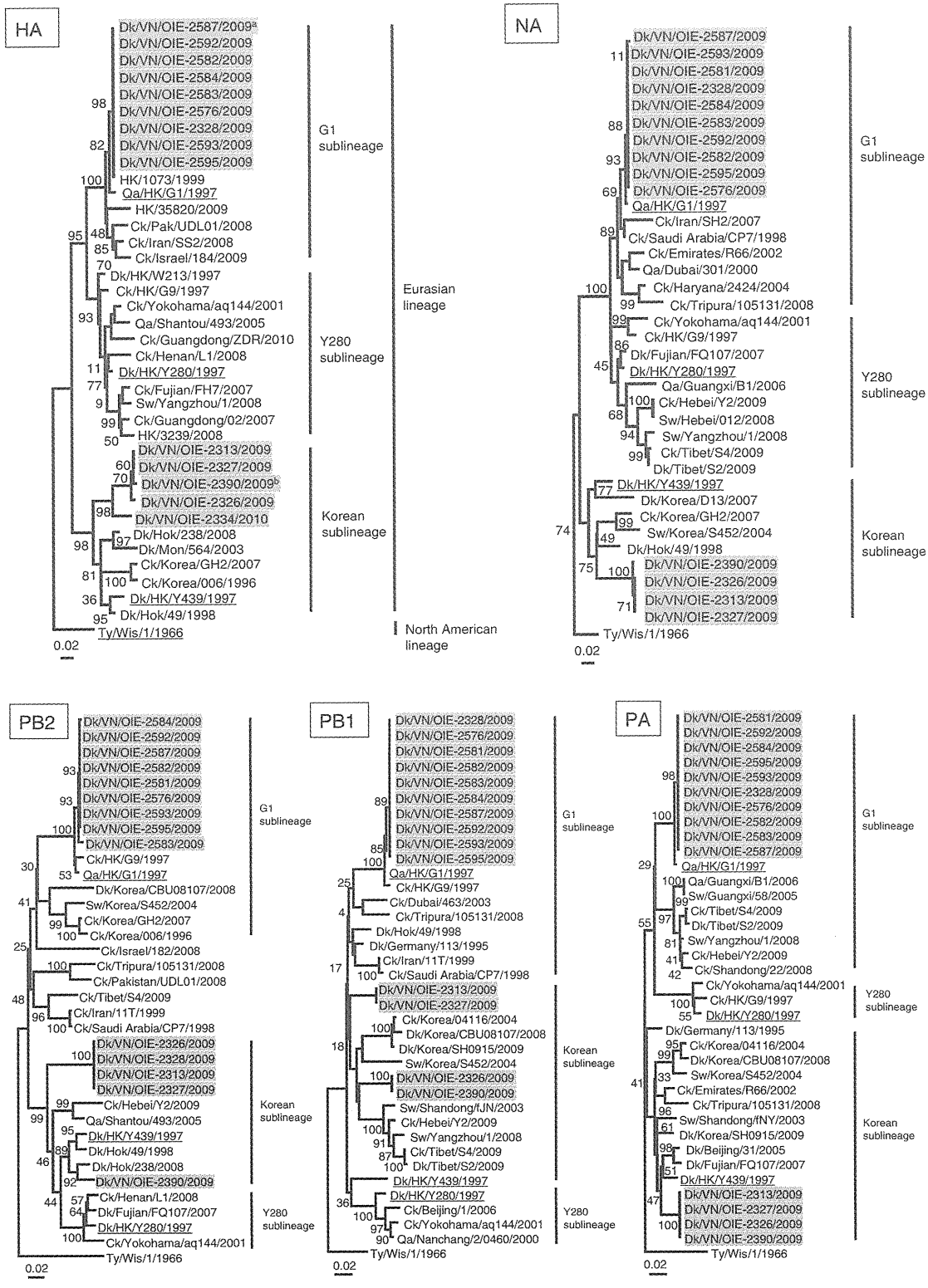


Fig. 1 Sampling points in southern Vietnam in the present study. Location of Bac Lieu province in Vietnam (**a**). Magnification of the circle in Fig. 1a and sampling points in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Bac Lieu province (**b**). Avian influenza viruses were isolated from domestic ducks in households A-E, live-bird markets F-I, and slaughterhouse J in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Vietnam


HA
Dk/VN/OIE-2587/2009[a]
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2595/2009
HK/1073/1999
Qa/HK/G1/1997
HK/35820/2009
Ck/Pak/UDL01/2008
Ck/Iran/SS2/2008
Ck/Israel/184/2009
Dk/HK/W213/1997
Ck/HK/G9/1997
Ck/Yokohama/aq144/2001
Qa/Shantou/493/2005
Ck/Guangdong/ZDR/2010
Ck/Henan/L1/2008
Dk/HK/Y280/1997
Ck/Fujian/FH7/2007
Sw/Yangzhou/1/2008
Ck/Guangdong/02/2007
HK/3239/2008
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Dk/VN/OIE-2390/2009[b]
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2334/2010
Dk/Hok/238/2008
Dk/Mon/564/2003
Ck/Korea/GH2/2007
Ck/Korea/006/1996
Dk/HK/Y439/1997
Dk/Hok/49/1998
Ty/Wis/1/1966
0.02
G1 sublineage
Y280 sublineage
Korean sublineage
Eurasian lineage
North American lineage
NA
Dk/VN/OIE-2587/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2595/2009
Dk/VN/OIE-2576/2009
Qa/HK/G1/1997
Ck/Iran/SH2/2007
Ck/Saudi Arabia/CP7/1998
Ck/Emirates/R66/2002
Qa/Dubai/301/2000
Ck/Haryana/2424/2004
Ck/Tripura/105131/2008
Ck/Yokohama/aq144/2001
Ck/HK/G9/1997
Dk/Fujian/FQ107/2007
Dk/HK/Y280/1997
Qa/Guangxi/B1/2006
Ck/Hebei/Y2/2009
Sw/Hebei/012/2008
Sw/Yangzhou/1/2008
Ck/Tibet/S4/2009
Dk/Tibet/S2/2009
Dk/HK/Y439/1997
Dk/Korea/D13/2007
Ck/Korea/GH2/2007
Sw/Korea/S452/2004
Dk/Hok/49/1998
Dk/VN/OIE-2390/2009
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Ty/Wis/1/1966
0.02
G1 sublineage
Y280 sublineage
Korean sublineage
PB2
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2587/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2595/2009
Dk/VN/OIE-2583/2009
Ck/HK/G9/1997
Qa/HK/G1/1997
Dk/Korea/CBU08107/2008
Sw/Korea/S452/2004
Ck/Korea/GH2/2007
Ck/Korea/006/1996
Ck/Israel/182/2008
Ck/Tripura/105131/2008
Ck/Pakistan/UDL01/2008
Ck/Tibet/S4/2009
Ck/Iran/11T/1999
Ck/Saudi Arabia/CP7/1998
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Ck/Hebei/Y2/2009
Qa/Shantou/493/2005
Dk/HK/Y439/1997
Dk/Hok/49/1998
Dk/Hok/238/2008
Dk/VN/OIE-2390/2009
Ck/Henan/L1/2008
Dk/Fujian/FQ107/2007
Dk/HK/Y280/1997
Ck/Yokohama/aq144/2001
Ty/Wis/1/1966
0.02
G1 sublineage
Korean sublineage
Y280 sublineage
PB1
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2587/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2595/2009
Qa/HK/G1/1997
Ck/HK/G9/1997
Ck/Dubai/463/2003
Ck/Tripura/105131/2008
Dk/Hok/49/1998
Dk/Germany/113/1995
Ck/Iran/11T/1999
Ck/Saudi Arabia/CP7/1998
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Ck/Korea/04116/2004
Dk/Korea/CBU08107/2008
Dk/Korea/SH0915/2009
Sw/Korea/S452/2004
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2390/2009
Sw/Shandong/fJN/2003
Ck/Hebei/Y2/2009
Sw/Yangzhou/1/2008
Ck/Tibet/S4/2009
Dk/Tibet/S2/2009
Dk/HK/Y439/1997
Dk/HK/Y280/1997
Ck/Beijing/1/2006
Ck/Yokohama/aq144/2001
Qa/Nanchang/2/0460/2000
Ty/Wis/1/1966
0.02
G1 sublineage
Korean sublineage
Y280 sublineage
PA
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2595/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2587/2009
Qa/HK/G1/1997
Qa/Guangxi/B1/2006
Sw/Guangxi/58/2005
Ck/Tibet/S4/2009
Dk/Tibet/S2/2009
Sw/Yangzhou/1/2008
Ck/Hebei/Y2/2009
Ck/Shandong/22/2008
Ck/Yokohama/aq144/2001
Ck/HK/G9/1997
Dk/HK/Y280/1997
Dk/Germany/113/1995
Ck/Korea/04116/2004
Dk/Korea/CBU08107/2008
Sw/Korea/S452/2004
Ck/Emirates/R66/2002
Ck/Tripura/105131/2008
Sw/Shandong/fNY/2003
Dk/Korea/SH0915/2009
Dk/Beijing/31/2005
Dk/Fujian/FQ107/2007
Dk/HK/Y439/1997
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2390/2009
Ty/Wis/1/1966
0.02
G1 sublineage
Y280 sublineage
Korean sublineage

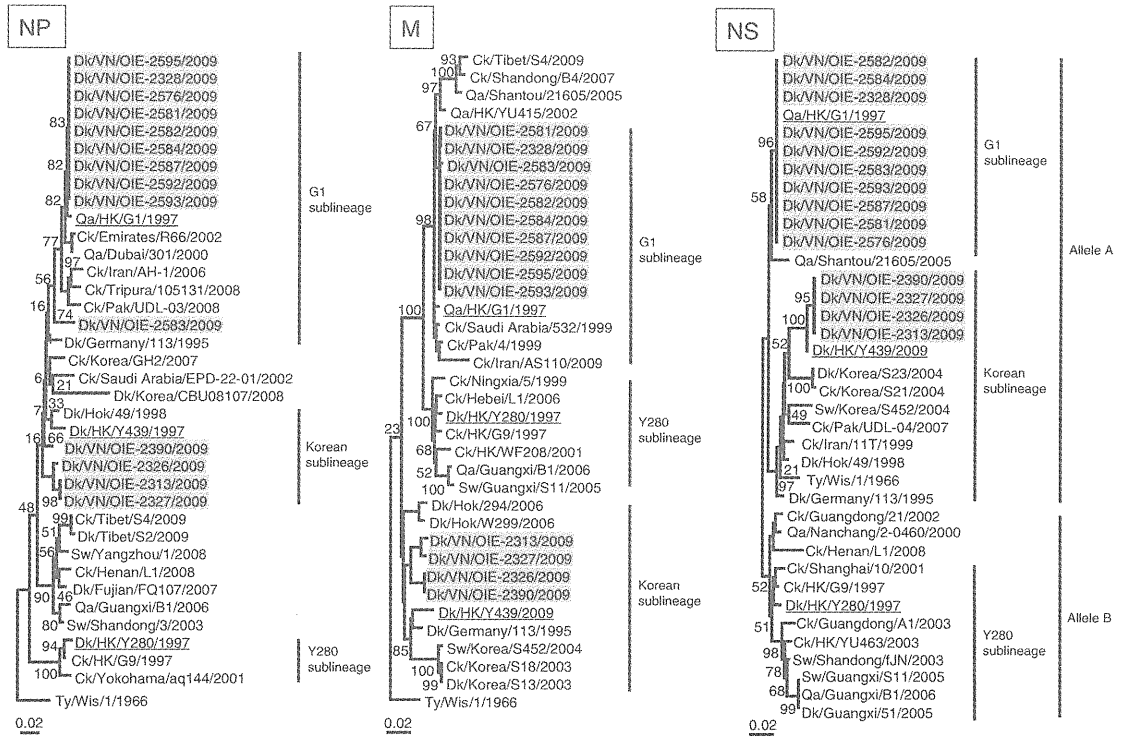


Fig. 2 continued

Clinical signs were not observed during the experiments in any of the chickens. Virus was not recovered from the brains, tracheas, lungs, or colons of chickens inoculated with the three H9N2 viruses at 3 d.p.i., while anti-H9 HA antibodies were detected in the sera of the chickens on 14 d.p.i. (data not shown), indicating that virus replication had occurred at a low level.

Clinical signs were not observed during the experiment in any of the pigs. Viruses were recovered from the nasal swabs of pigs inoculated with each of the three H9N2 isolates, and anti-H9 HA antibodies were detected in the sera of pigs at 14 d.p.i (Table 4). Antibodies were not detected in the sera of pig #1 inoculated with Dk/VN/OIE-2327/2009, indicating that the pig was not infected with influenza viruses.

Body weight fell in mice inoculated with Dk/VN/OIE-2583/2009, with 15-20% loss from 4 to 8 d.p.i (Fig. 3). Viruses were recovered from the lungs of mice inoculated with Dk/VN/OIE-2328/2009 and Dk/VN/OIE-2583/2009 at 3 d.p.i. (Table 5), and anti-H9 HA antibodies were detected in the sera of all mice at 14 d.p.i (Table 6).

Discussion

Recently, H9N2 viruses of the G1, Y280, and Korean sublineages have been isolated from wild birds and poultry worldwide [2, 3, 8, 29, 40]. H9N2 viruses have been isolated from pigs and humans in China [4, 39] and Korea, suggesting that the H9N2 virus is a candidate to cause pandemic influenza in humans. Live-bird markets provide an ideal environment for genetic reassortment and interspecies transmission of influenza viruses [24, 26, 28, 38]. In Asia, H9N2 influenza viruses had been isolated only from feral ducks until 1988 [37], but since then, H9N2 viruses have been isolated from domestic ducks and chickens [5]. H9N3 viruses belonging to the Korean sublineage have been isolated from domestic ducks in Vietnam [30]. In the present study, it was found that H9 viruses belonging to the Korean and G1 sublineages are circulating in domestic ducks in Vietnam, and one of these H9N2 viruses, belonging to the G1 sublineage but possessing the PB2 gene of the Korean sublineage, was isolated from domestic ducks. Thus, genetic reassortment has occurred between viruses of the G1 and Korean sublineages in the poultry population in Vietnam.

In this study, H9N2 viruses did not replicate well in chickens and ducks. It has been reported that H9N2 viruses isolated from ducks replicate slightly in chickens [34], suggesting that the similar results in this study were due to the low susceptibility of chickens to H9N2 viruses. It has also been reported that H9N2 viruses isolated from ducks replicate in only some of the organs in ducks, and viruses of low titer are recovered from tracheal and cloacal swabs [11, 32]. The present results were similar to those of the previous reports. In this animal experiment, we collected

Table 2 Cross-reactivity between antisera and H9 viruses by HI test[a]

Lineage	Sublineage	Virus	Antiserum to					
			Korean		G1	Y280		North American
			Dk/Hok/49/1998	Dk/Hok/13/2000	Qa/HK/G1/1997	Dk/HK/Y280/1997	Ck/HK/G9/1997	Ty/Wis/1/1966
Eurasian	Korean	Dk/Hok/49/1998	2,560	2,560	80	320	320	640
		Dk/Hok/13/2000	5,120	2,560	40	320	320	320
		Dk/VN/OIE-2313/2009	640	2,560	640	640	320	320
		Dk/VN/OIE-2322/2009	1,280	1,280	320	320	320	160
		Dk/VN/OIE-2323/2009	640	1,280	640	640	320	320
		Dk/VN/OIE-2325/2009	640	1,280	640	640	160	320
		Dk/VN/OIE-2326/2009	1,280	2,560	640	320	320	160
		Dk/VN/OIE-2327/2009	640	1,280	320	640	320	320
		Dk/VN/OIE-2390/2009	1,280	1,280	320	640	320	320
		Dk/VN/OIE-2334/2010	5,120	5,120	320	640	320	640
	G1	Qa/HK/G1/1997	1,280	1,280	5,120	1,280	640	320
		Dk/VN/OIE-2328/2009	1,280	1,280	10,240	5,120	1,280	320
		Dk/VN/OIE-2448/2009	1,280	1,280	5,120	1,280	1,280	640
		Dk/VN/OIE-2574/2009	1,280	2,560	2,560	5,120	1,280	160
		Dk/VN/OIE-2575/2009	1,280	2,560	5,120	2,560	1,280	320
		Dk/VN/OIE-2576/2009	640	1,280	5,120	2,560	1,280	160
		Dk/VN/OIE-2580/2009	1,280	2,560	2,560	2,560	640	320
		Dk/VN/OIE-2581/2009	1,280	1,280	5,120	2,560	640	320
		Dk/VN/OIE-2582/2009	640	1,280	2,560	1,280	1,280	160
		Dk/VN/OIE-2583/2009	1,280	2,560	5,120	2,560	1,280	320
		Dk/VN/OIE-2584/2009	1,280	640	1,280	1,280	1,280	160
		Dk/VN/OIE-2585/2009	2,560	1,280	2,560	1,280	640	160
		Dk/VN/OIE-2586/2009	1,280	1,280	2,560	2,560	1,280	160
		Dk/VN/OIE-2587/2009	1,280	2,560	5,120	5,120	640	160
		Dk/VN/OIE-2590/2009	2,560	2,560	1,280	1,280	320	160
		Dk/VN/OIE-2591/2009	1,280	2,560	1,280	1,280	640	320
		Dk/VN/OIE-2592/2009	640	1,280	5,120	1,280	640	320
		Dk/VN/OIE-2593/2009	640	640	2,560	1,280	320	160
		Dk/VN/OIE-2594/2009	1,280	1,280	2,560	5,120	1,280	160
		Dk/VN/OIE-2595/2009	1,280	1,280	2,560	2,560	1,280	320
	Y280	Dk/HK/Y280/1997	2,560	5,120	5,120	20,480	20,480	40
		Ck/HK/G9/1997	1,280	2,560	2,560	10,240	40,960	320
		Dk/HK/W213/1998	1,280	2,560	2,560	20,480	40,960	80
North American		Ty/Wis/1/1966	320	320	<20	20	80	640

[a] Homologous reactions are underlined

laryngopharyngeal swabs because it was hard to collect tracheal swabs daily from the ducks inoculated with H9N2 viruses in the safety cabinet. Furthermore, the strain of ducks used in experimental infection (Chelly Valley) may not be identical to that of domestic ducks in Vietnam. These factors might affect the titer of recovered virus.

In mice, H9N2 viruses replicate in the lungs, and body weight losses are observed [16, 28]. In this experiment, viruses replicated efficiently in the lungs of mice inoculated with Dk/VN/OIE-2328/2009 and Dk/VN/OIE-2583/2009. Body weight losses were observed in the mice inoculated with Dk/VN/OIE-2583/2009 and not in those with Dk/VN/OIE-2328/2009, indicating that Dk/VN/OIE-2583/2009 replicated more efficiently than Dk/VN/OIE-2328/2009 at the early stage of infection in mice. The genetic analysis suggested that the PB2 genes may be responsible for the higher replication rate in mice, since the sublineages of the PB2 gene are different in these two

Table 3 Virus titers of the laryngopharyngeal and cloacal swabs and antibody responses of ducks inoculated with H9N2 viruses[a]

Virus	Animal no.	Swab	Virus titer on the following d.p.i.[b] (log EID_{50}/ml)								Serum antibody titer[c]
			0	1	2	3	4	5	6	7	
Dk/VN/ OIE-2327/2009	#1	Laryngopharyngeal	–	–	–	0.7	1.5	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	
	#2	Laryngopharyngeal	–	–	–	–	–	–	–	–	160
		Cloacal	–	–	–	–	–	–	–	–	
	#3	Laryngopharyngeal	–	–	–	–	–	–	–	–	160
		Cloacal	–	–	–	–	–	–	–	–	
Dk/VN/ OIE-2328/2009	#4	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	
	#5	Laryngopharyngeal	–	–	–	0.8	–	–	–	–	160
		Cloacal	–	–	–	–	–	–	–	–	
	#6	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	
Dk/VN/ OIE-2583/2009	#7	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	0.7	–	–	
	#8	Laryngopharyngeal	–	1.7	–	1.3	–	–	–	–	40
		Cloacal	–	–	–	–	–	–	–	–	
	#9	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	

[a] Laryngopharyngeal and cloacal swabs of three inoculated ducks were collected daily from 1 to 7 d.p.i

[b] Bar (–) indicate that virus was not detected

[c] HI antibody titers to the inoculated viruses at 14 d.p.i

Table 4 Virus isolation from nasal swabs of pigs inoculated with H9N2 viruses[a]

Virus	Animal no.	Virus titer on the following d.p.i. (log EID_{50}/ml)								Serum antibody titer
		0	1	2	3	4	5	6	7	
Dk/VN/OIE-2327/2009	#1	-	-	-	-	-	-	-	-	-
	#2	-	-	2.7	1.5	2.5	2.7	1.5	-	640
Dk/VN/OIE-2328/2009	#3	-	-	3.8	-	5.3	4.3	2.7	1.7	320
	#4	-	3.5	3.5	1.8	4.3	4.5	3.5	3.0	640
Dk/VN/OIE-2583/2009	#5	-	5.0	3.5	2.8	4.3	3.8	2.8	1.5	320
	#6	-	4.3	3.8	3.7	4.8	3.8	1.8	-	320

[a] Nasal swabs of two inoculated pigs were collected daily from 1 to 7 d.p.i., -:< 0.5. Antibody responses to the inoculated viruses in pigs at 14 d.p.i. were examined by ELISA

viruses. Further study is needed to clarify the pathogenicity of H9N2 viruses in mice.

Experimental infection studies revealed that pigs are highly susceptible to infection with avian influenza viruses of each of the known HA subtypes, and genetic reassortment can take place in pigs [19]. Thus, pigs have been suggested to serve as intermediate hosts to generate genetic reassortants [19]. Three H9N2 viruses were recovered from swabs from pigs in this experiment, and the results were similar to those of previous reports [6, 19]. Especially, viruses were recovered efficiently from nasal swabs from pigs inoculated with Dk/VN/OIE-2328/2009 and Dk/VN/OIE-2583/2009, which belong to the G1 sublineage and replicate efficiently in mice. In addition, H9N2 viruses isolated from humans in Hong Kong were genetically classified as belonging to the G1 sublineage [4, 7, 23, 33, 43], suggesting that H9N2 viruses belonging to the G1 sublineage have the potential to replicate efficiently in mammals. The findings indicate that H9N2 virus is one of the candidates for pandemic influenza in humans. Surveillance of influenza in wild birds, domestic birds, and pigs is important in order to prepare for pandemic influenza in humans.

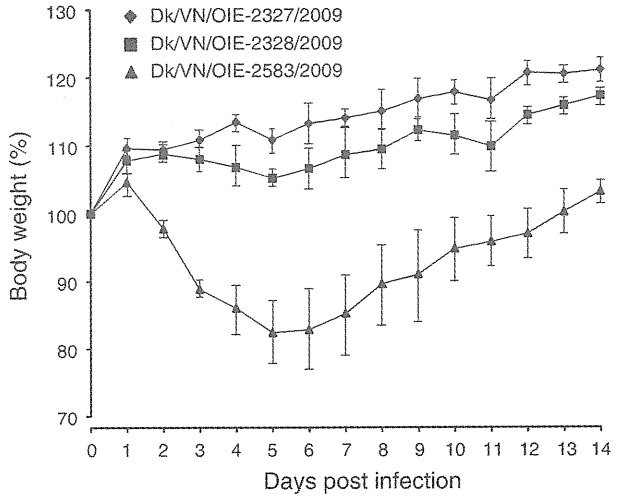


Fig. 3 Changes in body weight of mice inoculated with influenza viruses. Five mice were inoculated intranasally with Dk/VN/OIE-2327/2009, Dk/VN/OIE-2328/2009, and Dk/VN/OIE-2583/2009. The body weight of the mice was monitored for 14 days after inoculation with each influenza virus. Data are shown as averages of body weight changes in each group with the corresponding standard deviation

Table 5 Virus titers of the lungs of mice inoculated with H9N2 viruses

Virus	Animal no.	Virus titer[a] (log EID_{50}/g)
Dk/VN/OIE-2327/2009	#1	–
	#2	–
	#3	–
	#4	–
	#5	–
Dk/VN/OIE-2328/2009	#6	5.3
	#7	5.3
	#8	5.3
	#9	5.0
	#10	5.7
Dk/VN/OIE-2583/2009	#11	5.5
	#12	5.7
	#13	5.8
	#14	5.5
	#15	5.3

[a] Five mice were sacrificed at 3 d.p.i., and the lungs were collected for virus titration. –: virus was not detected

Table 6 Serum antibody responses of mice inoculated with H9N2 viruses

Virus	Animal no.	Serum antibody titer[a]
Dk/VN/OIE-2327/2009	#16	40
	#17	40
	#18	80
	#19	40
	#20	40
Dk/VN/OIE-2328/2009	#21	20
	#22	80
	#23	20
	#24	40
	#25	40
Dk/VN/OIE-2583/2009	#26	40
	#27	40
	#28	40
	#29	80
	#30	80

[a] HI antibody titer to the inoculated viruses at 14 d.p.i

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