Studies on the control of avian influenza virus infections in poultry and humans

家禽とヒトにおける鳥インフルエンザウイルス感染の制御に関する研究

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Abbreviations

AI	avian influenza
AIV(s)	avian influenza virus(es)
CI	confidence interval
DAH	Department of Animal Health, Vietnam
FAO	Food and Agriculture Organization of the United Nations
HA	hemagglutinin
HI	hemagglutination-inhibition
HPAI	highly pathogenic avian influenza
HPAIV(s)	highly pathogenic avian influenza virus(es)
KAP	knowledge, attitude, and practices
LBM(s)	live bird market(s)
LPAIV(s)	low pathogenic avian influenza virus(es)
MAbs	monoclonal antibodies
MDCK	Madin–Darby canine kidney
ML	maximum-likelihood
NA	neuraminidase
NCVD	National Center for Veterinary Diagnostics, Vietnam
NI	neuraminidase-inhibition
OIE	World Organization for Animal Health
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PPE	personal protective equipment
RT-PCR	reverse transcription polymerase chain reaction
SDAH	Sub-Department of Animal Health
SE	standard error
VAHIP	Vietnam Avian and Human Influenza Control and Preparedness Project
VI	virus isolation
WHO	World Health Organization

Notes

Contents of the present thesis were published in the following articles.

1. **Chu D-H, Okamatsu M, Matsuno K, Hiono T, Ogasawara K, Nguyen LT, Van Nguyen L, Nguyen TN, Nguyen TT, Van Pham D.** 2016. Genetic and antigenic characterization of H5, H6 and H9 avian influenza viruses circulating in live bird markets with intervention in the center part of Vietnam. Veterinary Microbiology **192:**194-203.

2. **Chu D-H, Stevenson M, Nguyen LV, Isoda N, Firestone S, Nguyen TN, Nguyen LT, Matsuno K, Okamatsu M, Kida H, Sakoda Y.** 2017. A cross-sectional study to quantify the prevalence of avian influenza viruses in poultry at intervention and non-intervention live bird markets in central Vietnam, 2014. Transboundary and Emerging Diseases, doi: 10.1111/tbed.12605.

3. **Chu D-H, Sakoda Y, Nishi T, Hiono T, Shichinohe S, Okamatsu M, Kida H.** 2014. Potency of an inactivated influenza vaccine prepared from A/duck/Mongolia/119/2008 (H7N9) against the challenge with A/Anhui/1/2013 (H7N9). Vaccine **32:**3473-3479.

Preface

Influenza A viruses are zoonotic pathogens that are widely distributed among mammalian hosts such as humans, pigs, and horses, as well as avian species such as chickens, ducks, many other poultry and wild birds [1, 2]. These viruses belong to genus *Influenzavirus A* of family *Orthomyxoviridae* [3]. Influenza A viruses are classified into subtypes on the basis of the antigenic specificity of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins; they are classified into 16 HA (H1−H16) subtypes and 9 NA (N1−N9) subtypes. Influenza A viruses infecting chickens are categorized into two pathotypes based on their virulence to chickens; namely, highly pathogenic avian influenza virus (HPAIV) and low pathogenic avian influenza virus (LPAIV). The HAs of HPAIVs have at least a pair of di-basic amino acid residues at their cleavage site, which permits cleavage activation by ubiquitous proteases such as furin and PC6, leading to systemic infection in chickens [4, 5]. In a different way, the HAs of LPAIVs are cleaved only by trypsin-like proteases expressed in the respiratory and intestinal tracts, causing local infection in chickens [6]. In ducks, the viruses replicate in the columnar epithelial cells which form crypts in the colon of ducks without showing clinical signs and are excreted in feces [7]. It is known that the pathogenicity of influenza A viruses for chickens ranges from asymptomatic to systemic infections with low to high mortality and HA subtypes of HPAIVs are mostly restricted to H5 and H7 [6]. Since late 2003, H5N1 HPAIVs have seriously affected poultry in the world [8]. HPAIVs are generated when non-pathogenic viruses circulating among water birds are transmitted to chickens via domestic water and terrestrial birds, where they acquire pathogenicity in chickens via multiple infections and replication in the chicken population [1]. In general, HPAIVs are less pathogenic to

ducks compared with chickens [9]. Thus, it has been widely postulated that ducks play crucial roles in the widespread dissemination of HPAIVs in poultry.

In the previous study, an active surveillance of avian influenza (AI) was conducted in the domestic bird in Vietnam from 2008 to 2012 by World Organization for Animal Health (OIE) Tokyo and the OIE Reference Laboratory for Highly Pathogenic Avian Influenza and Low Pathogenic Avian Influenza in Hokkaido University. The results of surveillance program showed that the prevalence of avian influenza viruses (AIVs) was substantially higher in live bird markets (LBMs) than in backyard farms, and H5N1 viruses were isolated from apparently healthy domestic ducks and chickens in the LBMs [10, 11]. The findings suggest that in Vietnam, common LBMs are more responsible for the amplification, maintenance, circulation, and transmission of AIVs than backyard farms. It was reported that closing LBMs effectively reduced the risk of virus transmission to the public [12-14]. However, banning LBMs in developing countries remains challenging because changing the traditional market style should take a long time. Therefore, government intervention to improve the biosecurity and hygiene measures employed at LBMs has been thought to represent a promising strategy to minimize the transmission of viruses in Asian countries including Vietnam. In the present study, surveillance of AIVs was conducted in Vietnamese LBMs but focusing on the evaluation of AIV prevalence in two phenotypes of LBMs, with intervention and non-intervention from 2013 to 2015. The results of antigenic and genetic analyses of the isolates of H5, H6, and H9 subtypes through the surveillance were described in Chapter I. Genetic diversity and antigenic stability of these viruses are also discussed. The present results should provide a better understanding of the AIVs circulating in LBMs and the efficacy of current control measures which applying in LBMs.

In Chapter II, the data of Chapter I were used for epidemiology study to identify characteristics associated with the presence of AIVs in poultry submitted for sale at intervention and non-intervention LBMs. The results demonstrate the relative importance of factors influencing the poultry submitted for sale at LBMs is a critical first step towards the design of evidence-based better measures to reduce the number of AIV positive birds (and therefore the risk of virus infection) within LBMs.

In March 2013, the 1st case of H7N9 influenza virus infection in humans was reported in China. Afterward, the number of human cases and deaths has continued to increase in China even though LBM closure had been conducted in real H7N9 outbreaks [15]. In addition, humans are immunologically naïve to the H7N9 subtype, for which the seasonal influenza vaccine is not effective. Therefore, the development of an H7N9 influenza virus vaccine is an urgent issue [16]. In Chapter III, it is described that H7 virus strains stocked in the influenza virus library in our laboratory were analyzed antigenically and phylogenetically to select a proper vaccine strain in case of an influenza pandemic. A/duck/Mongolia/119/2008 (H7N9) was selected and inactivated whole virus vaccine was prepared. The efficacy of the vaccine against the challenge with A/Anhui/1/2013 (H7N9) was assessed in mice.

Chapter I

Genetic and antigenic characterization of H5, H6, and H9 avian influenza viruses circulating in live bird markets with intervention in central Vietnam

Introduction

Transmission of AIVs among wild and domestic birds is an important target of control measures aiming to minimize the risk of influenza infection to both human and animal health worldwide [10, 11, 17]. Vietnam has a large population of poultry (approximately 308 million), and a majority of these animals (approximately 80%) are raised under backyard conditions at households in rural areas without biosecurity application [18]. In Vietnam, H5N1 HPAIVs have caused a large number of outbreaks in poultry since 2003 [19]. In our previous study, we conducted surveillance of AIVs in LBMs and households that raise poultry in a large number of Vietnamese provinces in 2009. We found that the prevalence of AIVs was substantially higher in LBMs than in backyard farms, and H5N1 viruses were isolated from apparently healthy domestic ducks and chickens in LBMs [10, 11]. The findings suggest that in Vietnam, LBMs are more responsible for the amplification, maintenance, circulation, and transmission of AIVs than backyard farms. Therefore, continuous surveillance of AIVs in LBMs in Vietnam is essential to understand the distribution of AIVs and to minimize the risk to public and animal health.

LBMs are ubiquitous and integral parts of the poultry industry in Vietnam and other developing countries in Asia [20, 21]. In China, LBMs have become a major source of human infection with H5 HPAIVs and H7 LPAIVs [13, 14, 21]. When keeping live birds overnight in LBMs was banned, the rate of virus isolation from birds declined [22]. In addition, it was reported that closing LBMs effectively reduced the risk of virus transmission to the public [12-14]. However, banning LBMs in developing countries remains challenging because changing the traditional market style should take a long time. Therefore, government intervention to improve the biosecurity measures employed at LBMs has been thought to represent a promising strategy to minimize the

transmission of viruses in Asian countries including Vietnam. To support for the evaluation of local authority on the current intervention, we surveyed the prevalence of AIVs at nine LBMs with or without the intervention of Vietnam Avian and Human Influenza Control and Preparedness Project (VAHIP) in Thua Thien Hue province, located in the central region of Vietnam. The VAHIP was a project funded by the World Bank aiming to reduce the risk of AIV transmission to humans [23]. The project developed the new model of LBM in selected provinces in Vietnam by deploying infrastructure for the poultry markets and operating periodic disinfection in these intervention LBMs. In this study, various subtypes of AIVs were isolated during surveillance of LBMs with or without intervention in August and December, 2014. The representative isolates were phylogenetically and antigenically analyzed to characterize the genetic and antigenic variation of the AIVs circulating in LBMs in Vietnam.

Materials and methods

Sample collection

The surveillance was conducted in August and December, 2014. Oropharyngeal, cloacal swabs and fecal samples from domestic birds and water troughs (environmental samples) were collected at nine LBMs (Figure 1). At three of the nine LBMs, biosecurity infrastructures had been established by the VAHIP program in Thua Thien Hue province, Vietnam (intervention LBMs; Figure 1a and Table 1). The other six LBMs were conventional markets at which no particular biosecurity infrastructure was established and at which poultry and other animals were usually mixed together in low biosecurity conditions (non-intervention LBMs; Figure 1b and Table 1).

All collected samples were stored in sterile tubes with transport medium (minimum essential medium, Nissui, Japan) containing 10,000 U/ml penicillin G (Meiji Seika, Japan), 10 mg/ml streptomycin (Meiji Seika, Japan), 0.3 mg/ml gentamicin (Schering Plough, USA), 250 U/ml nystatin (Sigma, USA), and 0.5% bovine serum albumin fraction V (Roche, Switzerland) at −80°C until use.

Isolation and identification of AIVs

Samples were resuspended in virus transport medium and centrifuged at 2,000 rpm for 5 min. The supernatant was inoculated into the allantoic cavity of a 10-day-old chicken embryo. After incubation at 35°C for 30–48 h, allantoic fluids exhibiting hemagglutination activity were collected for subtyping of influenza viruses by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests with antisera to the reference influenza virus strains [24].

a)

b)

Figure 1. Representative pictures of the intervention LBM and non-intervention LBM in Thua Thien Hue province, Vietnam.

a) Intervention LBM was deployed by VAHIP project, which has a good infrastructure with the biosecurity equipments. Only poultry with high density was gathered for selling in intervention LBM. **b)** Non-intervention LBM is the conventional market where no particular biosecurity infrastructure was established and at where poultry and other animals were usually mixed together in low biosecurity condition.

Table 1. Viruses isolated from LBMs in Thua Thien Hue province, Vietnam in 2014

Type of LBMs	Name of LBMs	Latitude/Longitude	Number of samples	Number of isolates	Subtype of isolates (number of isolates)
Intervention	An Lo	16.545962/107.452388	500	18	H3N2 (1) H4N6 (1) H6N6 (1) H9N2 (10) H9N6 (2) H11N7 (3)
	No	16.511793/107.600336	500	61	H3N2 (4) H3N6 (1) H5N6 (8) H6N2 (9) H6N6 (3) H9N2 (36)
	Thuy Phuong	16.433257/107.635256	496	13	H3N2 (2) H4N6 (1) H6N2 (1) H6N6 (5) H9N2 (3) H11N6 (1)
Non-intervention	Phu Bai	16.408326/107.677989	300	40	H6N2 (3) H6N6 (1) H9N2 (35) H11N7 (1)
	Phu Da	16.434797/107.710014	200	1	H3N2 (1)
	Than Phu	16.424635/107.656268	199	12	H3N2 (1) H6N6 (5) H9N2 (3) H9N6 (3)
	Quang Phuoc	16.574310/107.519840	212	6	H9N2 (6)
	Tay Ba	16.537018/107.560679	338	25	H3N2 (9) H6N2 (1) H9N2 (15)
	Vinh Thanh	16.431796/107.783475	300	2	H6N6 (1) H9N2 (1)

Sequencing and phylogenetic analysis

Viral RNA was extracted from the 250 µl of allantoic fluids by TRIzol LS Reagent (Life Technologies, USA) following the manufacturer's protocol and reverse transcribed with the Uni12 primer [25] and M-MLV Reverse Transcriptase (Life Technologies, USA). Full-length cDNAs of the eight gene segments were amplified by polymerase chain reaction (PCR) with Ex-Taq (TaKaRa, Shiga, Japan) and gene-specific primer sets [25]. Direct sequencing of each gene segment was performed using 3500 Genetic Analyzer (Life Technologies, USA).

For phylogenetic analysis, nucleotide sequences of the isolates, together with those from a public database, were aligned using Clustal W. Phylogenetic trees were constructed using the maximum-likelihood (ML) method with 1,000 bootstrap replicates using MEGA 5.0 software [26]. The genome sequences identified in this study have been registered in GenBank/EMBL/DDBJ (Table 2).

Antigenic analysis

Polyclonal antisera were prepared from chickens immunized with reference AIV strains that had been inactivated with formalin [24]. Antigenic analysis of H5, H6 and H9 viruses was performed using polyclonal antisera by HI test.

Pathogenicity of an H5N6 AIV in chickens

To assess the pathogenicity of the representative H5N6 virus in chickens, 0.2 ml of the 1:10-diluted fresh allantoic fluid of chicken embryos infected with A/duck/Vietnam/HU1-1151/2014 (H5N6) was inoculated intravenously into four 7-week-old chickens (*Gallus gallus*). Each chicken was housed in a self-contained isolator unit (Tokiwa Kagaku, Japan) in a BSL3 biosafety facility in the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Table 2. Representative H5, H6, and H9 viruses isolated in LBMs in Thua Thien Hue province, Vietnam in 2014

HA subtypes	Type of LBMs	Name of LBMs	Isolates	Accession numbers							
				PB2	PB1	PA	HA	NP	NA	M	NS
H5	Intervention	No	A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6)	LC069835	LC069867	LC069898	LC041323	LC069953	LC041324	LC070009	LC070040
			A/duck/Vietnam/HU1-1151/2014 (H5N6)	LC041310	LC041311	LC041312	LC041313	LC086330	LC041314	LC041315	LC041316
			A/environment/Vietnam/HU1-1434/2014 (H5N6)	LC069834	LC069866	LC069897	LC041321	LC069952	LC041322	LC070008	LC070039
			A/duck/Vietnam/HU1-1507/2014 (H5N6)	LC069832	LC069864	LC069895	LC041317	LC069950	LC041318	LC070006	LC070037
			A/duck/Vietnam/HU1-1511/2014 (H5N6)	LC069833	LC069865	LC069896	LC041319	LC069951	LC041320	LC070007	LC070038
H6	Intervention	An Lo	A/duck/Vietnam/HU1-1245/2014 (H6N2)	LC069813	LC069846	LC069877	LC069908	LC069932	LC069963	LC069988	LC070019
		No	A/environment/Vietnam/HU1-1423/2014 (H6N2)	LC069836	LC041334	LC041335	LC041336	LC041337	LC041338	LC041339	LC041340
			A/environment/Vietnam/HU1-1426/2014 (H6N2)	LC069837	LC069868	LC069899	LC041341	LC069954	LC041342	LC070010	LC070041
	Non-intervention	Than Phu	A/duck/Vietnam/HU1-637/2014 (H6N6)	LC069817	LC069850	LC069881	LC069912	LC069936	LC069967	LC069992	LC070023
H9	Intervention	An Lo	A/chicken/Vietnam/HU1-3/2014 (H9N2)	LC069838	LC069869	LC069900	LC041350	LC069955	LC069980	LC070011	LC070042
			A/duck/Vietnam/HU1-225/2014 (H9N2)	LC069842	LC069873	LC069904	LC069928	LC069959	LC069984	LC070015	LC070046
		No	A/chicken/Vietnam/HU1-1286/2014 (H9N2)	LC069840	LC069871	LC069902	LC069926	LC069957	LC069982	LC070013	LC070044
			A/environment/Vietnam/HU1-1424/2014 (H9N2)	LC069841	LC069872	LC069903	LC069927	LC069958	LC069983	LC070014	LC070045
			A/duck/Vietnam/HU1-1512/2014 (H9N2)	LC041343	LC041344	LC041345	LC041346	LC086331	LC041347	LC041348	LC041349
	Non-intervention	Phu Bai	A/chicken/Vietnam/HU1-381/2014 (H9N2)	LC069839	LC069870	LC069901	LC069925	LC069956	LC069981	LC070012	LC070043
		Than Phu	A/duck/Vietnam/HU1-675/2014 (H9N2)	LC069820	LC069853	LC069884	LC069915	LC069939	LC069970	LC069995	LC070026
		Quang Phuoc	A/chicken/Vietnam/HU1-786/2014 (H9N2)	LC069843	LC069874	LC069905	LC069929	LC069960	LC069985	LC070016	LC070047

Ethics statements

Animal experiments were authorized by the Institutional Animal Care and Use Committee of Hokkaido University (approval number: 13-0108), and all experiments were performed according to the guidelines of the committee.

Results

Identification of AIVs circulating in intervention and non-intervention LBMs

A total of 178 viruses were identified from 3,045 cloacal and oropharyngeal samples of domestic birds and environmental samples (Table 1). In total, 19 H3, 2 H4, 8 H5, 30 H6, 114 H9, and 5 H11 AIVs were isolated from the samples collected at nine LBMs. At the individual market level, the prevalence of AIVs in No market was the highest (12.2%; 95% CI: 9.6 to 15.4) in the group of intervention LBMs as well as that of Phu Bai market (13.3%; 95% CI: 9.9 to 17.6) in the group of non-intervention LBMs. At the type of LBMs level, the prevalence of AIVs in intervention LBMs (6.1%; 95% CI: 5.0 to 7.5) was similar to that in non-intervention LBMs (5.6%; 95% CI: 4.5 to 6.8; $\chi^2 = 0.532$; $df = 1$; $P = 0.53$). The subtypes of AIVs isolated in LBMs with intervention were H3N2 (7), H3N6 (1), H4N6 (2), H6N2 (10), H6N6 (9), H9N2 (49), H9N6 (2), H11N6 (1), and H11N7 (3). The subtypes of AIVs isolated in non-intervention LBMs were H3N2 (11), H6N2 (4), H6N6 (7), H9N2 (60), H9N6 (3), and H11N7 (1). Eight H5N6 viruses were isolated from apparently healthy ducks (5), Muscovy ducks (2), and an environmental sample at the "No" market with intervention. To assess the pathogenicity of the H5N6 viruses, a representative H5N6 virus, A/duck/Vietnam/HU1-1151/2014 (H5N6) was inoculated intravenously into four 7-week-old chickens. All chickens died within 1 day of infection indicating this H5N6 virus is HPAIVs.

Genetic and antigenic analysis of H5 viruses

The full-length nucleotide sequences of the HA genes of H5 representative viruses isolated in this study were phylogenetically analyzed and divided into two lineages: Eurasian and North American. The H5 viruses in the Eurasian lineage were identified on the basis of the nomenclature defined by WHO/OIE/FAO H5N1 Evolution Working

Group 2014 [27]. The HA genes of six representative H5N6 Vietnam isolates were classified into the clade 2.3.4.4, which was newly established in 2014 [27] and is closely related to the A/environment/Zhenjiang/C13/2013 (H5N6) virus isolated in China [28] (Figure 2). All the H5N6 viruses contain multiple basic amino acids, R and K, at the proteolytic cleavage site of the hemagglutinin protein, PLREKRRKR/GLF, identical to the typical cleavage site motif of H5 HPAIVs. The residues at position 190, 225, 226, and 228 (H3 numbering) are associated with influenza virus receptor specificity. In these viruses, those residues were E, G, Q, and G, respectively; all of them are of avian-type motif [29].

Four representative strains of the H5 isolate were antigenically analyzed by cross HI test (Table 3). Chicken antiserum against the reference virus of the clade 2.3.4.4, A/chicken/Kumamoto/1-7/2014 (H5N8) [30], effectively inhibited hemagglutination of A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6), A/duck/Vietnam/HU1-1151/2014 (H5N6), and A/muscovy duck/Vietnam/HU2-26/2014 (H5N6) and partly inhibited hemagglutination of A/environment/Vietnam/HU1-1434/2014 (H5N6) causing a 4-fold reduction in HI titer. In contrast, these viruses exhibited low HI titers in comparison with homologous titers in reactions with the antisera of different clades (HI titer from 20 to 80).

Genetic and antigenic analysis of H6 viruses

The H6 HA genes were phylogenetically divided into two lineages: Eurasian and North American. The H6 viruses in the Eurasian lineage were clustered into five different sublineages: Group II, W312, Group III, Early and Group I, as described in our previous study [11]. All H6 viruses isolated in Vietnam in 2014 were found to belong to the Group II sublineage (Figure 3), as were the viruses previously isolated in Vietnam between 2010 and 2012 and those isolated in China between 2003 and 2011.

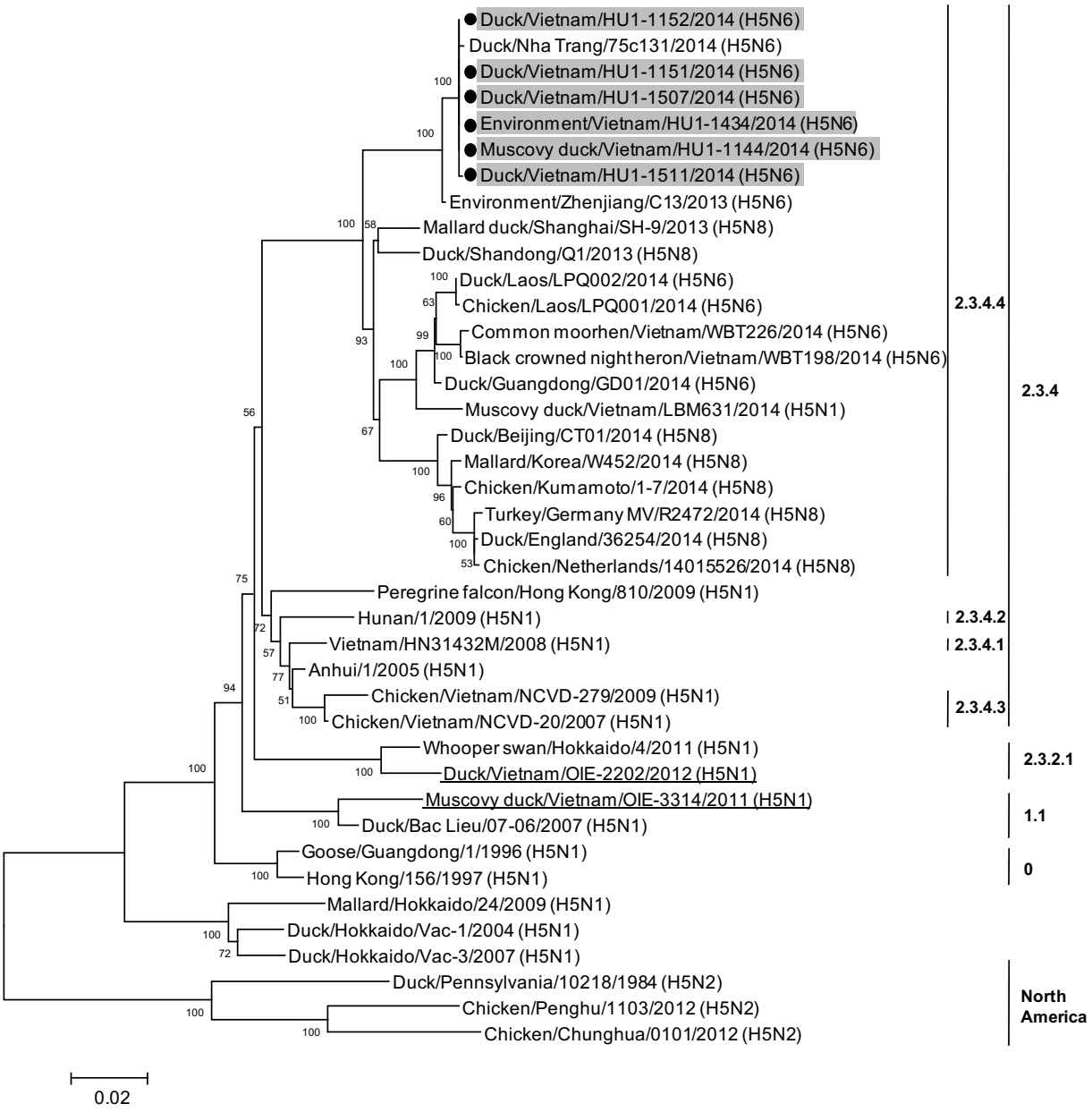

Figure 2. Phylogenetic tree for the influenza virus H5 HA genes.

Full-length HA genes of six H5 subtype viruses, and reference strains were analyzed using the ML method with MEGA 5.0 software and divided into two lineages: Eurasian and North American. The Eurasian H5 viruses were identified on the basis of the nomenclature defined by WHO/OIE/FAO H5N1 Evolution Working Group, 2014 [27]. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray. The viruses isolated in our previous study are underlined. The black circle indicates the viruses isolated in an intervention LBM.

Table 3. Cross-reactivity of H5 influenza viruses with antisera by HI test

Lineage	Viruses	Clade	Antiserum to						
			Vac-3	Mon/05	Hok/08	HK/09	Km/14	Yama/04	Ibr/05
Eurasia	A/duck/Hokkaido/Vac-3/2007 (H5N1)	−	10,240	10,240	640	1,280	1,280	5,120	2,560
	A/whooper swan/Mongolia/3/2005 (H5N1)	2.2	80	640	80	40	20	1,280	80
	A/whooper swan/Hokkaido/1/2008 (H5N1)	2.3.2.1	80	1,280	1,280	80	80	1,280	40
	A/peregrine falcon/Hong Kong/810/2009 (H5N1)	2.3.4	40	20	40	2,560	40	160	20
	A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6)	2.3.4.4	80	40	20	640	640	80	<20
	A/duck/Vietnam/HU1-1151/2014 (H5N6)	2.3.4.4	40	20	20	640	640	40	<20
	A/environment/Vietnam/HU1-1434/2014 (H5N6)	2.3.4.4	20	20	20	160	160	20	<20
	A/muscovy duck/Vietnam/HU2-26/2014 (H5N6)	2.3.4.4	40	40	20	640	640	80	<20
	A/chicken/Kumamoto/1-7/2014 (H5N8)	2.3.4.4	20	20	20	320	640	80	<20
	A/chicken/Yamaguchi/7/2004 (H5N1)	2.5	640	10,240	640	160	160	10,240	320
North America	A/chicken/Ibaraki/1/2005 (H5N2)	−	160	640	20	20	<20	2,560	10,240

The H5 isolates identified in the present study are shown in bold.

Vac-3, A/duck/Hokkaido/Vac-3/2007; Mon/05, A/whooper swan/Mongolia/3/2005; Hok/08, A/whooper swan/Hokkaido/1/2008; HK/09, A/peregrine falcon/Hong Kong/810/2009; Km/14, A/chicken/Kumamoto/1-7/2014; Yama/04, A/chicken/Yamaguchi/7/2004; Ibr/05,

Homologous titers are underlined.

"−" indicates that the virus does not belong to clade 0–9.

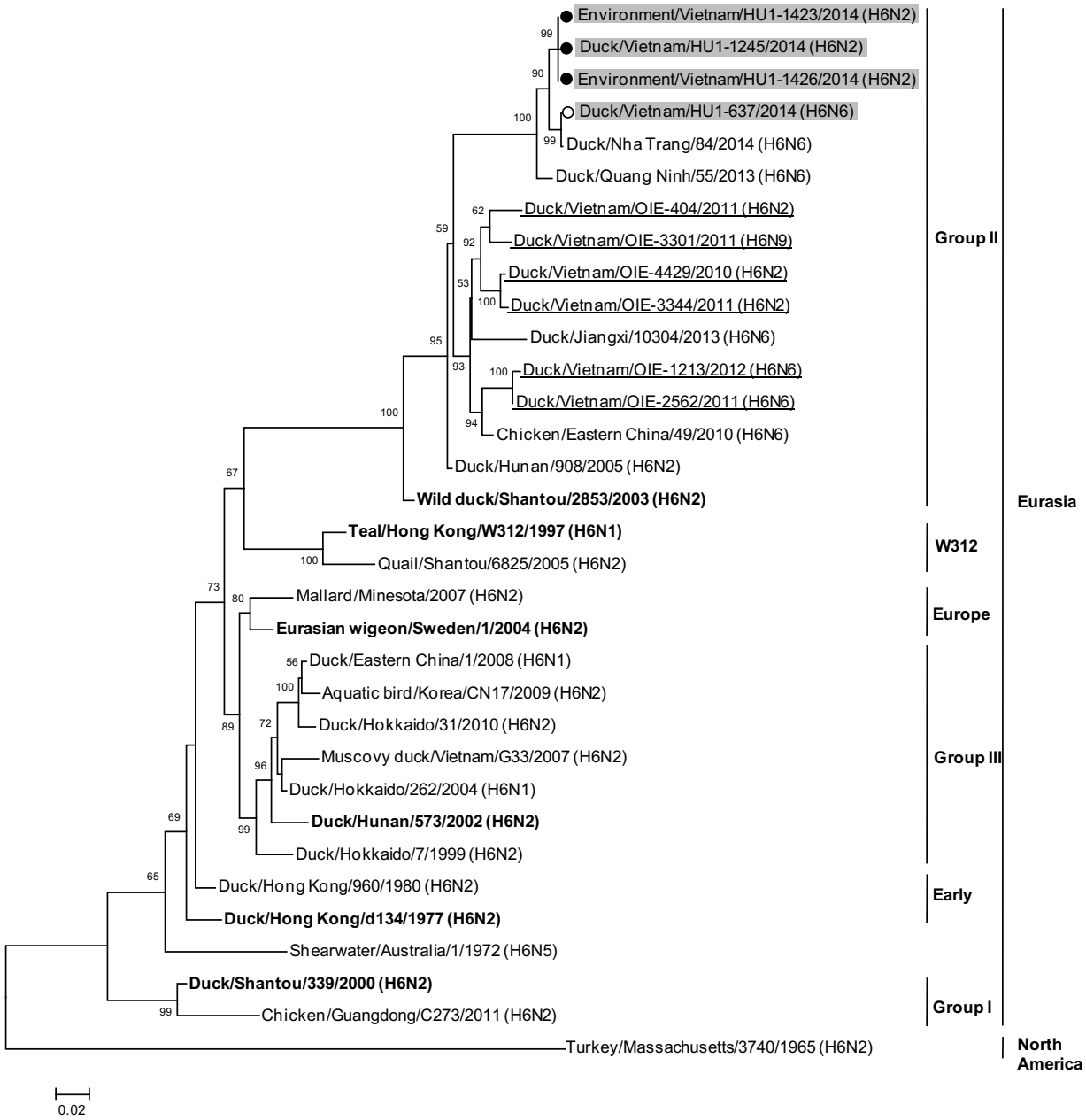

Figure 3. Phylogenetic tree for the influenza virus H6 HA genes.

Nucleotides 51–943 (893 bp) of the HA genes of four viruses of H6 subtype and reference strains were analyzed using the maximum-likelihood method with MEGA 5.0 software and divided into two lineages: Eurasian and North American. The Eurasian H6 viruses were clustered into five different sublineages: Group II, W312, Group III, Early, and Group I [11]. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray, and the representative of each sublineage is indicated in bold. The viruses isolated in a previous study are underlined. The viruses isolated at intervention LBMs are indicated by black circles, and the virus isolated at a non-intervention LBM is indicated by a white circle.

However, the H6 viruses isolated in this study and in our previous study occupied different clusters.

Four strains representative of the H6 isolates were antigenically analyzed by cross HI test using a panel of chicken antisera against four viruses of different sublineages (Table 4). These H6 viruses weakly reacted with A/duck/Vietnam/OIE-4429/2010 (H6N2) antiserum, a virus of the same genetic sublineage, Group II. In reaction with viruses of other sublineages, these H6 viruses exhibited HI titers 4-fold lower than the homologous groups. In addition, these H6 viruses share equivalent HI titers with homologous hyperimmune serum against A/duck/Vietnam/HU1-637/2014 (H6N6) virus, while A/duck/Vietnam/OIE-4429/2010 (H6N2) virus reacted weakly with the antiserum with 16-fold lower HI titers. These results suggest that the antigenicity of the H6 viruses we isolated differed from viruses isolated in Vietnam between 2010 and 2012 [11].

Genetic and antigenic analysis of H9 viruses

The HA genes of H9 isolates were phylogenetically divided into two lineages: Eurasian and North American lineages. All H9 viruses isolated at LBMs in Thua Thien Hue province in 2014 were classified as Y280 sublineage of the Eurasian lineage [10, 11]. These viruses were genetically related to the A/chicken/Vietnam/OIE-1611/2012 (H9N2) virus isolated in the North Vietnam in 2012 and other viruses isolated from poultry in China in 1997 and 2012, which were also classified into the Y280 sublineage (Figure 4).

Representative isolates of the H9 isolates were antigenically analyzed by cross HI test (Table 5). All Vietnam isolates reacted with the antiserum of A/duck/Hong Kong/Y280/1997 (H9N2) virus, which belonged to the Y280 sublineage. These Vietnamese H9N2 viruses reacted weakly with antisera of other H9N2 viruses classified

Table 4. Cross-reactivity of H6 influenza viruses with antisera by HI test

Lineage	Sublinage	Viruses	Antiserum to				
			HK/960/80	VN/OIE-4429/10	VN/HU1-637/14	Aus/1/72	Mas/3740/65
Eurasia	Early	A/duck/Hong Kong/960/1980 (H6N2)	5,120	640	640	640	640
	Group II	A/duck/Vietnam/OIE-4429/2010 (H6N2)	640	5,120	640	160	2,560
		A/duck/Vietnam/HU1-637/2014 (H6N6)	640	1,280	10,240	80	640
		A/duck/Vietnam/HU1-1245/2014 (H6N2)	160	160	2,560	20	160
		A/environment/Vietnam/HU1-1423/2014 (H6N2)	320	640	2,560	40	320
		A/environment/Vietnam/HU1-1426/2014 (H6N2)	320	640	2,560	80	320
–		A/shearwater/South Australia/1/72 (H6N5)	2,560	40	80	1,280	640
North American		A/turkey/Masachusetts/3740/65 (H6N2)	2,560	1,280	40	1,280	5,120

The H6 isolates identified in the present study are shown in bold.

HK/960/80, A/duck/Hong Kong/960/1980 (H6N2); VN/OIE-4429/10, A/duck/Vietnam/OIE-4429/2010 (H6N2); VN/HU1-637/14, A/duck/Vietnam/HU1-637/2014 (H6N6); Aus/1/72, A/shearwater/South Australia/1/72 (H6N5); Mas/3740/65, A/turkey/Masachusetts/3740/65 (H6N2).

Homologous titers are underlined.

"–" indicates that the virus does not belong to any lineage.

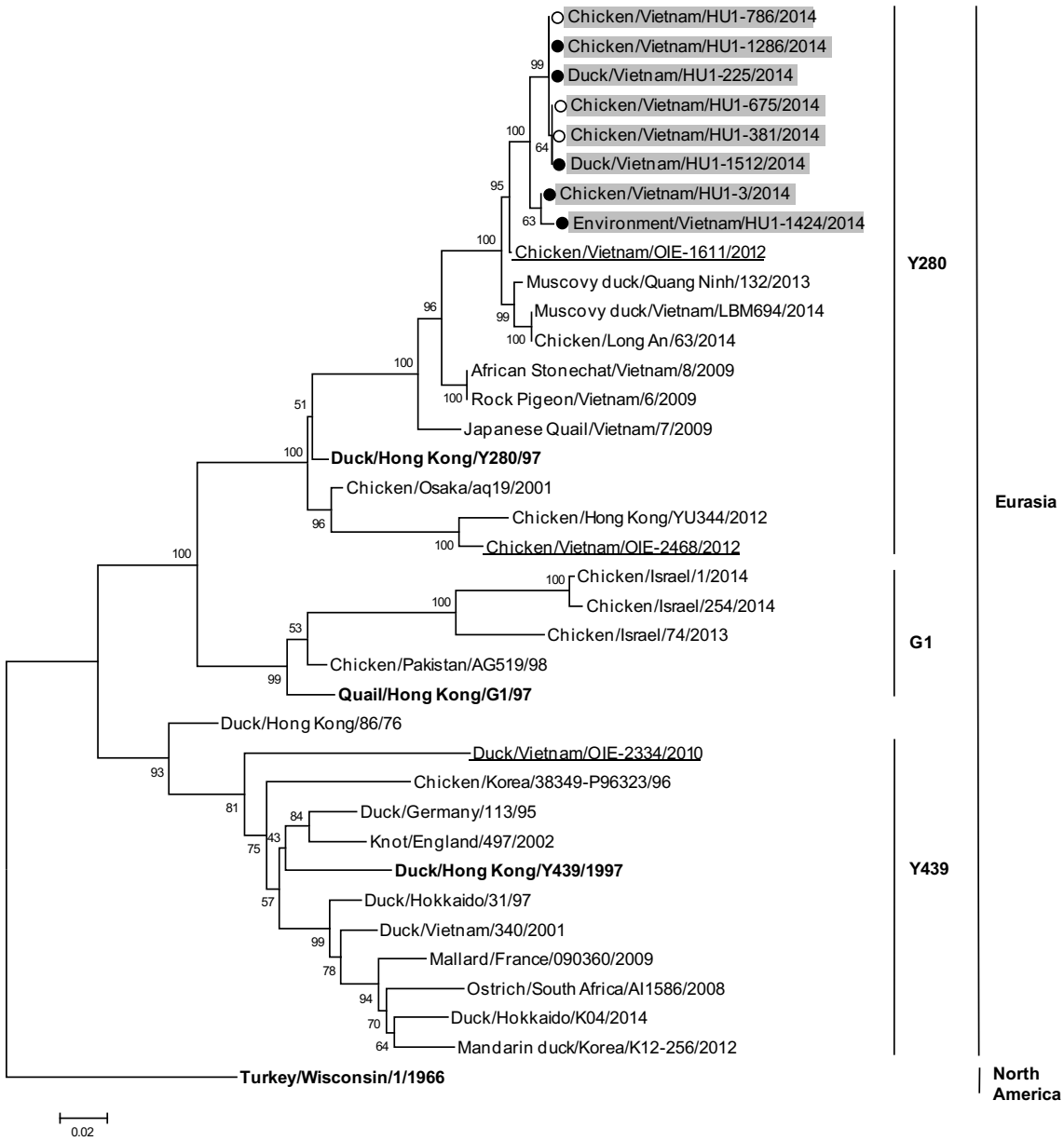

Figure 4. Phylogenetic tree for the influenza virus H9 HA genes.

Full-length HA genes of eight viruses of H9N2 subtype, and reference strains were analyzed using the maximum-likelihood method with MEGA 5.0 software and divided into two lineages: Eurasian and North American lineages. The Eurasian H9 viruses were clustered into three different sublineages: Y280, G1, and Y439 [9, 10]. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray, and the representative of each sublineage is indicated in bold. The viruses isolated in a previous study are underlined. The viruses isolated in intervention LBMs are indicated by black circles, and the viruses isolated in non-intervention LBMs are indicated by white circles. The H9N2 subtype of each virus strain is omitted.

Table 5. Cross-reactivity of H9N2 influenza viruses with antisera by HI test

Lineage	Sublinage	Viruses	Antiserum to			
			HK/Y280/97	HK/G1/97	Hok/49	Wis/66
Eurasia	Y280	A/duck/Hong Kong/Y280/1997	10,240	1,280	2,560	80
		A/chicken/Vietnam/HU1-3/2014	10,240	1,280	1,280	80
		A/duck/Vietnam/HU1-225/2014	2,560	1,280	640	80
		A/chicken/Vietnam/HU1-381/2014	10,240	640	1,280	40
		A/chicken/Vietnam/HU1-786/2014	20,480	1,280	2,560	80
		A/chicken/Vietnam/HU1-1286/2014	10,240	640	640	80
		A/environment/Vietnam/HU1-1424/2014	10,240	320	1,280	40
		A/duck/Vietnam/HU1-1512/2014	10,240	640	2,560	40
	G1	A/quail/Hong Kong/G1/1997	1,280	5,120	640	80
	Y439	A/duck/Hokkaido/49/1998	640	80	2,560	320
North America	−	A/turkey/Wisconsin/1/1966	80	40	320	320

The H9 isolates identified in the present study are shown in bold.

HK/Y280/97, A/duck/Hong Kong/Y280/1997; HK/G1/97, A/quail/Hong Kong/G1/1997; Hok/49, A/duck/Hokkaido/49/1998; Wis/66, A/turkey/Wisconsin/1/1966.

Homologous titers are underlined.

"−" indicates that the virus does not belong to any sublinage.

into different sublineages, such as the G1 or North American lineages, and reacted moderately with antisera to Y349 sublineage virus. These suggest that the antigenicities of the H9N2 viruses isolated in Vietnam have been stable in the poultry population.

The neuraminidase genes of AIVs isolated in LBMs

For neuraminidase (NA) gene segments, the names of groups were defined based on previous studies [11, 31]. The N2 NA genes of the H6 and H9 AIVs were phylogenetically categorized into two groups, Group II and Y280 respectively (Figure 5a). All N6 NA genes of the H5 viruses belonged to Group II and of the H6 viruses belonged to Group I (Figure 5b).

Genetic diversity of AIVs isolated in LBMs

The six internal gene segments of the AIVs were then phylogenetically analyzed to investigate the genetic diversity of AIVs circulating in Vietnamese LBMs (Figure 6). For each internal gene segment, the names of groups were defined as previously described [11, 31]. In this study, the six internal gene segments were also classified into H6 Group I, H6 Group II, H6 Group III, JX8264 like, and Gs/Gd like, indicating that these viruses were closely related to the viruses isolated in China. For instance, the PB2 and PA genes of the A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6) were classified as JX8264 like. The NP, M, and NS genes of this H5N6 virus were grouped into Gs/Gd like and H6 Group I where Chinese viruses were also grouped into. Phylogenetic analysis also indicated that the PB2, PB1, PA, and NS internal genes of H9N2 viruses, A/chicken/Vietnam/HU1-1050/2014 (H9N2) and A/chicken/Vietnam/HU1-976/2014 (H9N2), were classified into the same groups: Vietnam II, Vietnam, and JX8286 like. However, other gene segments, NP and M genes, were classified into different groups (Figure 6).

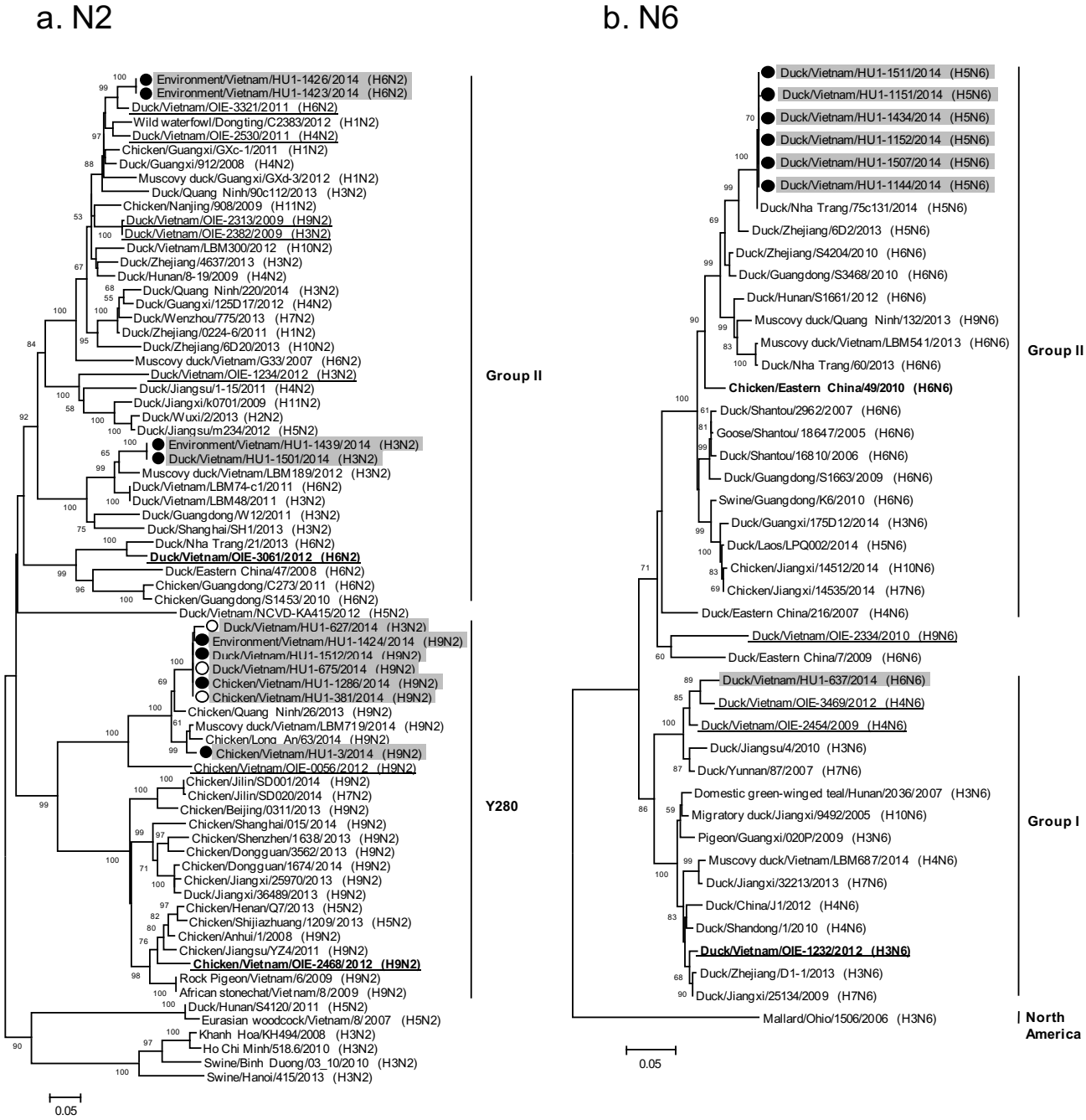

Figure 5. Phylogenetic tree for the NA genes of influenza viruses isolated in LBMs. Nucleotide 29–1411 (1383 bp) of N2 (a) and 83–740 (658 bp) of N6 (b) were used for the ML phylogenetic analysis. The NA genes of viruses isolated in LBMs were clustered into two different sublineages: Y280 and Group II for N2 genes and Group I and Group II for N6 genes. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray, and the representative of each sublineage is indicated in bold. The virus isolated in our previous study is underlined. The viruses isolated at intervention LBMs are indicated by black circles, and the viruses isolated at non-intervention LBMs are indicated by white circles.

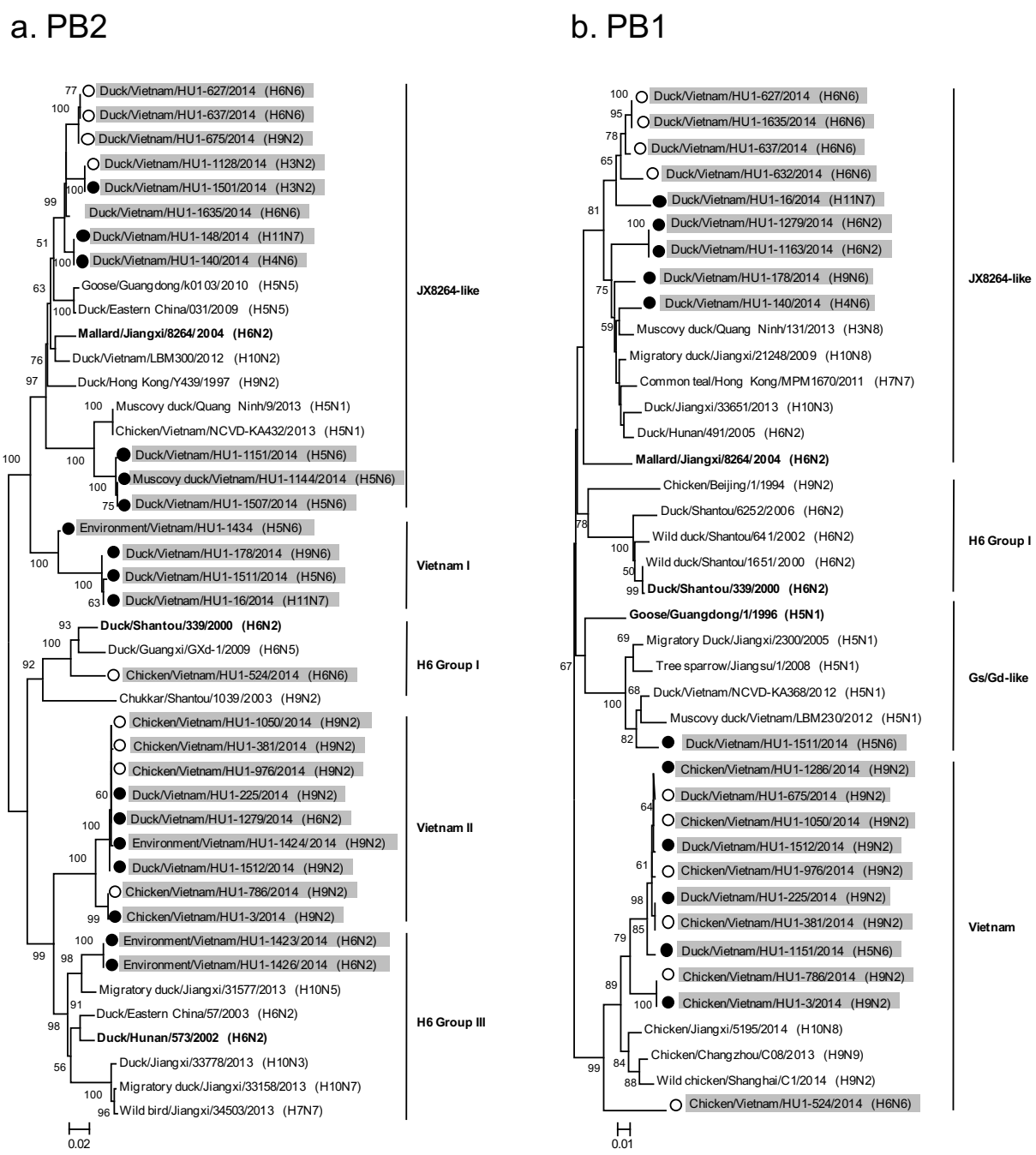

Figure 6. Phylogenetic tree for the six internal genes of influenza viruses isolated in LBMs.

Nucleotide 1089–1923 (835 bp) of PB2 (a), 1169–1883 (715 bp) of PB1 (b), 813–1520 (708 bp) of PA (c), 1–643 (643 bp) of NP (d), 1–825 (825 bp) of M (e), and 27–839 (813 bp) of NS (f) were used for the ML phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are indicated are highlighted in gray, and the representative of each sublineage is indicated in bold. The viruses isolated at intervention LBMs are indicated by black circles, and the viruses isolated at non-intervention LBMs are indicated by white circles.

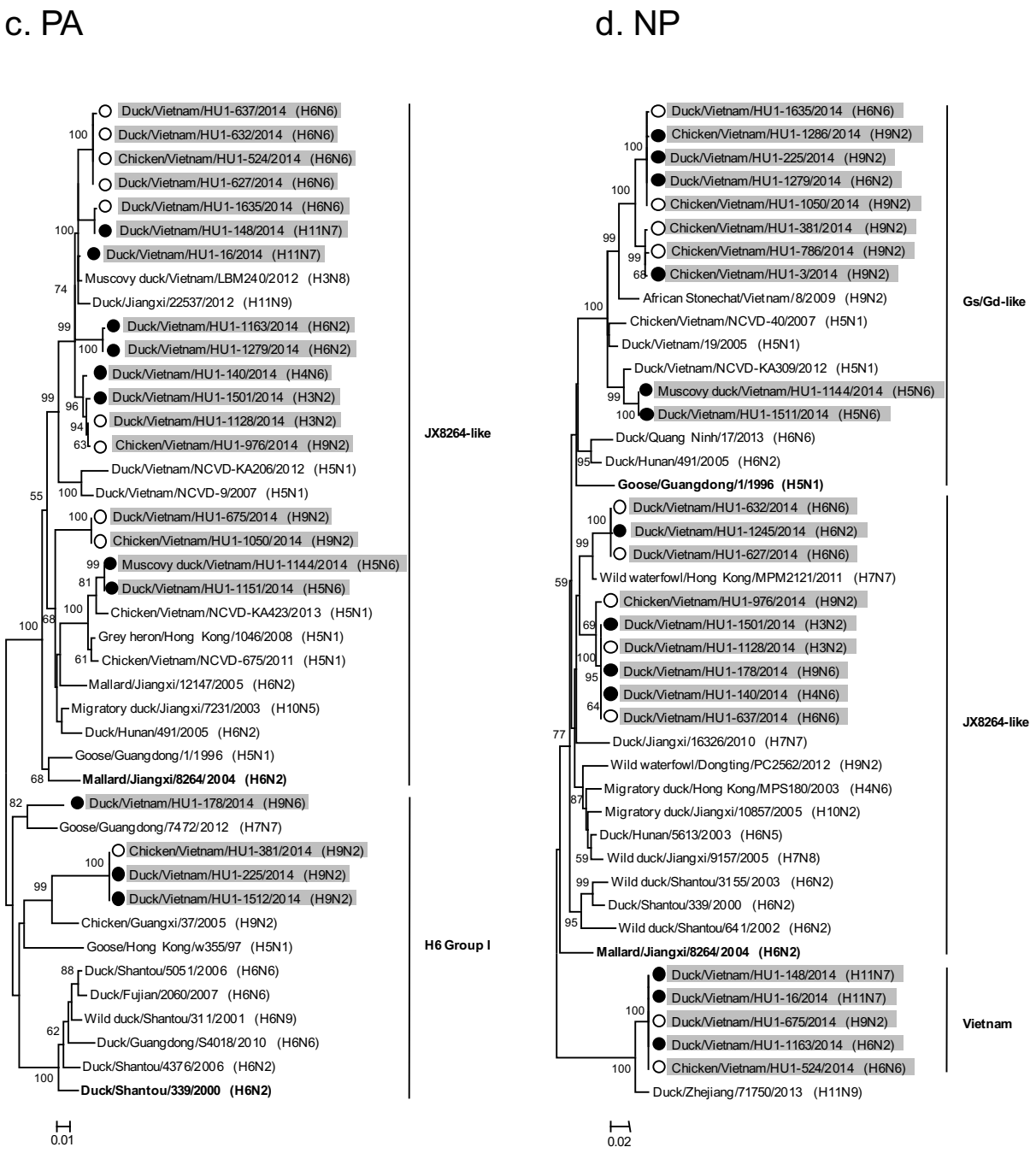

Figure 6 *(cont.)*. **Phylogenetic tree for the six internal genes of influenza viruses isolated in LBMs.**

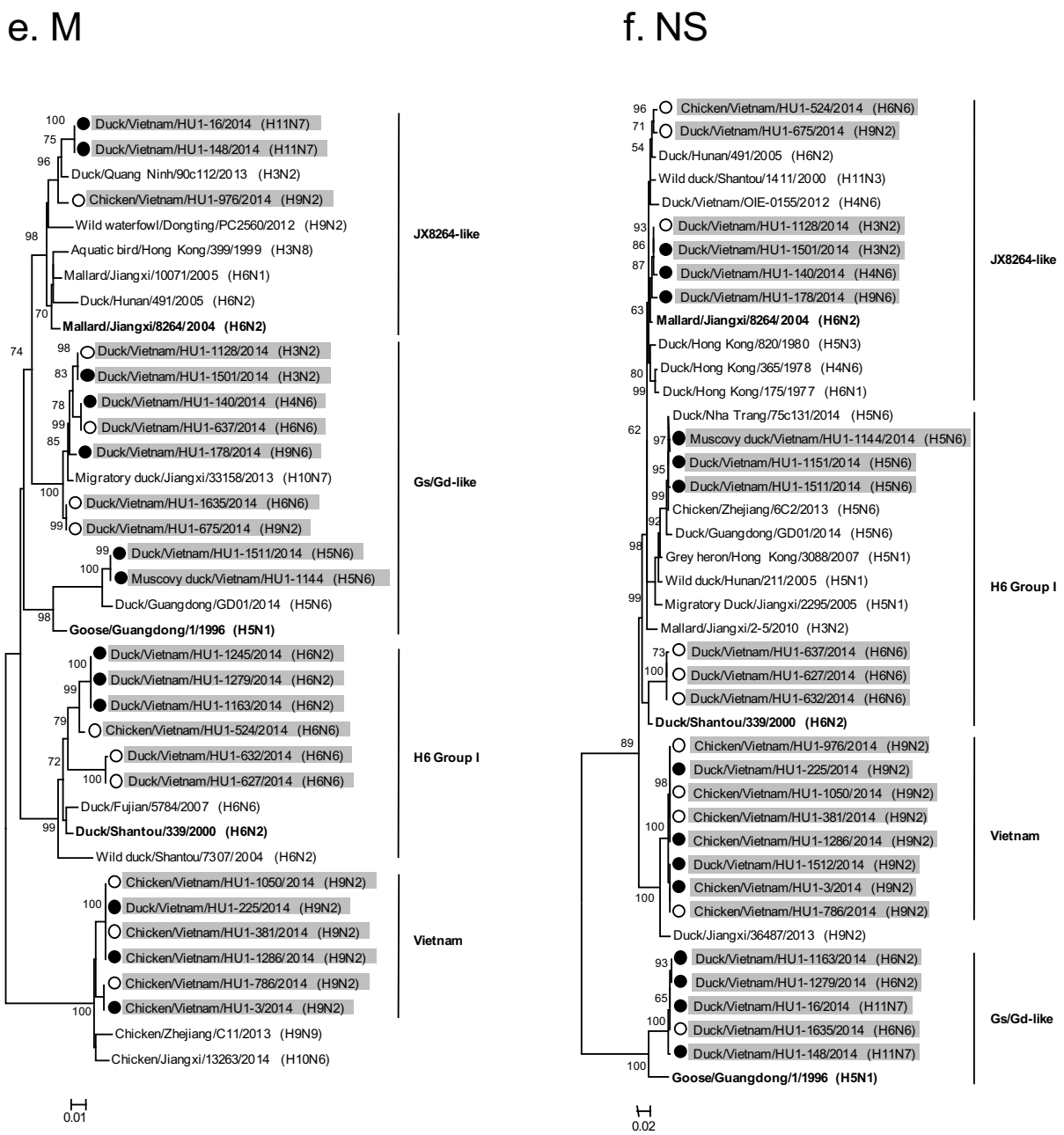

Figure 6 *(cont.)*. **Phylogenetic tree for the six internal genes of influenza viruses isolated in LBMs.**

Discussion

LBMs provide an ideal environment for genetic reassortment events and interspecies transmission of AIVs [11, 13, 14, 21]. In this study, 178 AIVs were isolated from poultry in both intervention and non-intervention LBMs. The H5N6 viruses were isolated from apparently healthy domestic ducks, muscovy ducks, and an environmental sample at an intervention LBM, indicating that subclinical HPAIV infections are endemic in domestic birds. These results remind us that LBMs play an important role as a hotspot for the transmission of AIVs, including HPAIVs, in Vietnam. Although closure of LBMs or banning the storage of poultry overnight at the markets represents a highly effective method to reduce amplification and persistence of viruses in LBMs [12-14, 22], it is hard to change the LBMs system in Vietnam due to well-established traditional cultures and the behavior of customers. Therefore, biosecurity measures were developed with the intention of reducing AI transmission. In this study, we conducted surveillance of AIVs in both intervention and non-intervention LBMs in a central province of Vietnam. We sampled viruses on two occasions and found the AIV prevalence at intervention LBMs to be 6.1%. The prevalence of AIVs at non-intervention LBMs did not differ significantly from that at intervention LBMs, which was 5.6%. All the viruses identified in this study were isolated from apparent healthy LBM poultry. In addition, various subtypes of AIVs were identified at intervention LBMs. The H6 and H9 viruses isolated in intervention LBMs were genetically similar to H6 and H9 viruses isolated at non-intervention LBMs. Although disinfection was reported to be performed twice per day at intervention LBMs, AIVs still contaminated the environment. These results indicate that the current intervention LBMs remain several limitations for the control of AI contamination such as problems of periodic disinfection procedure and concentration of chemical disinfection. The intervention

LBMs are generally larger capability to hold poultry than these of non-intervention LBMs, meaning that intervention LBMs can contain poultry originating from many different sources, including neighboring provinces. In addition, KAP surveys indicated that the sources of poultry in intervention LBMs in Thua Thien Hue province are widely distributed from local area to other provinces. Therefore, the source of poultry may play an important role in AIV transmission at LBMs, and this factor should be further studied. As a next step, a risk analysis will be conducted to identify the factors contributing to the appearance of various subtypes of AIVs at intervention LBMs.

The H5N1 HPAIVs were first detected in Vietnam in 2001, and outbreaks have been regularly reported since the end of 2003 [19]. The H5 viruses circulating in Vietnam were classified into seven major genetic clades: 1.1, 2.3.2.1, 2.3.4.1, 2.3.4.2, 2.3.4.3, 7.1, and 7.2 [11, 32, 33]. In this study, we report for the first time the characterization of H5N6 viruses isolated in an intervention LBM in a center province of Vietnam. The HA genes of the H5N6 viruses isolated from poultry and an environmental sample at an intervention LBM in Thua Thien Hue province were classified as clade 2.3.4.4. Genetic analysis indicated that these H5N6 viruses were closely related to an H5N6 virus isolated in Zenjiang, China and differed slightly from the H5N6 viruses isolated in Lao PDR, indicating an existing genetic diversity within this new clade 2.3.4.4 [34]. The H5 viruses found in northern Vietnam may have been introduced from China. In April, 2014, the first outbreak of H5N6 was found in a flock of chickens in Lang Son province, located near the border with China. Then another outbreak was reported in a flock of ducks in Ha Tinh province, located in central part of Vietnam, and China [35]. In Vietnam and China, AI vaccines are used to control H5 HPAIV infections in poultry [36, 37]. However, immunological selection pressure has driven development of antigenic variants of H5 HPAIVs [38, 39]. The H5N6 HPAIVs we identified were also antigenically distinct from the clade 2.3.4 virus used in the Re-5

vaccine, which has been applied in China and Vietnam [36, 37]. Recently, several human H5N6 AIV infections were reported, and avian-originated H5N6 viruses were also isolated from healthy pigs in China, although the H5N6 AIV has not adapted to the swine population yet [40, 41]. In Thua Thien Hue province, Vietnam, piglets, and poultry are often housed together in non-intervention LBMs. This LBM system may facilitate influenza virus reassortment and transmission of influenza viruses between poultry and from poultry to pigs or humans. Therefore, it will be important to monitor swine influenza, and strict controls should be applied to limit interspecies transmission of influenza viruses.

H6 and H9 AIVs are widely distributed among poultry and wild birds in Asia [42, 43]. H9N2 viruses have become the most prevalent subtype detected in poultry populations in China since 2004 [44] and in Vietnam since 2009 [10, 11]. In this study, H6 and H9 viruses isolated from domestic ducks, chickens, and environment samples were genetically identical in both types of LBMs in Thua Thien Hue province, Vietnam. These viruses were phylogenetically closely related to viruses previously isolated in poultry in China. The HA genes of H9 viruses did not differ from those previously isolated in 2011 and 2012, indicating that these viruses were maintained in poultry in Vietnam. Antigenic analysis of H6 viruses indicated that all the Vietnamese H6 viruses we isolated in 2014 exhibited low cross reactivity with antiserum of A/duck/Vietnam/OIE-4429/2010 (H6N2), another Group II virus. In addition, genetic analysis also indicated that the H6 viruses isolated in this study differed from other Vietnamese H6 viruses isolated in our previous study [11]. These results suggest that the antigenicity of these Vietnamese H6 viruses isolated in 2014 differs from those isolated in Vietnam between 2010 and 2012. Perhaps the H6 viruses circulating in Vietnam in 2014 exhibited altered antigenicity as a result of repetitive infections of the

poultry population. Further studies should be conducted to characterize the antigenic variation of H6 viruses circulating in Vietnam.

In Vietnam, domestic birds are mainly raised in households in a free-range manner and are transported to LBMs by their owners or poultry sellers. AIVs are transmitted and spread within the poultry population. Although domestic birds are vaccinated against H5N1 in Vietnam [19, 37, 45], HPAIVs have silently spread in the poultry population. Our study provides initial evidences for the improvement of intervention strategies in study LBMs. The intervention with supporting a good infrastructure as "a good hardware" need to comprise well with "a good software" like increasing of education level of people involved in poultry trade and improvement of hygiene procedures by local authority at LBMs. Active surveillance program of AI and hygiene practice performance monitoring in LBMs will be essential to eradicate HPAI from Vietnam as well as Asian countries.

Summary

A total of 3,045 environmental samples and oropharyngeal and cloacal swabs from apparently healthy poultry have been collected at three LBMs at which practices were applied to reduce AIV transmission (intervention LBMs) and six conventional LBMs (non-intervention LBMs) in Thua Thien Hue province in 2014 to evaluate the efficacy of the intervention LBMs. The 178 AIVs, including H3 (19 viruses), H4 (2), H5 (8), H6 (30), H9 (114), and H11 (5), were isolated from domestic ducks, muscovy ducks, chickens, and the environment. The prevalence of AIVs in intervention LBMs (6.1%; 95% CI: 5.0 to 7.5) was similar to that in non-intervention LBMs (5.6%; 95% CI: 4.5 to 6.8; $\chi^2 = 0.532$; $df = 1$; $P = 0.53$) in the study area. Eight H5N6 HPAIVs were isolated from apparently healthy ducks, Muscovy ducks, and an environmental sample in an intervention LBM. The HA genes of the H5N6 HPAIVs belonged to the genetic clade 2.3.4.4, and the antigenicity of the H5N6 HPAIVs differed from the H5N1 HPAIVs previously circulating in Vietnam. Phylogenetic and antigenic analyses of the H6 and H9 viruses isolated in both types of LBMs revealed that they were closely related to the viruses isolated from domestic birds in China, Group II of H6 viruses and Y280 lineage of H9 viruses. These results indicate that the interventions currently applied in LBMs are insufficient to control AI. A risk analysis should be conducted to identify the key factors contributing to AIV prevalence in intervention LBMs.

Chapter II

A cross-sectional study to quantify the prevalence of avian influenza viruses in poultry at intervention and non-intervention live bird markets in central Vietnam, 2014

Introduction

LBMs are known to be reservoirs and transmission hubs for AIVs [46]. In Southeast Asian countries, LBMs are ubiquitous and integral components of the semi-intensive poultry industries that are common in this part of the world [20, 21]. In Vietnam, LBMs are found in most populated centers, providing the means by which the majority of the population access fresh poultry for immediate consumption [47]. LBMs tend to be small scale operations where poultry are mixed together with other animals under conditions of relatively poor infrastructure, mostly trading poultry derived from household and semi-commercial enterprises situated closely to the area in which markets are located [48]. In LBMs, it is common for a range of subtypes of AIVs to be mixed as a result of different poultry types and species being brought together from different geographical locations [49]. In Southeast Asia, HPAIVs are known to circulate in LBMs [20, 32, 46, 48, 50] and it has been hypothesized that LBMs may facilitate the emergence and spread of new viral reassortants due to close contact amongst the infected birds [51]. Furthermore, it has also been shown that, in China, human infections with AIVs, in particular, of the subtypes H5N1 and H7N9 are associated with recent exposure to poultry in LBMs [21, 49].

An effective strategy for reducing the likelihood of AIV transmission to the general public is to close LBMs indefinitely [13, 14, 52]. This approach was used in the outbreak of HPAI that occurred in the Hong Kong Special Administrative Region of the People's Republic of China in 1997 [53]. Although this strategy is effective for reducing the risk of AIV infection, it is an unpopular approach with poultry consumers [54] and difficult in terms of promoting effective long term control of AI because poultry sellers that are displaced from LBMs that have been closed tend to rapidly establish 'black market' poultry trading locations (Vietnam Department of Animal

Health, personal communication, 2014). For these reasons, a less draconian approach has been to adopt interventions aimed to improve LBM biosecurity and hygiene. In this way, the risk of AIV infection within LBMs can be minimized and, at the same time, poultry trade can be permitted to continue. In Chapter I, the characteristics of LBMs with improved infrastructure ('intervention LBMs', n = 3) were compared with those operating in a routine manner (n = 6) under the VAHIP in Thua Thien Hue province, in the central region of Vietnam [23]. The study showed that H5N6 HPAIVs were isolated from apparently healthy ducks, Muscovy ducks and an environmental sample in one of the intervention LBMs. Although the number of LBMs that took part in the study was small, it appears that physical improvements in the market biosecurity and hygiene had little apparent effects on the prevalence of AIVs amongst poultry present for sale at those markets.

In this study, the data of Chapter I was used to identify characteristics associated with the presence of AIVs in poultry submitted for sale at seven LBMs in Thua Thien Hue province, Vietnam. Our specific aims were to: (1) document the prevalence of AIVs in seven LBMs that took part in this study and compare the prevalence of AIVs positive samples amongst intervention and non-intervention markets; and (2) quantify bird-, poultry seller- and market-level characteristics that rendered individual birds more likely to be AIV isolation positive at the time of sale. Identifying the relative importance of factors influencing the poultry submitted for sale at LBMs is a critical first step towards the design of evidence-based better measures to reduce the number of AIV positive birds (and therefore the risk of virus infection) within LBMs in Vietnam.

Materials and methods

Study design and study area

A cross-sectional study was carried out in seven LBMs in four districts of Thua Thien Hue province (Figure 7 and Table 6), Vietnam, in August and December, 2014. At three of the seven LBMs, biosecurity had been improved as part of the VAHIP program in Thua Thien Hue province. In intervention LBMs, there was a good standard of infrastructure with poultry from different sources physically separated by the seller; besides, disinfection procedures were performed twice, in the morning and evening, on a given sale day. The other four (non-intervention) LBMs were wet markets at which no particular intensive biosecurity interventions were carried out and at which poultry and other animals were mixed together under relatively low biosecurity conditions; details are described in Chapter I.

Each of the LBMs was visited by the investigators in Vietnam Department of Animal Health (DAH) and Sub-Department of Animal Health (SDAH) staff of Thua Thien Hue province on two occasions: August and December 2014. At the time of each market visit, a list of all poultry sellers present was obtained from the market manager. Each poultry seller was contacted with the investigators and samples were taken from individual birds for AIV isolation. At the conclusion of sampling, a questionnaire was administered to the poultry sellers.

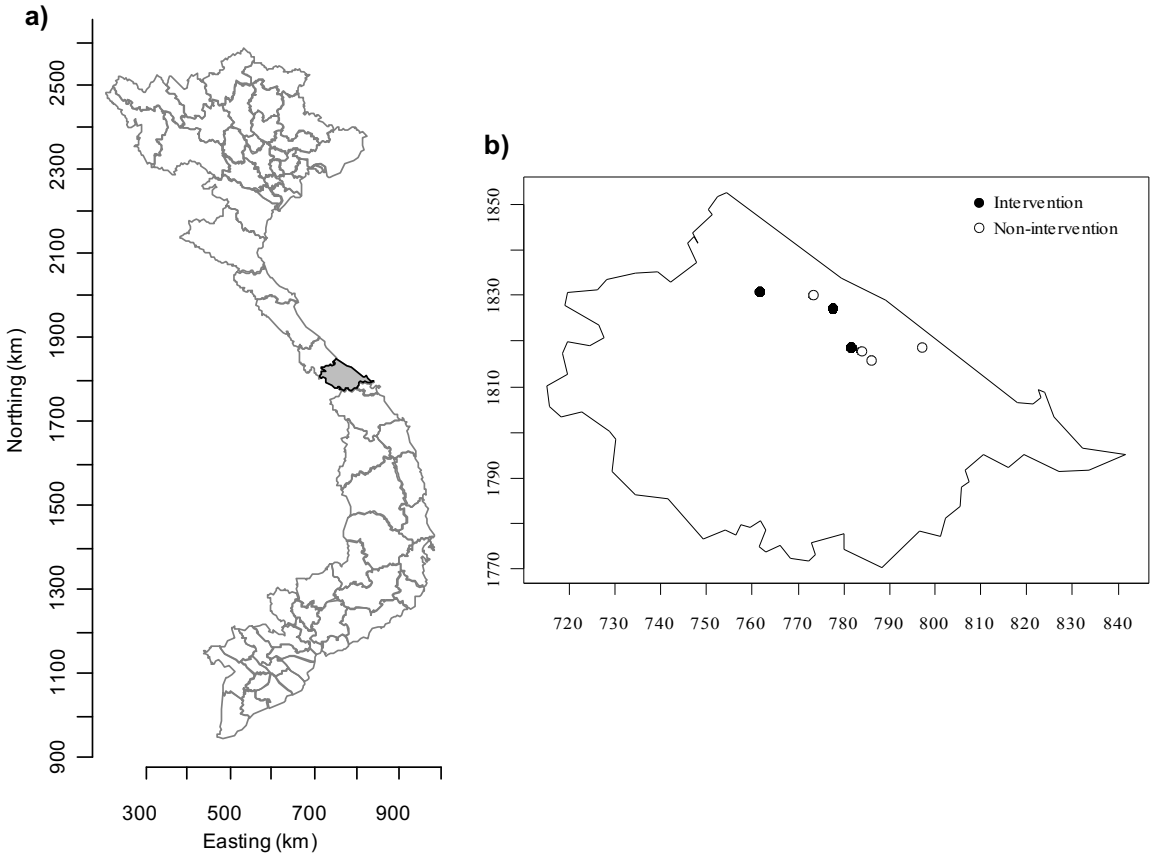

Figure 7. Location of the seven intervention- and non-intervention live bird markets described in this study.

a) Research location – Thua Thien Hue province in center of Vietnam;

b) Locations of the intervention- and non-intervention live bird markets in Thua Thien Hue province.

Table 6. Structure of the data from 1,629 individual bird samples from 83 sellers in seven live bird markets

Level	Number	Number per unit at next-higher level	
		Mean	Range
District[a] (highest level)	4	—	—
Live bird market	7	2	1 – 3
Seller	83	21	6 – 32
Birds sampled	1,629	20	2 – 142

[a] Each district had mean number, 2 (range 1–3) live bird markets.

Laboratory procedures

Oropharyngeal, cloacal swabs and fecal samples were collected from chickens, ducks, and Muscovy ducks for each poultry seller on each of the two sampling rounds. For each bird, the oropharyngeal and cloacal swabs were collected in a sterile tube with transport medium, as described by in Chapter I. Samples were then transported to the National Center for Veterinary Diagnostics (NCVD), Hanoi, Vietnam. At the NCVD, the samples were tested for the presence of influenza type A viruses (M gene detection) using real time RT-PCR. All samples were then prepared for transfer to the Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan. The shipment of samples containing AIVs were classified into Biological Substance, Category B, following the instructions of the International Air Transport Association (IATA) in Dangerous Goods Regulation Manual [55]. At the Laboratory of Microbiology, all samples were submitted for virus isolation (VI). After which, representative isolates, such as H5, H6, and H9 AIVs, were phylogenetically and antigenically analyzed to characterize the genetic and antigenic variation of AIVs currently circulating in LBMs in Vietnam, further detail is provided in Chapter I.

Questionnaire and interview

A questionnaire developed to solicit details of KAP concerning AIVs was developed by the authors in conjunction with staffs from the DAH, Vietnam (a copy of the questionnaire is available in Appendix). The questionnaire was developed in Vietnamese and was comprised of 45 open and two closed questions organized into the following sections: (1) demographic details of the seller; (2) a description of the source and type of poultry on sale on the given market day; and (3) details of AI biosecurity measures typically used by the seller.

A total of 83 face-to-face interviews with poultry sellers were carried out over the two sampling rounds in the seven markets (45 in intervention and 38 in non-intervention LBMs). Questionnaire survey was administered by trained interviewers from the SDAH of Thua Thien Hue and the District Veterinary Stations of each of the districts in which the study markets were located. Interviews were carried out with assistance from SDAH veterinarians located in communes adjacent to each market. DAH staff provided technical supervision and assistance during each market visit. One average, the length of time taken to visit all of the sellers within a market and to complete sampling and administration of the questionnaires was two days (minimum 1 day; maximum 7 days).

Data management

Questionnaire responses for each sampling round were entered into a relational database with a numeric poultry seller identifier (assigned at the time of interview in the first round) used as a unique key. The results of AIVs isolation were entered into this database as a separate table. The two tables were linked within the database using the unique poultry seller identifier.

Statistical analyses

The prevalence of AIVs at the individual bird level was calculated as the total number of individual bird samples that were AIV positive as the numerator and the total number of birds sampled as the denominator.

Unconditional associations between questionnaire responses (the explanatory variables) and the outcome of interest (the presence or absence of AIV in an individual bird) were computed using the odds ratio. Explanatory variables with unconditional associations at the $P < 0.2$ level (2-sided) were selected for multivariable modelling.

A fixed-effects logistic regression model was developed where the probability of a bird being AIV positivity was parameterized as a function of the *m* explanatory variables with unconditional associations significant at P < 0.2, as described above. Given $p_i = P(Y_i = 1)$ and assuming that Y_i are mutually independent, this model takes the form:

Equation 1:

$$log\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_1 x_{1i} + \cdots + \beta_m x_{mi} + \epsilon_i$$

In Equation 1, β_0 represents the intercept term and $\beta_1, \ldots, \beta_m$ represent the regression coefficients for each of the *m* explanatory variables included in the model. Explanatory variables that were not statistically significant were removed from the model one at a time, beginning with the least significant, until the estimated regression coefficients for all explanatory variables retained were significant at an alpha level of less than 0.05. Explanatory variables that were excluded at the initial screening stage were tested for inclusion in the final model and were retained in the model if they changed any of the estimated regression coefficients by more than 20%. Biologically plausible two-way interactions were tested and none were significant at an alpha level of 0.05.

To account for lack of independence arising from the hierarchical structure of the data, that is, individual birds clustered within seller and sellers clustered within sampling rounds and markets we extended the model shown in Equation 1 to a mixed-effects model:

Equation 2:

$$log\left(\frac{p_{ijk}}{1-p_{ijk}}\right) = \beta_0 + \beta_1 x_{1ijk} + \cdots + \beta_m x_{mijk} + M_k + S_{jk} + \epsilon_{ijk}$$

In Equation 2, p_{ijk} represents the probability of being influenza A virus isolation positive for the *i*th bird from the *j*th seller in the *k*th market. Parameter M_k is a zero mean random effect term with variance σ_M^2 representing the influence of the *k*th market on the probability of being AIV positive. Similarly, parameter S_{jk} is a zero mean random effect term with variance σ_S^2 representing the influence of the *j*th seller in the *k*th market on the probability of being AIV positive. Our reason for including S_{jk} and M_k in the model was to account for unexplained extra-binomial variation operating at the seller- and market-level on AIV risk.

Frequency histograms of the residuals from the multilevel model and plots of the residuals versus predicted values were constructed to check that the assumptions of normality and homogeneity of variance had been met. In the multilevel model, the level 1 (individual bird) variance was constrained to 1 (that is, no extra-binomial variation was permitted). Because this variance was expressed on the binomial rather than the logit scale, the estimates of the proportion of variation of each level of the hierarchy (market, seller, and bird) were computed assuming the level 1 variance on the logit scale was $\pi^2/3$, where π = 3.1416. This calculation is based on interpreting the presence or absence of virus isolation as the result of an underlying latent process with a continuous, logistic distribution.

Descriptive analyses, the unconditional measures of association and the fixed-effects logistic regression models were carried out using R version 3.2.3 (R Development Core Team 2016). The mixed-effects model was developed with MLwiN [56] using the R2MLwiN package in R [57].

Results

Descriptive statistics and unconditional associations

Table 6 describes the structure of the data. The final dataset comprised details from 1,629 birds from 83 sellers in seven LBMs in seven communes in four districts of Thua Thien Hue province. The average number of birds sampled per seller over the study period was 20 (minimum 2; maximum 142). The average number of sellers per market was 21 (minimum 6; maximum 32).

Table 7 presents the 16-questionnaire derived explanatory variables that were associated with VI positivity at $P < 0.2$. Most of the birds sampled were sold by women (1,558 of 1,629; 96%) and the odds of birds sold by women sellers being AIV positive was 0.57 (95% CI 0.27 to 1.22) times that of birds sold by male sellers. A relatively small proportion of birds were sold by sellers with high school education (71 of 1,629) and the odds of birds sold by those with high school education being AIV positive was 2.68 (95% CI 1.17 to 5.90) times that of birds sold by those with no formal education. Most of the birds submitted for sale were sourced from the same commune as the commune in which the market was located (1,128 of 1,629; 69%) and the odds of birds sourced from the same commune being AIV positive was 0.36 (95% CI 0.25 to 0.53) times that of birds sourced from different communes. Most (1,050 of 1,629; 64%) birds were handled by their sellers without gloves and a similar proportion (1,037 of 1,629; 64%) were handled without the seller washing their hands afterwards. While the number of birds sold by sellers who had attended an AI training course was relatively high (968 of 1,629; 60%) only 11% (180 of 1,629) were sold by sellers who were confident of the clinical signs of AI and a high proportion (1,331 of 1,629; 82%) of birds were sold by those who believed that control of AI would have a positive effect on their business. A total of 1,144 of the 1,629 birds (70%) were sold at three intervention

Table 7. Unconditional associations between the outcome variable (virus isolation positive) and the sixteen explanatory variables

Variable	VI positive	Birds	OR (95%CI)	P-value
Gender:				
Female	105	1,558	1.00	Reference
Male	8	71	1.76 (0.76 – 3.56)	0.15
Education:				
None	18	281	1.00	Reference
Elementary	35	497	1.11 (0.62 – 2.03)	0.73
Middle school	49	780	0.98 (0.57 – 1.75)	0.94
High school	11	71	2.68 (1.17 – 5.90)	0.01
Number of years trading:				
1–5 years	7	294	1.00	Reference
6–10 years	79	922	3.84 (1.88 – 9.25)	< 0.01
Over 10 years	27	413	2.87 (1.30 – 7.23)	0.01
Do you source birds from the same commune as the market?				
No	60	501	1.00	Reference
Yes	53	1,128	0.36 (0.25 – 0.53)	< 0.01
What is the cause of avian influenza				
Unknown	34	627	1.00	Reference
Bacteria	1	10	1.94 (0.10 – 10.8)	0.53
Virus	78	992	1.49 (0.99 – 2.28)	0.06
Do you separate ducks and chickens at				
No	99	1,490	1.00	Reference
Yes	14	139	1.57 (0.84 – 2.75)	0.13
Do you wash your hands with soap after				
No	58	1,037	1.00	Reference
Yes	55	592	1.73 (1.18 – 2.54)	< 0.01
Do you wear gloves when handling				
No	81	1,050	1.00	Reference
Yes	32	579	0.70 (0.45 – 1.06)	0.10
Are you confident of the clinical signs of				
No	49	828	1.00	Reference
Not sure	58	621	1.64 (1.10 – 2.44)	0.01
Yes	6	180	0.55 (0.21 – 1.20)	0.17
Do you believe personal protective equipment will protect you from AI?				
No	28	599	1.00	Reference
Not sure	56	695	1.79 (1.13 – 2.89)	0.01
Yes	29	335	1.93 (1.13 – 3.32)	0.02
What benefit will AI control have for				
Very little	9	166	1.00	Reference
Not sure	1	132	0.13 (0.01 – 0.72)	0.06
A lot	103	1,331	1.46 (0.77 – 3.16)	0.29
Why do you not use PPE?				
No answer	57	818	1.00	Reference
Cost money	5	143	0.48 (0.17 – 1.12)	0.13
Inconvenience	51	668	1.10 (0.74 – 1.63)	0.62
Are your poultry kept at the market overnight?				
No	45	769	1.00	Reference
Yes	68	860	1.38 (0.94 – 2.05)	0.10
Have you attended a course on AI?				
No	57	661	1.00	Reference
Yes	56	968	0.65 (0.44 – 0.95)	0.03
Market type:				
Non–intervention	40	485	1.00	Reference
Intervention	73	1,144	0.76 (0.51 – 1.14)	0.18
Sampling round:				
The 1st (August 2014)	64	1,078	1.00	Reference
The 2nd (December 2014)	49	551	1.55 (1.05 – 2.27)	0.03

markets which had a higher volume of sale than the non-intervention markets.

Figure 8 demonstrates the variation of AIV isolation positivity prevalence amongst intervention and non-intervention LBMs. After the 2nd round of sampling, there was no reduction of AIV prevalence, in either the intervention or non-intervention LBMs.

Multivariable logistic regression analyses

Estimated regression coefficients for the effect of the district in which the market was located and estimates of the variability of the market- and seller-level random effect terms from the mixed effects model are provided in Table 8. In the mixed-effects model, district was retained as an explanatory variable because *a priori* it was considered to comprise part of the hierarchical structure of the data. None of the explanatory variables that were associated with the risk of being AIV positive at the $P < 0.2$ level were statistically significant in the final mixed-effects model.

After adjusting for the effect of the district in which a given market was located, the proportions of variance at the individual market-, seller-, and individual bird-level were $(0.4041 \div (0.4041 + 3.3652 + \pi^2/3) = 0.06$, $(3.3652 \div (0.4041 + 3.3652 + \pi^2/3) = 0.48$ and $(\pi^2/3 \div (0.4041 + 3.3652 + \pi^2/3) = 0.46$, respectively. There were relatively large numbers of sellers and individual birds where AIV likelihood was positively associated with unmeasured seller-level as well as bird-level effects. The identifiers of the 83 sellers were sorted in order of their estimated random effect terms and an error bar plot produced showing the point estimate of the seller-level random effect (and its 95% confidence interval) as a function of seller rank (Figure 9).

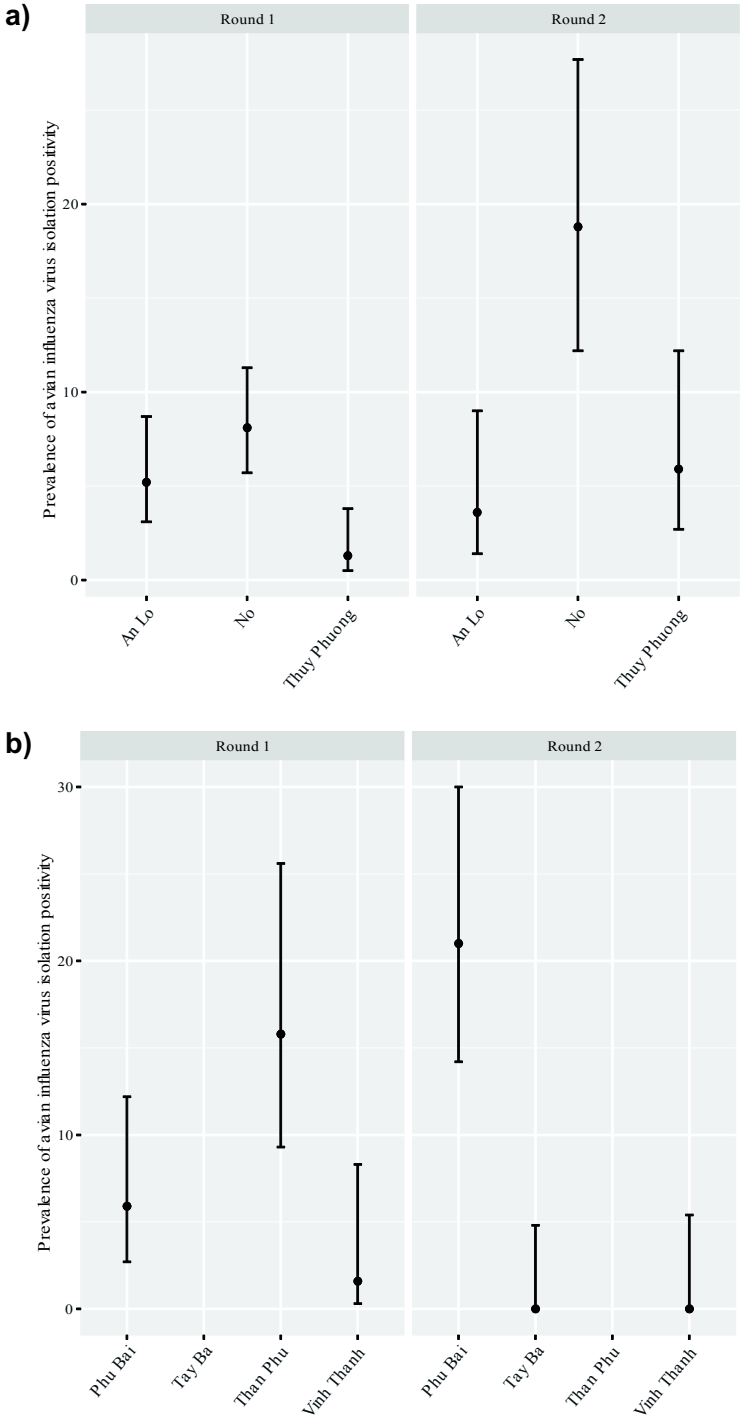

Figure 8. Error bar plots showing avian influenza virus prevalence (and its 95% confidence interval) in the intervention- and non-intervention live bird markets over the two rounds of sample collection.

a) Prevalence of avian influenza viruses in the intervention live bird markets; **b)** Prevalence of avian influenza viruses in the non-intervention live bird markets.

Table 8. Estimated regression coefficients from a mixed-effects logistic regression model

Explanatory variable	VI positive[a]	Total[b]	Coefficient (SE)	*P*-value	OR (95% CI)
Fixed effects					
Intercept	113	1,629	-2.5280 (0.4291)	< 0.01	—
District:					
Huong Thuy	48	606	Reference		1.00
Phong Dien	17	436	-0.6857 (0.7063)	0.33	0.50 (0.13 – 2.01)[c]
Phu Vang	48	587	0.0193 (0.6470)	0.97	1.02 (0.29 – 3.62)
Random effects[d]			Variance	SE	
Market	113	1,629	0.4041	0.3812	
Seller	113	1,629	3.3652	0.6935	

[a] Number of bird samples were positive with avian influenza virus isolation.

[b] Total of bird samples.

[c] Interpretation: The proportion of AI virus isolation positive poultry from sellers from Phong Dien was 0.50 (95% CI 0.13 – 2.10) times that of poultry whose sellers were from Huong Thuy.

[d] Variance and standard error of the variance of the random effect terms.

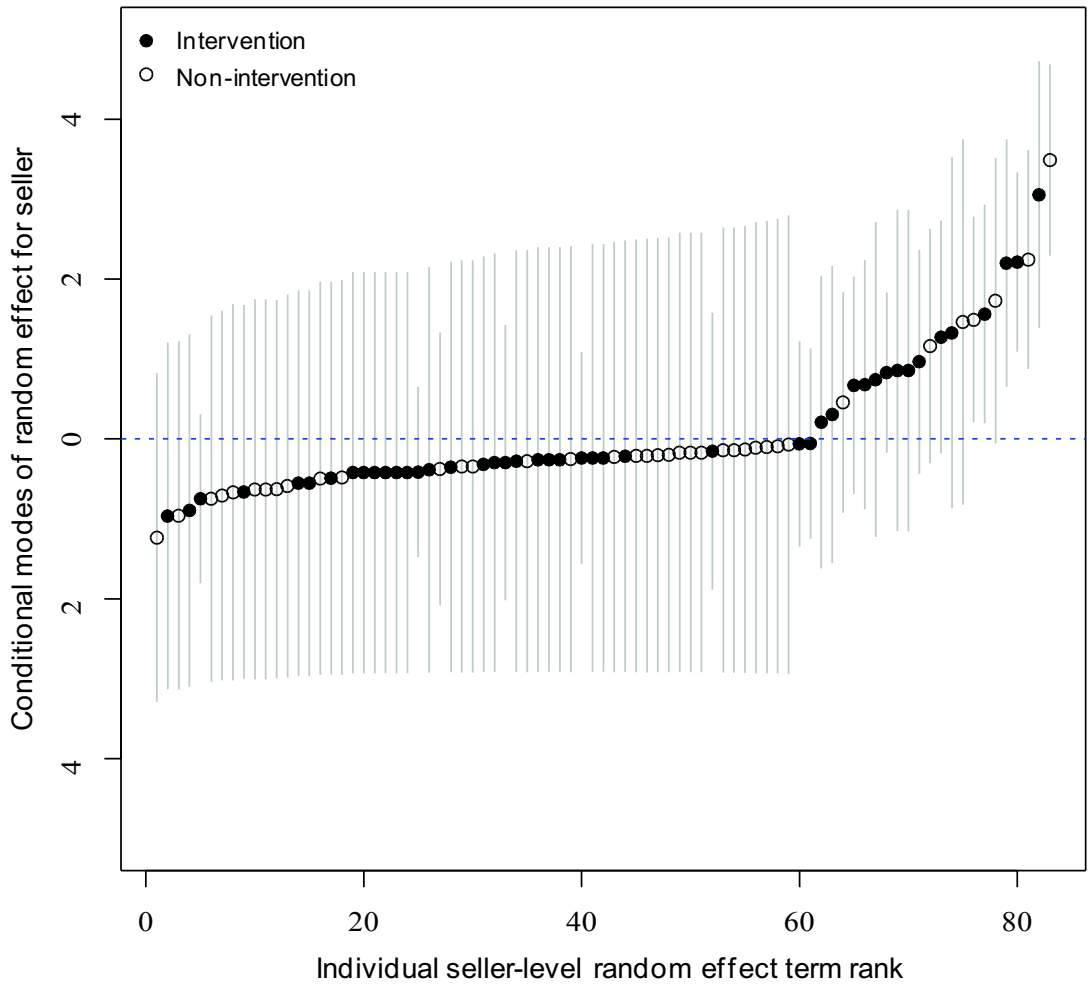

Figure 9. Caterpillar plot showing the point estimate of the individual seller-level random effect terms (and their 95% confidence intervals) for the 83 sellers included in this study.

Discussion

This was a cross-sectional study to quantify the prevalence of AIVs in poultry submitted for sale at seven LBMs in Thua Thien Hue province, central Vietnam. Across the two sampling rounds, a total of 113 out of the 1,629 sampled birds were positive for the AIVs, with a prevalence of 6.9 (95% CI 5.8 to 8.3) AIV positive birds per 100 birds submitted for sale. AIV positivity varied by market and sampling round (Figure 8) with the intervention markets having a relatively low prevalence of AIVs in the first round and marked variation in positivity prevalence in the second. For the non-intervention markets, the prevalence of AIV positivity was variable across both sampling rounds. Our ability to draw definitive conclusions from these data is limited given the relatively small numbers of markets in the intervention and non-intervention groups in each sampling round. At the very least, it is evident that AIV positivity amongst poultry submitted for sale at LBMs varies over time and the prevalence of AIV positivity in birds sampled from LBMs on one occasion will not necessarily be similar to the prevalence of positivity on a second occasion. If sampling of live birds for AIV isolation is to be carried out in future studies, and ignoring the effect of clustering of AIV positivity at the seller-level we estimate that at least 580 birds need to be sampled and tested at the 95% level of confidence under the estimation of 6.9% of the expected prevalence introduced from the present study and desired absolute precision of 2.1% which is equal to 30% of the expected prevalence [58].

While some questionnaire responses were significantly associated with AIV positivity at the unconditional level, adjustment for confounding using the mixed-effects logistic regression model rendered none of the questionnaire variables significantly associated with AIV positivity. There are two explanations for these findings. Firstly, it is possible that a considerable amount of confounding was present

in the data which meant that after adjustment the association between each of the fixed-effect explanatory variables and the study outcome was no longer statistically significant. A second explanation is that the number of birds sampled in our study provided insufficient power to detect associations between certain explanatory variables and the outcome at the alpha level of 0.05 [59], as indicated, more birds were sold in intervention than non-intervention LBMs (Table 7). Although this was more than likely to be the case for some explanatory variables where the prevalence of exposure for AIV positive and negative birds was similar (e.g. gender, where the proportion of AIV positive birds sold by females was 0.93 and the proportion of AIV negative birds sold by females was 0.96) it was not so for others, for example whether or not sellers sourced their birds from the same commune as the LBM (Table 7).

In the multivariable model the inclusion of market-, seller- and individual bird random effect terms was useful in terms of providing an indication of the proportions of variance in AIV positivity that was explained by unmeasured effects operating at each of the three levels. This extension to the model was informative because it provided the opportunity to distinguish the influence of the individual bird, the seller and the market in which birds were sold on the risk of being AIV positive. Our mixed-effects logistic regression model shows that only 6% of the variation in AIV positivity risk was at the market level whereas 48% and 46% of the variation in AIV positivity risk was at the seller and individual bird level, respectively (Table 8). These findings indicate that characteristics of the seller (apart from those measured in the questionnaire) and the birds themselves should be much more likely to contribute the AIV positivity prevalence than market location characters. Furthermore, of the 45 interviewed sellers selling their birds in intervention LBMs in which the odds of the sellers in the intervention group being AIV positive bird was 3.59 (95% CI 1.39 to 9.96) times that of those in the non-intervention group of 38 sellers. Our inference from these findings is

that the emphasis of AI control efforts needs to be at the individual seller-level rather than the market-level. Furthermore, to be effective, interventions need to recognize that sellers at LBMs are a diverse group demographically (Table 8) and, ideally, intervention measures should target specific demographic groupings. Encouragingly, at the bivariate level (at least), those sellers that attended a training course had a reduced risk of having AIV positive birds.

If it is assumed that AIVs enter a market via poultry submitted for sale by individual sellers, it is perhaps not surprising that only 6% of the variation in AIV positivity risk was due to factors operating at the market-level. This finding is biologically plausible, since birds enter a market on a given sale day from a number of geographic locations and it is reasonable to expect that the risk of virus entry into a market depends largely on the location from which birds are sourced. LBMs are licensed or registered under local law to operate from a fixed address and must have a certificate for tracking the source of birds introduced into the market on a given day. Because LBMs are the congregation point for relatively large numbers of (presumably) AI naïve birds they represent ideal surveillance points for estimation of AI prevalence [60]. Poultry remains in the LBMs environment for a relatively short period of time (typically one to two days) so the risk of within-market spread of AIVs is likely to be small. The length of time birds is kept in an LBM, the effectiveness of disinfection and biosecurity procedures may therefore contribute to the prevalence of AIV positivity, although based on our findings the contribution of market effects on AIV positivity prevalence was relatively small. We expected that within market transmission of AIVs to be less in the intervention LBMs. However, field observations showed that there were periodic lapses in cleaning procedures including incomplete coverage of disinfectant and use of disinfectants diluted at incorrect concentrations.

A limitation of this study is that our observations were based on a cross-sectional survey in which LBMs were sampled on only two occasions and the interval between the two sampling rounds was relatively short (approximately 3 months). Reports from market managers and sellers about their biosecurity practices in the LBMs were not verified. Although around 60% of birds were handled by sellers who did not use gloves it is likely that this proportion has been underestimated because of obsequiousness on behalf of questionnaire respondents (that is, sellers altering their responses to a given question to conform with the perceived expectations of the person administering the questionnaire).

Summary

In Vietnam, LBMs are found in most populated centers, providing the means by which fresh poultry can be purchased by consumers for immediate consumption. LBMs are aggregation points for large numbers of poultry and therefore it is common for a range of AIVs to be mixed within LBMs as a result of different poultry types and species being brought together from different geographical locations. We conducted a cross-sectional study in seven LBMs in four districts of Thua Thien Hue province in August and December, 2014. The aims of this study were to: (1) document the prevalence of AI in LBMs (as measured by VI); and (2) quantify individual bird-, seller-, and market-level characteristics that rendered poultry more likely to be positive for AIVs at the time of sale. A questionnaire soliciting details of knowledge, attitude and AI practices was administered to poultry sellers in study markets. At the same time, swabs and fecal samples were collected from individual poultry and submitted for isolation of AIVs. The final dataset comprised samples from 1,629 birds from 83 sellers in the seven LBMs. A total of 113 birds were positive for VI; a prevalence of 6.9 (95% CI 5.8 to 8.3) AIV positive birds per 100 birds submitted for sale. After adjusting for clustering at the market- and individual seller-level, none of the explanatory variables solicited in the questionnaire were significantly associated with AIV isolation positivity. The proportions of variance at the individual market-, seller-, and individual bird-level were 6%, 48% and 46%, respectively. We conclude that the emphasis of AI control efforts in Vietnam should be at the individual seller- as opposed to the market-level.

Chapter III

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

Introduction

In March 2013, the first cases of human infection with an H7N9 influenza A virus were identified in China. As of November 21, 2016, 800 cases of human infection and 322 deaths have been reported, and the number of cases continues to increase in China [15]. The HA and the 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 [61, 62]. Characterization of the H7N9 influenza virus indicated that it has the potential to infect humans [63, 64]. Currently, no vaccine is available for the prevention of H7N9 influenza virus infection. The urgent need for the development of such a vaccine for humans has been acknowledged by the WHO [16].

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 [65-67]. 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 [68-71]. In addition, whole virion influenza vaccines are shown to be more effective than ether-split vaccines [71, 72].

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) in mouse model.

Materials and methods

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 [73]. Dk/Mon/119/08 was applied to plaque purification twice in Madin–Darby canine kidney (MDCK) cells to improve the propagation efficiency. All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and infectious allantoic fluids were stored at −80°C until use.

MDCK cells were maintained in minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% calf serum and used for titration of viral infectivity.

Sequencing and phylogenetic analysis

Viral RNAs were extracted from the allantoic fluids of chicken embryos infected with viruses using TRIzol LS Reagent (Life Technologies, Foster City, CA, USA) and reverse-transcribed using the Uni12 primer [25] and M-MLV reverse transcriptase (Life Technologies). The cDNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Inc., Shiga, Japan). Primers used in this study are as follows; BmHA-1F, 5′-primer TATTCGTCTCAGGGAGCAAAAGCAGGGG-3′; NS-890R, 5′-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3′; H7-368F, 5′-primer CAGGCGGAATTGACAAGGAG-3′; and H7-1141R, 5′-primer

TGCAGCAGTTCCCTCTCCTTGTGC-3′ [16]. PCR products were reacted with gene-specific primers and using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Sequences of the DNA templates were determined using the 3500/3500xL genetic analyzer (Life Technologies). Sequencing data was analyzed using GENETYX version 11 (Genetyx Corporation, Tokyo, Japan). For phylogenetic analysis, sequence data obtained for the genes together with those from GenBank/EMBL/DDBJ and GISAID database (Table 9) were analyzed using the neighbor-joining method [74] with MEGA 5.0 software [26].

Antigenic analysis

The antigenic properties of Dk/Mon/119/08 and Anhui/1/13 were assessed using hyper-immunized chicken antisera against five H7 viruses by HI test according to a standard protocol [75]. HI titers were expressed as the reciprocals of the highest serum dilutions that showed complete HI.

Anhui/1/13, Dk/Mon/119/08, and other H7 influenza viruses were antigenically compared by the fluorescent antibody (FA) method using monoclonal antibodies (MAbs) to the H7 HA according to the method of Sakabe *et al*. [76]. In brief, MDCK cells infected with H7 influenza viruses were fixed with cold 100% acetone at 8 h after inoculation. The reactivity patterns of the H7 viruses with MAbs were investigated with an FITC-conjugated goat anti-mouse IgG (MP Biomedicals, Santa Ana, CA, USA) using a fluorescence microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany).

Vaccine preparation

Dk/Mon/119/08 and Anhui/1/13 were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and propagated at 35°C for 48 hours. The viruses in the allantoic fluids were purified by differential centrifugation and sedimentation

Table 9. H7 viruses used in this study

Virus	Strain name	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.

through a sucrose gradient [24]. The protein concentration was measured using the BCA Protein Assay Reagent (Thermo Fisher Scientific K. K., Waltham, MA, USA). The purified virus was inactivated with 0.1% formalin at 4°C for 7 days. The HA content was standardized according to the method of Ninomiya *et al*. [77]. On the basis of this method, dose of HA concentration was estimated 14.7 μg in 50 μg of vaccine.

Potency test of vaccine against Anhui/1/13 in mice

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

Results

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 (H7N9), A/duck/Mongolia/128/2008 (H7N9), and A/duck/Mongolia/129/2010 (H7N9) 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 (H7N9) and Anhui/1/13 (H7N9) were antigenically analyzed by the neutralization tests (Table 10). The infectivity of Anhui/1/13 was neutralized by all the antisera to H7 AIVs, particularly with the antiserum to Dk/Mon/119/08 (H7N9) 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 (H7N9), 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 11). The results revealed that HA of Dk/Mon/119/08 (H7N9) is antigenically related closely to that of Anhui/1/13 (H7N9).

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 9), were phylogenetically analyzed using the neighbor-joining method (Figure 10). The H7N9 viruses isolated from humans were closely related to H7 LPAIVs. 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.

Table 10. Cross-reactivity of H7N9 viruses with antisera by neutralization test

Lineage	Viruses	Antiserum to[a]				
		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/HongKong/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**

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

Table 11. 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*., [86]).

[c] MAbs to the HA of Duck/Hokkaido/Vac-2/04 (H7N7) (Sakabe *et al*., [76]).

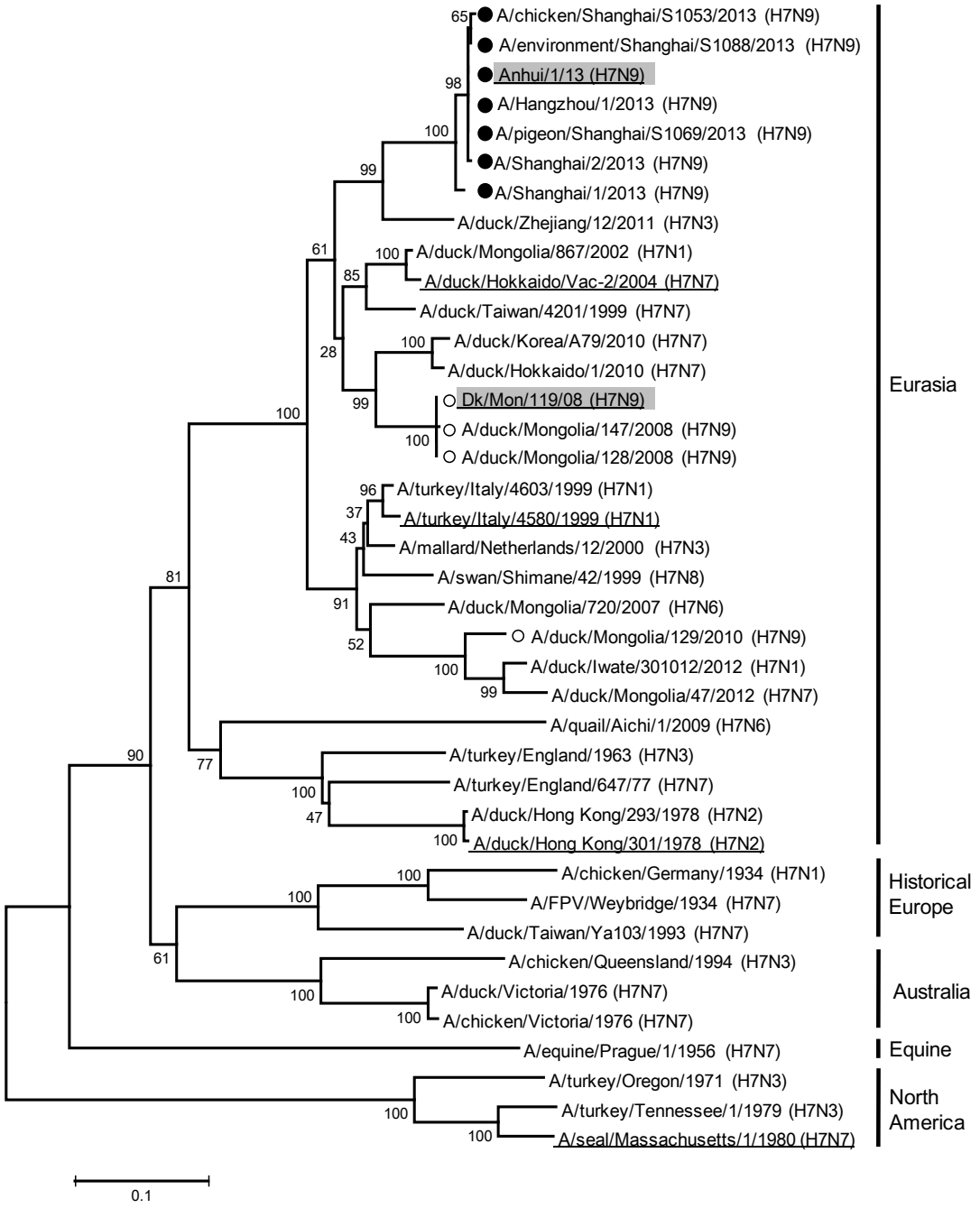

Figure 10. Phylogenetic tree of H7 HA of influenza viruses.

Full-length nucleotide sequences of the HA genes were used for the 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 a bootstrap analysis with 1,000 replicates. Viruses were used to generate vaccines are highlighted. H7N9 influenza viruses in China are indicated by black circles and those stocked in the virus library are indicated by white circles. H7 viruses used for neutralization tests are underlined.

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 vaccinated mice against Anhui/1/13 were measured (Table 12). 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 12). 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 significant 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 Figure 11. 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 13 and Figure 12). 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 13). 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 (Figure 12). The virus titers in the lungs of the mice vaccinated twice with either 50 μg or 10 μg 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.

Table 12. 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.

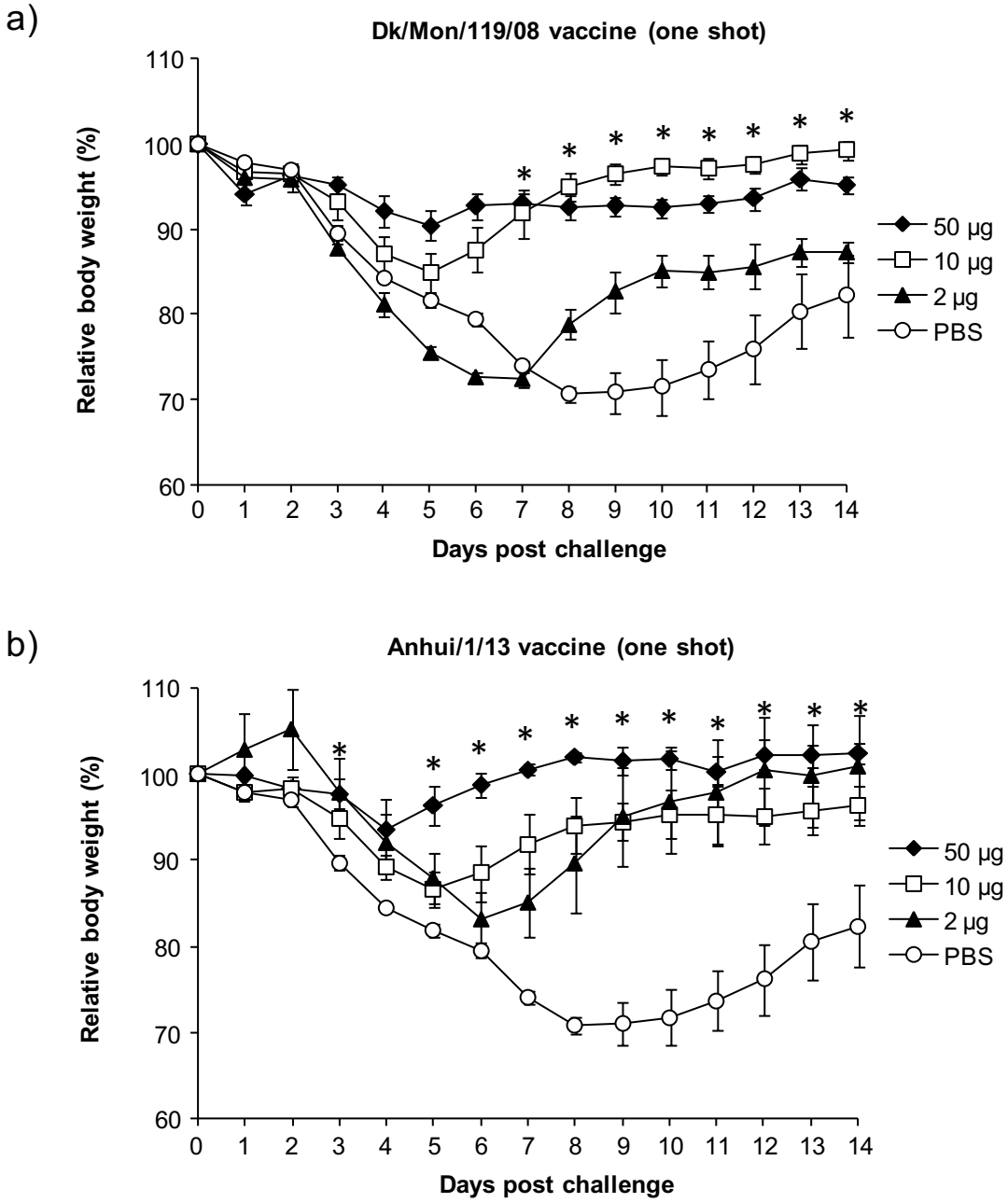

Figure 11. Changes in body weight of mice vaccinated once following challenge with Anhui/1/13.

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

Table 13. 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.

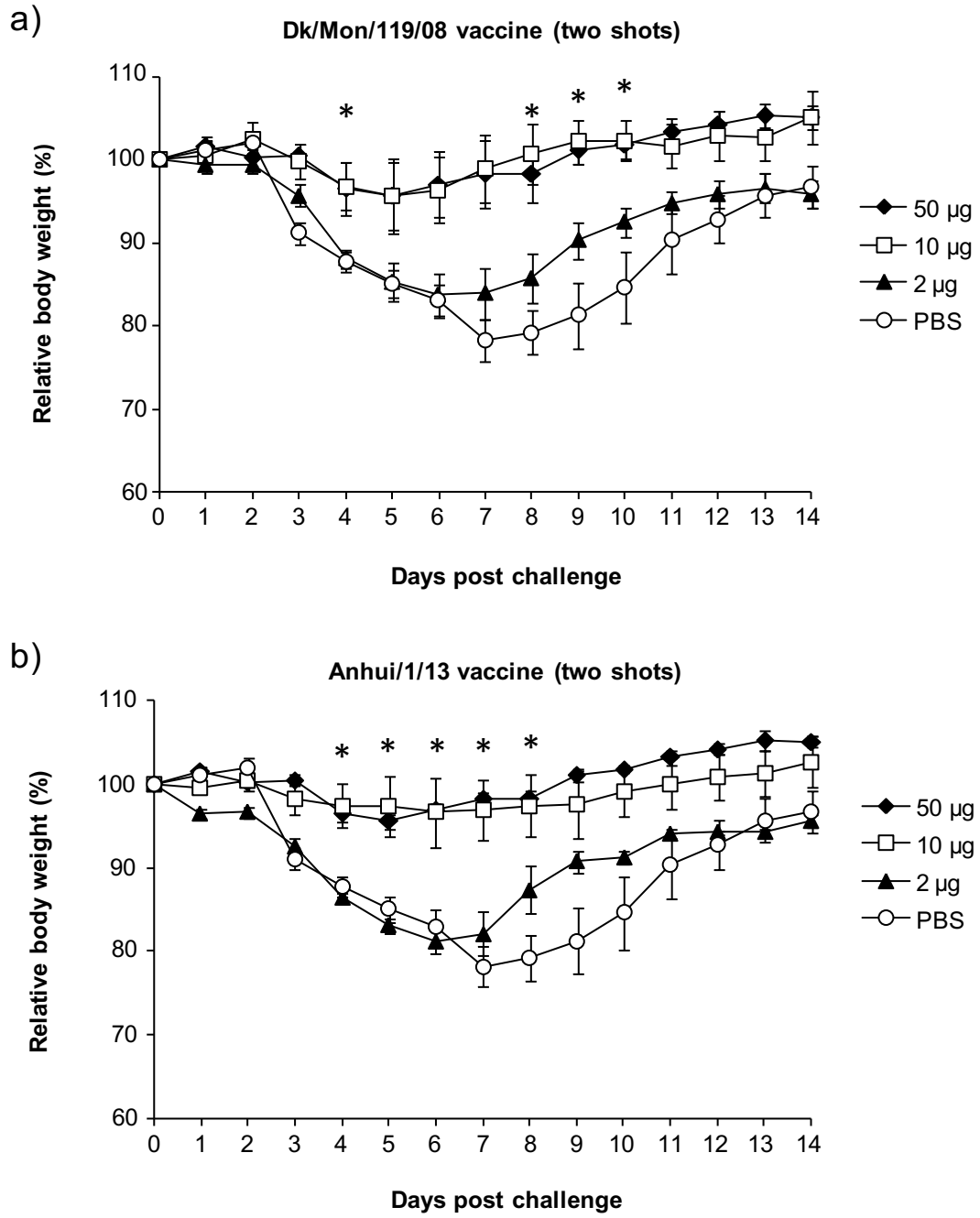

Figure 12. Changes in body weight of mice vaccinated twice following challenge with Anhui/1/13.

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

Discussion

Before 2013, the infections with H7 subtype virus were reported in humans, including A/Netherlands/33/03 (H7N7), A/New York/107/2003 (H7N2), and Seal/Mass/1/80 (H7N7), as a result of direct transmission from animal to human or laboratory accidents [78-81]. 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 the genes of avian origin [61]. Some isolates of the H7N9 influenza viruses bind equally well to human- and avian-type receptors [63]. Although there is no case of human-to-human transmission of H7N9 influenza viruses, these viruses may have the potential to cause pandemic influenza in humans. Consequently, vaccines are required for H7N9 influenza virus infection in humans.

In the early stage of influenza pandemic, the antigenicity, pathogenicity and growth ability of novel virus may not be known. In this study, we compared antigenically and genetically H7 AIVs in the library with Anhui/1/13 virus. Anhui/1/13 is antigenically similar to H7 AIVs isolated from ducks (Table 10). Kida *et al*. showed that antigenic drift extensively occurs in human strains, whereas the hemagglutinins of duck viruses were antigenically highly conserved [82]. Therefore, the pandemic virus strains emerging in humans are considered to be antigenically similar to that of AIV strains in the library. The Dk/Mon/119/08 replicated efficiently in embryonated chicken eggs and was low pathogenic in chicken embryos (data not shown). The HA of Dk/Mon/119/08 was antigenically similar to that of A/Anhui/1/13. Therefore, Dk/Mon/119/08 should be an ideal vaccine strain for H7N9 virus infection.

Whole virus particle vaccines have been reported to induce protective immunity more effectively than ether-split vaccines [71, 72], and the influenza H7N9 virus-like particle vaccine was effective in mice against a challenge with H7N9 influenza virus

isolated from humans [83]. In the present study, an inactivated whole particle H7N9 influenza vaccine was prepared from an H7N9 AIV, Dk/Mon/119/08, present in the influenza virus library [73]. In the mice injected once with Anhui/1/13 vaccine or Dk/Mon/119/08 vaccine, virus titers in the lungs of mice after the challenge with A/Anhui/1/13 virus were lower compared with those of control animals. One shot of Dk/Mon/119/08 vaccine conferred significant immunity in mice against the challenge with Anhui/1/13. Two shots of the vaccines induced stronger immunity to prevent the body weight loss and to reduce virus replication in the lungs of mice than those of one shot of vaccines (Table 13 and Figure 12). These results indicate that Dk/Mon/119/08 vaccine induced enough immunity to prevent the impact of the disease.

Vaccination is one of the important control measures against influenza. Approximately 6 months is required to produce vaccines [84, 85]. To prepare for future influenza pandemics, we have conducted surveillance of AI since 1977. The pathogenicity, antigenicity, genetic information, and yield in embryonated chicken eggs of the virus strains in the library have been assessed. AIVs of 144 combinations of 16 HA and 9 NA subtypes are stocked in our influenza virus library. The present whole virus vaccine prepared from an influenza virus from the library should be useful as a vaccine strain in the case of the emergence of influenza pandemic.

Summary

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 AIV, 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.

Conclusion

LBMs are ubiquitous and integral parts of the poultry industry in Vietnam and other developing countries in Asia. In LBMs, it is common for a range of subtypes of AIVs to be mixed as a result of different poultry types and species being brought together from different geographical locations. In Southeast Asia, HPAIVs are known to circulate in LBMs and it has been hypothesized that LBMs may facilitate the emergence and spread of new viral reassortants due to close contact amongst the infected birds. Furthermore, it has also been aware that, in China, human infections with AIVs, in particular, of the subtypes H5N1, H5N6, and H7N9 associated with recent exposure to poultry in LBMs even though LBM closure had been conducted in real AIVs outbreaks. In addition, the number of human cases infected with H7N9 AIVs has increased in China based on WHO notifications from 2013 to 2016. However, termination of LBMs in developing countries remains challenging because changing the traditional market style should take a long time. Therefore, government intervention to improve the biosecurity measures employed at LBMs has been thought to represent a promising strategy to minimize the transmission of viruses in Asian countries including Vietnam.

To support the evaluation of local authority on the current intervention, we surveyed the prevalence of AIVs in either intervention or non-intervention. In Chapter I, various subtypes of AIVs were isolated during surveillance of LBMs with or without intervention. The 178 AIVs, including H3 (19 viruses), H4 (2), H5 (8), H6 (30), H9 (114), and H11 (5), were isolated from domestic ducks, Muscovy ducks, chickens, and the environment. The prevalence of AIVs in intervention LBMs (6.1%; 95% CI: 5.0 to 7.5) was similar to that in non-intervention LBMs (5.6%; 95% CI: 4.5 to 6.8; χ^2 = 0.532; *df* = 1; *P* = 0.53) in the study area. Eight H5N6 HPAIVs were isolated from

apparently healthy ducks, muscovy ducks, and an environmental sample in an intervention LBM. The HA of the H5N6 HPAIVs belonged to the genetic clade 2.3.4.4, and the antigenicity of the H5N6 HPAIVs differs from the H5N1 HPAIVs previously circulating in Vietnam. Phylogenetic and antigenic analyses of the H6 and H9 viruses isolated in both types of LBMs revealed that they were closely related to the viruses isolated from domestic birds in China, Group II of H6 viruses and Y280 lineage of H9 viruses. These results indicate that the interventions currently applied in LBMs are insufficient to control AI. A risk analysis should be conducted to identify the key factors contributing to AIVs prevalence in intervention LBMs.

To identify the factors contributing to AIVs circulation in both types of LBMs, Chapter II focused on quantification of bird-, poultry seller- and market-level characteristics that rendered individual birds more likely to be AIV isolation positive at the time of sale. Identifying the relative importance of factors was influencing the poultry submitted for sale at LBMs in Vietnam. The present results demonstrate that after adjusting for clustering at the market- and individual seller-level none of the explanatory variables solicited in the questionnaire were significantly associated with AIV positivity. A relatively small component of the variation in AIV positivity risk was at the individual market-level. It indicates that the emphasis of AI control efforts should be at the seller-level rather than market-level.

In Chapter III, to prepare for the emergence of H7N9 influenza pandemic, an inactivated whole particle H7N9 influenza vaccine was selected from an H7N9 AIV, A/duck/Mongolia/119/2008 (H7N9), present in the influenza virus library. In the mice injected once with A/Anhui/1/2013 (H7N9) vaccine or A/duck/Mongolia/119/2008 (H7N9) vaccine, virus titers in the lungs of mice were lower compared with those of control animals after the challenge with A/Anhui/1/2013 (H7N9) virus. One shot of A/duck/Mongolia/119/2008 (H7N9) vaccine conferred protective immunity in mice

against the challenge with A/Anhui/1/2013 (H7N9) virus. Two shots of the vaccines induced stronger immunity to prevent the body weight loss and to reduce virus replication in the lungs of mice than those of one shot of vaccines. These results indicate that A/duck/Mongolia/119/2008 (H7N9) vaccine induced enough immunity to prevent the impact of the disease. 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.

These present studies provide a better understanding of AIVs circulation and current AI control strategies for poultry and humans. It is also support to the early preparation of influenza pandemic.

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Appendix

Baseline questionnaire for HPAI investigation on the knowledge, attitude, and practices of seller at live bird market

INFORMATION TO READ TO RESPONDENT:

Good morning/afternoon/evening. I am ……, a veterinarian of.…….

We are planning a study from August 2014 and March 2015 to identify potential risk factors of avian influenza. The information will help finding appropriate control and prevention strategies for HPAI in Vietnam.

Participation in this survey will take approximately 40 minutes. Your participation in this research is voluntary. There is a possibility that you may feel uncomfortable with the questions, but you may stop whenever you want or skip the question.

There are no risks for participating.

If at any time during the interview you are not clear about the question, be sure to ask me.

If you have any questions later, please contact

- Dr. Nguyen Van Hung, Director of Thua Thien Hue Sub-Department of Animal Health

- Dr. Chu Duc Huy, Vietnam Department of Animal Health

- Or Dr. Nguyen Van Long, Vietnam Department of Animal Health

(For poultry seller/shop keeper)

Date of investigation	_ _ / _ _ / _ _ _ _
Name of investigator	

Address

Province	
District	
Commune	

Name of the market	
Type of market	
Name of shop owner/seller	
Phone number of seller	
Order collected sample	

X co-ordinate: …… Y co-ordinate: ……

A. GENERAL INFORMATION

1. How old are you?
   - ☐ Under 20
   - ☐ 21-30
   - ☐ 31-40
   - ☐ 41-50
   - ☐ Over 50

2. What is your gender?
   - ☐ Male
   - ☐ Female

3. What is your highest education?
   - ☐ None
   - ☐ Elementary
   - ☐ Middle school
   - ☐ High school
   - ☐ College
   - Other ……..

4. How long have you involved in poultry trading?
   - ☐ Under 1 year
   - ☐ 1-5 years
   - ☐ 6-10 years
   - ☐ Over 10 years

5. What type of birds do you usually sell?
   - ☐ Chickens
   - ☐ Ducks
   - ☐ Muscovy duck
   - ☐ Pigeons
   - ☐ Quails
   - ☐ Other……

6. What is the approximate number of birds that you sell every day?

Chickens: Pigeons: ……
……..
Ducks: Quails: ……
……
Muscovy duck: …….. Other: ……..……

7. Which month in the year was the best seller of your poultry?

Please specify the month:……..

8. Where do you usually buy your poultry?

☐ Same commune

☐ Different commune ☞

Address 1: Prov……..,
Dist…….,Comm……
Address 2: Prov……..,
Dist…….,Comm……
Address 3: Prov……..,
Dist…….,Comm……

☐ Trader in the same commune

☐ Trader in different commune ☞

Address 1: Prov……..,
Dist…….,Comm……
Address 2: Prov……..,
Dist…….,Comm……
Address 3: Prov……..,
Dist…….,Comm……

9. What is the reason for buying the poultry from that source?

☐ Price ☐ Relationship
☐ Convenience ☐ Other:……..

B. KNOWLEDGE

(Do not read the answers in this part)

1. Have you ever heard about AI (bird flu)?

☐ Yes ☐ No

If **No**, terminate the interview

2. What is the causative agent of AI?

☐ Virus ☐ Bacteria ☐ Parasite ☐ Don't know

3. Which animals can be infected with AI?

☐ Only chicken ☐ Poultry ☐ Mammals ☐ Don't know

4. From where did you learn about AI?

☐ TV
☐ Radio
☐ Newspaper
☐ Brochure
☐ Market manager
☐ Animal health worker
☐ Training course
☐ Other….

5. Do you think that AI can be prevented? (if the answer is Not sure/Don't know, skip Q6)

☐ Yes ☐ Not sure ☐ Don't know

6. In your opinion, can you tell me something that you think you could do to prevent AI in your poultry when you introduce the new flocks or handling/slaughtering them?

☐ Vaccine
☐ Keep poultry in good condition (clean area)
☐ Separate species
☐ Keep separately all poultry from other poultry for at least 2 weeks
☐ Wash hand with soap before and after taking care of poultry and other animal
☐ Wear gloves
☐ Wear a mask
☐ Other.....

7. In your opinion, how is AI spread among poultry?

☐ Contact with infected bird
☐ Contact with contaminated equipment
☐ Contact with virus brought by people, their clothing or footwear
☐ Other….
☐ Don't know

8. In your opinion, how is AI spread in humans?

☐ Contact with infected or sick bird
☐ Contact with contaminated equipment
☐ Eat duck blood pudding
☐ Other….
☐ Don't know

9. Have you ever seen the infected poultry with AI showing clinical signs?

☐ Yes
☐ No
☐ Not sure

10. Which infected avian species will show the clinical signs?

- ☐ Chicken
- ☐ Duck
- ☐ Muscovy duck
- ☐ Not at all

11. Do you know the clinical signs of AI in poultry?

- ☐ Sleepiness
- ☐ Dark/red/blue comb and wattles
- ☐ Swollen and puffy looking eyes
- ☐ Sudden death in large number
- ☐ Ruffled feathers
- ☐ Diarrhea
- ☐ Other….
- ☐ Don't know

12. What do you do with your poultry that you suspect have AI?

- ☐ Keep them in a closed building/separate from other poultry and animal
- ☐ Sell them
- ☐ Slaughter for food
- ☐ Throw them away in river or pond
- ☐ Kill them and bury them
- ☐ Burn them
- ☐ Report to local authority
- ☐ Give antibiotics
- ☐ Do nothing
- ☐ Other…….

13. What will you do if there is an outbreak of AI in the area where you purchase your poultry?

- ☐ Sell off all your poultry
- ☐ Follow animal health authority instruction
- ☐ Do nothing
- ☐ Other…..

14. Have you ever attended, been trained or participated in an activity that educated about bird flu?

- ☐ Yes — How many times?.....
- ☐ No — When is the latest time?.....

15. Do you know about Decree number 119/2013/NĐ-CP dated 09-10-2013 of Prime minister on the regulations of administrative sanctions in the field of animal health, livestock, animal feeds, and Circular number 53/2013/TT-BNNPTNT dated 12-12-2013 of the Ministry of Agriculture and Rural Development for terrestrial animal diseases reporting regulations?

- ☐ Yes
- ☐ No
- ☐ Not sure

C. ATTITUDES

1. If you thought you had a bird flu case in your cage or near your shop (other owner) would you report it? (If the answer is No/Not sure, skip Q2)
   - ☐ Yes
   - ☐ No
   - ☐ Not sure

2. To whom would you be more likely to report suspected cases of bird flu in poultry?
   - ☐ Market manager
   - ☐ Veterinarian
   - ☐ Local authority

3. Do you think you will be safe from bird flu without using PPE in handling the poultry?
   - ☐ Yes
   - ☐ No
   - ☐ Not sure

4. Bird flu issues are important for your business?
   - ☐ Yes
   - ☐ No
   - ☐ Not sure

5. How serious a problem do you think bird flu is in Vietnam or your region?
   - ☐ Very
   - ☐ Somewhat
   - ☐ Not very

6. Do you feel well informed about bird flu?
   - ☐ Yes
   - ☐ No

7. Do you agree with the current solutions of local authority for the control of AI?
   - ☐ Yes
   - ☐ No
   - ☐ Not sure

8. Do you think the programs of AI control will give you more benefits?
   - ☐ Very
   - ☐ Somewhat
   - ☐ Not sure

9. Do you think that for the control of AI is a part of your responsibility?
   - ☐ Yes
   - ☐ No
   - ☐ Not sure

10. Do you wish you could get more information about bird flu?
    - ☐ Yes

☐ No

11. What are the sources of information you think can get effectively on bird flu?

☐ TV
☐ Radio
☐ Poster, brochures
☐ Newpapers
☐ Market manager
☐ Animal health workers
☐ Family, friends, neighbors and colleagues
☐ Other…….

D. PRACTICES

1. Do you use the personal protective equipment (e.g. mask, gloves) when handling or slaughtering live birds? (should be checked directly by interviewer)

☐ Yes
☐ No

❖ If the answer is **Yes**: How often do you use?

☐ Every time
☐ Sometime

❖ If the answer is **No**: Why did not you use?

☐ Cost money
☐ It is not convenience
☐ I don't believe it help to protect from AI

2. Do you use the personal protective equipment (e.g. mask, gloves) when contacting with sick or dead birds?

☐ Every time
☐ Sometime
☐ Never

3. Do you use soap or disinfectant to clean your hands and equipment after finishing your work?

☐ Yes
☐ No

❖ If the answer is **Yes**: How often do you use?

☐ Every time
☐ Sometime

❖ If the answer is **No**: Why did not you use?

☐ Cost money ☐ I don't believe it help to protect from AI
☐ It is not convenience

4. Do you spray disinfectant on your vehicles before and after you use for transport poultry?
   ☐ Every time ☐ Never
   ☐ Sometime

5. Do you use the same vehicle to carry other products or humans (your family)?
   ☐ Every time ☐ Never
   ☐ Sometime

6. What will you do when you find the dead birds during your transportation? (select more than 1)
   ☐ Keep them in sealed plastic bags ☐ Burn them
   ☐ Sell them ☐ Report to animal health workers
   ☐ Slaughter for food ☐ Other…….
   ☐ Throw them away on the road ☐

7. Do you sanitate the lairage?
   ☐ Yes
   ☐ No

   ❖ If the answer is **Yes**: How often do you sanitate the lairage?
   ☐ Every day ☐ After selling batch
   ☐ Every week ☐ Never
   ☐ Every month

   ❖ If the answer is **Yes**: How do you sanitate lairage area?
   ☐ Cleaning by normal water ☐ By disinfection materials
   ☐ Cleaning by brush

   ❖ If the answer is **No**: Why did not you clean up?
   ☐ Cost money and waste time ☐ I don't believe it help to protect from AI
   ☐ It not my responsibility, it belong to market manager ☐ Not required

8. What will you do when you find the **sick birds** in your business area? (select more than 1 answer)

- ☐ Keep them in separate from other poultry
- ☐ Sell them as soon as possible
- ☐ Give them antibiotics
- ☐ Slaughter for food
- ☐ Report to animal health workers
- ☐ Do nothing

9. What will you do when you find the **dead birds** in your business area? (select more than 1 answer)
   - ☐ Keep them in separate from other poultry
   - ☐ Sell them
   - ☐ Slaughter for food
   - ☐ Throw them away
   - ☐ Bury or burn them
   - ☐ Report to animal health workers
   - ☐ Other…….

10. Do you separate the new imported birds?
    - ☐ Yes
    - ☐ No

11. Do you keep chickens separate with ducks or muscovy duck? (interviewer should observe the real situation)
    - ☐ Yes
    - ☐ No

12. Where and how did you keep the remaining birds of the day?
    - ☐ In your own shop
    - ☐ Bring them to your home and
      1. keep them separate with your living area ☐
      2. keep them together with other animals ☐
      3. keep them anywhere based on your convenience ☐

Thank you very much for participating in our survey.