Study on the control of high pathogenicity and low pathogenicity avian influenza in Vietnam

(ベトナムにおける高病原性および低病原性鳥インフルエンザの制御に関する研究)

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Table of contents

Table of figures and tables

Abbreviations

AI	avian influenza
AIV(s)	avian influenza virus(es)
ANOVA	analysis of variance
AUC	area under the ROC curve
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
DAH	Department of Animal Health, Vietnam
Gs/GD	A/Goose/Guangdong/1/1996 (H5N1)
HA	hemagglutinin
HCPC	hierarchical clustering on principal components
HI	hemagglutination inhibition
HPAI	high pathogenicity avian influenza
HPAIV(s)	high pathogenicity avian influenza virus(es)
LBM(s)	live bird market(s)
LPAI	low pathogenicity avian influenza
LPAIV(s)	low pathogenicity avian influenza virus(es)
M	matrix
MCA	Multiple correspondence analysis
min	minute
ml	milliliter
mg	milligram
µl	microliter
ML	maximum likelihood
NA	neuraminidase

NI	neuraminidase inhibition
NP	nucleoprotein
NS	nonstructural
OIE	World Organization for Animal Health
OR	odds ratio
PA	polymerase acidic
PB1	polymerase basic 1
PB2	polymerase basic 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDS(s)	poultry delivery station(s)
PPE	personal protective equipment
RAHO7	Regional Animal Health Office No. 7
RNA	ribonucleic acid
ROC	Receiver Operating Characteristic
RT-PCR	reverse transcription polymerase chain reaction
SDAH	Sub-Department of Animal Health, Vietnam
SE	standard error

Notes

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

1. **Le, K. T.,** Okamatsu, M., Nguyen, L. T., Matsuno, K., Chu, D. H., Tien, T. N., Le, T. T., Kida, H. and Sakoda, Y. Genetic and antigenic characterization of the first H7N7 low pathogenic avian influenza viruses isolated in Vietnam. **Infect. Genet. Evol. 2020, 78, 104117–104125. doi: 10.1016/j.meegid.2019.104117.**

2. **Le, K. T.,** Stevenson, M. A., Isoda, N., Nguyen, L. T., Chu, D. H., Nguyen, T. N., Nguyen, L. V., Tien, T. N., Le, T. T., Matsuno, K., Okamatsu, M. and Sakoda, Y. 2021. A systematic approach to illuminate a new hot spot of avian influenza virus circulation in South Vietnam, 2016–2017. **Transbound. Emerg. Dis. 2021, In print doi: 10.1111/tbed.14380.**

Preface

The poultry industry is a significant contributor to world total meat production, accounting for 132 million tons (39%) in 2019 [1]. The market value of the poultry sector has increased yearly; especially in developing countries, poultry production is the primary source of income for over 80% of rural households. Therefore, once a disease emerges then spreads widely and quickly, it can cause severe severe damage to the poultry industry. The high pathogenicity avian influenza (HPAI) was one of the most contagious diseases in poultry described in the World Organization for Animal Health (OIE) list A disease. Avian influenza (AI), specifically HPAI, is the biggest concern for the global poultry industry because of its high mortality, high transmissibility, and transboundary spread, resulting in a substantial negative impact on poultry production and significant escalation of disease control costs.

AI is caused by the infection of avian influenza viruses (AIVs), which belong to the *Alphainfluenzavirus* genus of the *Orthomyxoviridae* family. AIVs have a genome made up of eight negative-sense single-stranded ribonucleic acid (RNA) segments [2]. The viral RNA was contained in the capsid, and the envelope formed the particle, which ranged in size from 80 to 120 nm in diameter. Based on the antigenic characteristics of surface glycoproteins, AIVs are classified into 16 hemagglutinin (HA, H1–H16) and 9 neuraminidase (NA, N1–N9) subtypes [3-5], and all subtypes have been detected in wild aquatic birds, specifically wild ducks, which are identified as natural reservoirs of AIVs. Recently, the novel influenza A viruses wet genetically detected in bats and proposed as the H17N10 and H18N11 viruses [6]. Considering the pathogenicity of these viruses in chicken, AIVs are categorized into high pathogenicity AIVs (HPAIVs) and low pathogenicity AIVs (LPAIVs). While the pathogenicity of influenza A viruses in chickens ranges from asymptomatic to systemic infections with high mortality, its infection in ducks rarely shows any clinical signs [7]. The emergence of HPAIV from LPAIV has been reported worldwide [8-12], and the acquisition of high virulence of AIV in terrestrial birds was attributed to the consecutive infection in the terrestrial birds. Until now, this event has only been reported in H5 and H7 subtypes.

One of the most well-known HPAIVs is A/goose/Guangdong/1/1996 (H5N1) (Gs/GD), which was firstly isolated from sick domestic geese in Guangdong province,

China, in 1996. The first outbreak of H5 HPAIVs Gs/GD-lineage was reported in both poultry and humans in Hong Kong in 1997, resulting in the the huge economic loss including millions of chickens culled [13]. Since its emergence, the H5 HPAIVs Gs/GD-lineage has caused enormous damage to the global economy, especially for developing countries that lack many tools, such as facilities, infrastructure, and software like human resources, monitoring/reporting systems to support for the control strategy against the spread of this virus. From late 2003 to early 2004, outbreaks related to this virus were reported widely in Asia, including mainland China, South Korea, Japan, Taiwan, Vietnam, Thailand, Laos, Cambodia, and Indonesia [14]. In Vietnam, the first outbreaks of H5 HPAI Gs/GD-lineage in poultry were reported in late 2003, with hundreds of outbreaks reported annually [15]. Despite significant attempts to control H5 HPAI through intense countermeasures in several countries, outbreaks of H5 HPAI still occur in Asia [15]. Therefore, the control system in Vietnam, including timely reports and regular surveillance, was launched to minimize the risk of HPAI, in addition to the intensive studies promoted by the government and international organizations to support the AI prevention campaign. The first target for HPAI eradication is to reduce AIV prevalence in the poultry population [16], which will require a combination of five components: education, biosecurity, diagnostics and surveillance, stamping out the infected poultry, and vaccination [17,18].

Due to the implementation of the control measures, the number of H5 HPAI outbreaks had been remarkably reduced [19], but the H5 HPAIV still existed in the poultry population. According to the active surveillance of AIVs, the co-circulation of multi-AIV subtypes in the field has been previously reported [20-22]. HPAIVs were detected in the apparently healthy poultry, whereas LPAIVs, such as H9 and H6, were the major subtypes of AIVs detected in the poultry population. Furthermore, a high prevalence of AIVs was identified in live bird markets (LBMs) followed by backyard farms [20-22]. Unfortunately, the control measure applied in LBMs was ineffective in reducing AIV prevalence [23]. These findings suggest that there is another hot spot where AIV could be continuously circulated and introduced into LBM.

The traditional farming system in Vietnam was a combination of intensive rice cultivation and poultry production. Especially, free-grazing farming was a common model to maximize income using the leftover grains from rice paddies to feed ducks.

Moreover, ~50% of farm households in Vietnam's rural area were backyard farms that practice free-grazing of poultry under low biosecurity conditions [24]. Taken together, the risk of virus exposure from wild birds to domestic birds as well as human was relatively high in Vietnam [14,25]. It was consistent with the annual report from the Department of Animal Health of Vietnam (DAH) that most of H5 HPAI outbreaks were reported in backyard farms. AIV transmission should originate at the farms and spread through the live poultry trading network, and the structure of this network shows clear influences on the spread of AIVs. In detail, the genetic relationship of AIVs within LBM was closer than AIVs between LBMs, and AIVs isolated in the same province were also genetically closer than AIVs isolated in other provinces [26].

Based on the aforementioned issues, previous studies focusing on the evolution of H5 HPAIV strongly indicated a large antigenic distance from H5 HPAIVs isolated in Vietnam to their progenitors or a commercial vaccine antigen [27,28]. H5 HPAIVs that circulated in Vietnam from 2014 to 2017 were likely introduced from China in 2012–2013. These Vietnamese H5 HPAIVs shared genetic traits with other viruses isolated in neighboring countries, implying that transboundary spillover could occur continuously. Furthermore, the H5 HPAI outbreaks were associated with an increase in poultry movement during the Tet holiday, a high density of geese, and the occurrence of previous outbreaks [29]. While the identification of an HPAI outbreak could be notified by the observation of clinical signs and the laboratory diagnosis, LPAIV infection usually causes asymptomatic disease, and a lack of diagnosis for LPAI might result in the misidentification of an LPAI outbreak. In fact, the damage caused by LPAIV infection in farms has been reported globally, however, it is mainly due to egg and meat reduction [30,31]. Unfortunately, if the occurrence of an LPAI outbreak was missed due to misidentification, the necessary data for risk analysis may not be acquired. Collectively, the information on the evolution of LPAIVs, the factors associated with LPAIV infection, and the impact of LPAI in Vietnam remain unclear.

Thus, this study identified the genetic and antigenic characteristics of H6, H7, and H9 subtypes isolated during active surveillance, as described in Chapter I. Discussion on genetic diversity and antigenic stability of these viruses improves the knowledge of LPAIVs circulating in Vietnam. During this surveillance, an intensive investigation was conducted to discover the hidden contributor to the high prevalence of AIVs in LBMs.

By combining the virological and epidemiological studies, the poultry delivery station (PDS) was identified as a new hot spot for AIV circulation, with AIV prevalence even higher than LBM. The detail of this finding was described in Chapter II. In chapter III, a further study targeting LPAIVs was conducted to elucidate the risk factors associated with LPAIV infection in farms. By raising awareness of LPAIV, the outcome of this thesis may contribute to the improvement of the general AI control strategy.

Chapter I

Genetic and antigenic characterization of H6, H7, and H9 low pathogenicity avian influenza viruses isolated in Vietnam

Introduction

AIVs are grouped in the genus *Alphainfluenzavirus* of the *Orthomyxoviridae* family and carry eight negative-stranded RNA segments as their genome [2]. AIVs are categorized based on the antigenic differences of their surface glycoproteins; these glycoproteins comprise 16 HA and 9 NA subtypes. AIVs are also categorized into HPAIVs and LPAIVs, based on their pathogenicity in chickens. Wild aquatic birds are the natural reservoir of AIVs, being capable of harboring all subtypes of AIVs while not usually showing any clinical signs of AIV infection [7]. In this way, AIVs can be maintained and spread globally without being noticed or recognized. Because of their zoonotic potential, AIVs pose a major threat, not only to the global poultry industry but also to human public health [32].

Among AIVs subtypes detected in Asia, the H6 and H9 viruses were reported as the dominant subtypes in wild birds and poultry [33,34]. Although H6 and H9 AIVs subtypes are classified as LPAIVs which originally causes asymptomatic infection but they still possess the potential to cause severe respiratory distress due to the occasional coinfection with other pathogens in the field [35-38]. In addition, H6 and H9 LPAIVs contribute to the generation of the pandemic potential strains via reassortment [39]. Except for H5 and H7 subtypes, the zoonotic potentials were reported in the other LPAIVs such as H6N1 [40,41], H9N2 [42-44] and H10N3 [45] subtype viruses.

Moreover, the infections caused by H7 viruses in both wild birds and domestic poultry have been reported globally, including in Asian countries [46-51]. Recently, an H7N9 LPAIV, capable of causing fatal disease in humans, emerged in China (2013–present) and rapidly evolved into HPAIVs by the insertion of a multibasic cleavage motif on the HA [39,52]. Both H7N9 HPAIV and H7Nx LPAIVs can be continuously introduced into neighboring countries via the migration of wild birds, human movement, live-bird transportation, and the transportation of poultry products [53-55]. Because of the potential impact of H7 AIVs, the occurrence of any viruses belonging to subtype H7 is notifiable, regardless of their actual pathogenicity in chicken; this process aims to reduce the spread of the disease and eventually to achieve its eradication [56].

While no outbreaks of H7 HPAIV infection have been reported in Southeast Asian countries, the detection of H7 LPAIVs has been reported in domestic poultry and wild

birds, e.g., in Singapore (H7N1 in 1994–1995 [57]); Thailand (H7N1 in 2009–2010, H7N6 in 2010, and H7N4 in 2010–2011 [58,59]); Cambodia (H7N3, H7N4, H7N7, and H7N9 2017–2019 [59,60]); and Vietnam (H7N1 in 2012 [20]). Because of the potential for H5/H7 LPAIVs to evolve into HPAIVs during the circulation of these viruses in poultry [61] and the zoonotic potential, the circulation of H5/H7 LPAIVs in the poultry population should be minimized to enhance disease control efforts. Therefore, the Vietnamese government applied many interventions to minimize the transmission of AIVs along with the poultry trading network in Vietnam.

Under the support from OIE, the active surveillance program has been conducted since 2009 to control and prevent AIV infections in poultry in Vietnam [20-22]. During this surveillance program, many H6 and H9 LPAIVs were isolated from poultry, moreover, three H7N7 LPAIVs were isolated from the same flock of domestic ducks in 2018. The present study reports the genetic and antigenic characteristics of H6, H7, and H9 LPAIVs isolated in Vietnam, with the aim of providing a better understanding of the LPAIVs circulating in Asia.

Materials and Methods

Sample collection

From 2014 to 2018, surveillance for avian influenza was conducted in three provinces in Vietnam with detail as follow, Lang Son to detect the introduction from the bordering areas, Hue to monitor the circulation of viruses, and Vinh Long to assess the characteristics of domestic viruses (Figure 1). Oropharyngeal and cloacal swabs were obtained from domestic birds housed at biosecurity or backyard farms, LBMs, and PDSs. Transport medium was used for the preservation of field samples; this medium comprised the minimum essential medium containing penicillin, streptomycin, gentamicin, nystatin, and bovine serum. All samples were stored at −80°C until they could be tested.

Isolation and identification of AIVs

Each sample along with transport medium was inoculated into the allantoic cavity of a 10-day-old chicken embryo from conventional chicken flock tested free of avian influenza virus antibody. After incubation at 35°C for 30–48 h, allantoic fluid exhibiting hemagglutination activity was collected. The influenza virus subtypes were identified by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests with antisera to the reference influenza virus strains [62].

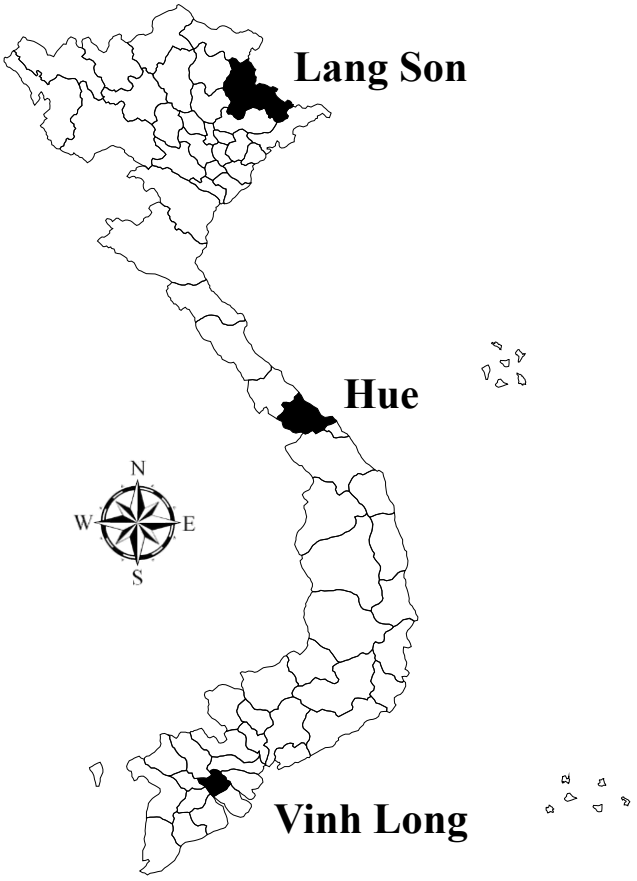


Figure 1. Location of Lang Son, Hue, and Vinh Long provinces where the avian influenza surveillance was conducted. Sampling provinces are indicated in black on the map of Vietnam.

Sequencing and phylogenetic analysis

Viral RNA was extracted from 250 µl of allantoic fluid using TRIzol LS Reagent (Life Technologies, USA) according to the manufacturer's protocol, followed by reverse transcription with the Uni12 primer [63] and Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies). Full-length complementary deoxyribonucleic acids (cDNA)s of the HA RNA gene segments were amplified by polymerase chain reaction (PCR) with Ex-Taq (Takara Bio, Japan) and gene-specific primer sets [63]. Direct sequencing of the HA gene segment was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500 Genetic Analyzer (Life Technologies). Next generation sequencing was applied to determine the whole genome sequences of the other seven gene segments as follows. MiSeq libraries were prepared using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA) and sequenced using a MiSeq system and MiSeq Reagent Kit v3 (600 cycles) (Illumina, USA). Sequence reads were assembled using CLC Genomics Workbench, version 12 (CLC Bio, Denmark; now Qiagen). The deduced amino acid sequence of the HA was interpreted from cDNA sequence information using GENETYX version 12 (Genetyx Corporation, Japan).

For phylogenetic analysis, nucleotide sequences of the isolates and those from a public database were aligned using Clustal W version 2.0 [64]. Phylogenetic trees were constructed using the maximum likelihood (ML) method, with 1,000 bootstrap replicates, using MEGA 7.0 software [65].

Estimating the major antigenic regions of H7 hemagglutinin

The amino acid sequence of an H7 HA derived from A/Netherlands/219/2003 (H7N7), was aligned with that of the H3 HA from A/Aichi/2/1968 (H3N2) to identify the antigenic sites in the study of Liu *et al.* [66]. The antigenic sites in the H7 HA of viruses in the present study [A/duck/Vietnam/HU10-48/2018 (H7N7), A/duck/Vietnam/HU10-64/2018 (H7N7), and A/duck/Vietnam/OIE-0178/2012 (H7N1)] and other viruses from public database were identified based on the corresponding location of epitopes (from A to E) of the H7 HA from A/Netherlands/219/2003 (H7N7) by using GENETYX version 12.

HI test and antigenic cartography

Polyclonal antisera were prepared as previously described during our research [48,54,67], by hyperimmunizing chickens against various viruses belonging to H6, H7, and H9 subtypes. Complete freund's adjuvant and incomplete freund's adjuvant (Becton, Dickinson and Company, USA) were mixed with 500 μg formalin-inactivated whole virus particles for the first and second immunization, respectively. Five days after a final immunization with a mixture of 500 μg formalin-inactivated whole virus particles in phosphate buffered saline (PBS), sera were collected and stored at −20°C until use. Then, the antigenic properties of the newly isolated H7 viruses were assessed using polyclonal antisera by performing a cross-HI test as follows. Antibodies were two-fold serially diluted with PBS in 96-well U-bottom microtiter plates and mixed with an amount of virus equivalent to four HA units, followed by incubation at room temperature (approximately 25°C) for 60 min. After adding 50 μl of 0.5% chicken red blood cells, the compound was gently mixed and incubated at room temperature for a further 45 min. HI titers were expressed as reciprocals of the highest serum dilutions that showed complete HI.

The resulting data containing cross-HI titers were used to estimate x/y coordinates of each antiserum and antigen in the antigenic cartography by using web-based software [68]. In an antigenic map, both vertical and horizontal axes represent antigenic distance. One antigenic-unit distance corresponded to a 2-fold dilution in the HI assay (e.g. 2 units correspond to a 4-fold dilution, 3 units correspond to an 8-fold dilution etc.). If the distance between two antigens was more than two antigenic units, the difference was considered significant.

Results

Identification of AIVs circulating in PDSs, LBMs, and farms

From 2014 to 2018, a total of 1,361 viruses were isolated from 15,431 cloacal and oropharyngeal samples of domestic birds and environmental samples (Table 1), returning an AIVs prevalence of 8.8 % (95% confidence interval [CI]: 8.4–9.3). In total, 69 of H3, 8 of H4, 213 of H5, 344 of H6, 3 of H7, 698 of H9, 9 of H10, 15 of H11, 1 of H12 and 1 of H13 AIVs were identified from samples collected at three provinces (Table 1). A total of 177 H5N1 HPAIVs clade 2.3.2.1 were isolated from apparently healthy chickens (39), ducks (47), Muscovy ducks (67), geese (3), and environment (21) in PDSs, LBMs, and farms. Thirty-six H5N6 HPAIVs were isolated from apparently healthy chickens (3), ducks (21), Muscovy ducks (10), environment (1), and hand of seller (1). In 2018, the first report of the detection of H5N6 HPAIVs clade 2.3.4.4 was confirmed in Vinh Long province.

Table 1. Summary of avian influenza virus surveillance in Vietnam from 2014 to 2018

Year	Region	Province	No. of samples	AIV positive	Prevalence (%) (95% CI)	Subtype (no. of isolates)	Reference
2014	Central	Hue	3,045	178	5.8 (5.0–6.7)	H3N2 (18), H3N6 (1), H4N6 (2), **H5N6 (8)**, H6N2 (14), H6N6 (16), H9N2 (109), H9N6 (5), H11N6 (1), H11N7 (4)	Chu *et al.* 2016
2015	Central	Hue	2,040	49	2.4 (1.8–3.2)	H3N1 (1), H3N8 (3), H4N2 (3), **H5N1 (4)**, **H5N6 (9)**, H6N1 (14), H9N2 (15)	Chu *et al.* 2016
	South	Vinh Long	1,400	243	17.4 (15.4–19.4)	H3N2 (1), H4N6 (1), **H5N1 (130)**, H6N6 (24), H9N2 (86), H11N9 (1)	
2016	South	Vinh Long	3,300	131	4.0 (3.3–4.7)	H3N2 (11), H3N8 (2), **H5N1 (5)**, H6N6 (69), H9N2 (31), H10N6 (7), H11N9 (5), H12N5 (1)	Le *et al.* 2021
2017	North	Lang Son	1,000	148	14.8 (12.7–17.2)	**H5N6 (6)**, H6N6 (3), H9N2 (139)	
	South	Vinh Long	1,800	167	9.3 (8.0–10.7)	H3N2 (2), **H5N1 (21)**, H6N6 (63), H9N2 (79), H10N3 (2)	
2018	North	Lang Son	1,000	306	30.6 (27.8–33.6)	H3N2 (29), **H5N6 (2)**, H6N6 (89), H9N2 (186)	
	South	Vinh Long	1,846	139	7.5 (6.4–8.8)	H3N2 (1), H4N6 (2), **H5N1 (17)**, **H5N6 (11)**, H6N6 (52), H7N7 (3), H9N2 (47), H9N6 (1), H11N1 (1), H11N9 (3), H13N9 (1)	Le *et al.* 2020
Total			15,431	1,361	8.8 (8.4–9.3)		

High pathogenicity avian influenza viruses are highlighted in bold.

Isolation and identification of H7N7 LPAIVs from domestic ducks in Vietnam

Three H7N7 viruses were isolated from apparently healthy ducks on the same farm in Vinh Long province in 2018 (Figure 1 and Table 1). Two H7N7 viruses were selected in this study and virus strain names were assigned as A/duck/Vietnam/HU10-48/2018 (H7N7) and A/duck/Vietnam/HU10-64/2018 (H7N7). Deduced amino acid sequences of the HA gene segments of these isolates were examined to estimate viral pathotypes. These H7N7 viruses had mono-basic amino acid motifs of PEPPKG/GLF at the HA cleavage site (Table 2), indicating that these isolates were likely to be LPAIVs.

Table 2. Amino acid sequence alignments of H7 viruses for the viral proteins related to the pathogenicity, receptor specificity, and antiviral susceptibility

Virus	Subtype	HA[§]				NA[*]		PB2			M2			
		Cleavage site	138	225	226	274	Stalk deletion	591	627	701	26	27	30	31
A/duck/Vietnam/HU10-48/2018	H7N7	PEPPKGR/GLF	A	G	Q	H	No	Q	E	D	L	V	A	S
A/duck/Vietnam/HU10-64/2018	H7N7	PEPPKGR/GLF	T	•	•	•	No	•	•	•	•	I	•	•
A/duck/Cambodia/b0120501/2017	H7N3	PEPPKGR/GLF	•	•	•	•	No	•	•	•	•	I	•	•
A/duck/Vietnam/OIE-0178/2012	H7N1	PEGPKGR/GLF	•	•	•	•	No	•	•	•	•	I	•	•
A/turkey/Italy/4580/1999	H7N1	PEIPKGSRVRR/GLF	T	•	•	•	Yes	•	•	•	•	•	•	•
A/duck/Taiwan/Ya103/1993	H7N7	PEIPKKREKR/GLF	•	•	•	•	Yes	•	•	•	•	•	•	•

[§] H3 numbering; [*] N2 numbering.
"•" indicates the same amino acids as A/duck/Vietnam/HU10-48/2018 (H7N7).
Viruses isolated in this study are highlighted in bold; Critical basic amino acids for cleavage activity are underlined.

Molecular characterization of viral amino acid sequences of H7N7 AIVs

The deduced viral amino acid sequences of the two H7N7 AIVs were examined to estimate the viral phenotypes. The amino acid residues at the receptor binding site in the HA protein are Q226 and G228 (Table 2), same as H7 avian progenitor virus, which indicates an avian-like receptor binding preference [69]. Additionally, these strains have several differences in the antigenic region from A to E compared with the reference strain [A/duck/Hokkaido/W19/2013 (H7N2)], which is shown in Table 3. In particular, the A/duck/Vietnam/HU10-64/2018 (H7N7) strain had one amino acid mutation at A138T in the 130-loop compared with A/duck/Vietnam/HU10-48/2018 (H7N7) [70].

The newly detected viruses have a full-length NA protein, with no amino acid deletions in the stalk region (Table 2), meaning that these viruses have not yet adapted to terrestrial birds [71]. No single mutations in the polymerase basic 2 (PB2) protein (i.e., Q591K, E627K, or D701N), which are markers of high pathogenicity of influenza viruses in mammals [72], were detected in the present H7N7 viruses. Furthermore, the retention of H274 in the NA and L26, V/I27, A30, and S31 in the matrix (M) 2 amino acid sequences suggests that both newly detected H7N7 viruses are sensitive to NA and M2 inhibitors [73,74].

Table 3. Amino acid sequence of antigenic sites for H7 viruses

Virus	Subtypes	Antigenic region[§]												
		A						B						
		122	135	138	140	144	152	128	158	160	186	188	189	193
A/Netherlands/219/2003	H7N7	T	T	A	R	G	K	S	T	A	G	T	T	K
A/duck/Vietnam/HU10-64/2018	H7N7	•	A	T*	K	•	•	•	•	•	•	A	•	•
A/duck/Vietnam/HU10-48/2018	H7N7	•	A	•	K	•	•	•	•	•	•	A	•	•
A/duck/Vietnam/NCVD-197/2009	H7N3	S	A	•	•	•	•	•	•	•	•	A	E	•
A/duck/Vietnam/OIE-0178/2012	H7N1	S	A	•	•	•	•	•	•	•	•	A	A	•
A/duck/Cambodia/b0120501/2017	H7N3	•	A	•	K	•	•	•	•	V	•	•	•	•
A/duck/Hokkaido/W19/2013	H7N2	•	A	•	•	•	•	•	•	•	•	•	•	M
A/duck/Hokkaido/Vac-2/2004	H7N7	I	A	•	•	•	•	•	•	•	•	•	•	•
A/Anhui/1/2013	H7N9	A	A	•	•	•	•	•	•	•	V	•	A	•
A/chicken/North Korea/7916/2005	H7N7	•	•	•	•	•	•	•	•	•	•	•	A	•
A/duck/Japan/AQ-HE29-22/2017	H7N9	P	V	•	•	•	•	N	•	•	V	•	A	•
A/turkey/Italy/4580/1999	H7N1	A	•	T	•	•	•	•	•	•	•	•	•	•
A/duck/Taiwan/Ya103/1993	H7N7	•	•	•	•	E	E	•	A	T	•	•	•	•
A/chicken/New South Wales/327/1997	H7N4	•	V	•	•	•	•	•	•	•	•	•	•	•
A/seal/Massachusetts/1/1980	H7N7	S	A	•	•	•	•	•	S	•	•	•	•	R

[§] H3 Numbering.

"•" indicates the same amino acids as A/Netherlands/219/2003 (H7N7).

"*" indicates the different amino acids between A/duck/Vietnam/HU10-64/2018 (H7N7) and A/duck/Vietnam/HU10-48/2018 (H7N7).

Viruses isolated in this study are shown in bold; Viruses isolated previously in Vietnam are underlined; high pathogenicity avian influenza viruses are shown in italic.

Table 3 *(cont)***.** Amino acid sequence of antigenic sites for H7 viruses

Virus	Subtypes	Antigenic region[§]														
		C														
		45	**46**	**48**	**50**	**53**	**54**	**273**	**276**	**278**	**280**	**297**	**299**	**307**	**310**	**312**
A/Netherlands/219/2003	H7N7	R	T	V	R	S	K	Q	A	E	D	I	S	R	K	E
A/duck/Vietnam/HU10-64/2018	H7N7	•	•	•	•	•	•	•	•	•	•	•	N	•	•	•
A/duck/Vietnam/HU10-48/2018	H7N7	•	•	•	•	•	•	•	•	•	•	•	N	•	•	•
A/duck/Vietnam/NCVD-197/2009	H7N3	•	•	T	•	•	•	•	•	•	•	•	•	•	•	R
A/duck/Vietnam/OIE-0178/2012	H7N1	•	•	T	•	•	•	•	•	•	•	•	•	•	•	S
A/duck/Cambodia/b0120501/2017	H7N3	•	•	•	•	•	•	•	•	•	•	•	•	•	R	•
A/duck/Hokkaido/W19/2013	H7N2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
A/duck/Hokkaido/Vac-2/2004	H7N7	•	•	I	•	•	•	•	•	•	•	•	•	•	•	G
A/Anhui/1/2013	H7N9	•	•	I	•	•	•	•	•	•	•	•	•	•	•	R
A/chicken/North Korea/7916/2005	H7N7	•	•	I	•	•	•	•	•	•	•	•	•	•	•	•
A/duck/Japan/AQ-HE29-22/2017	H7N9	•	•	T	•	•	•	•	•	•	N	•	•	•	•	R
A/turkey/Italy/4580/1999	H7N1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
A/duck/Taiwan/Ya103/1993	H7N7	•	•	T	K	•	•	•	•	•	E	•	•	K	•	G
A/chicken/New South Wales/327/1997	H7N4	Q	M	I	•	T	•	P	•	•	E	V	•	•	•	K
A/seal/Massachusetts/1/1980	H7N7	T	A	I	K	T	Q	P	S	G	•	•	•	•	•	P

Table 3 *(cont)***.** Amino acid sequence of antigenic sites for H7 viruses

Virus	Subtypes	Antigenic region[§]													
		D													
		103	**171**	**173**	**174**	**175**	**179**	**208**	**213**	**214**	**218**	**219**	**226**	**238**	**246**
A/Netherlands/219/2003	H7N7	V	T	K	D	P	I	N	F	V	G	A	Q	N	S
A/duck/Vietnam/HU10-64/2018	H7N7	•	•	R	•	•	V	K	•	•	•	•	•	D	G
A/duck/Vietnam/HU10-48/2018	H7N7	•	•	R	•	•	V	K	•	•	•	•	•	D	G
A/duck/Vietnam/NCVD-197/2009	H7N3	•	•	R	•	•	V	•	•	•	•	•	•	•	G
A/duck/Vietnam/OIE-0178/2012	H7N1	•	•	R	•	S	V	•	•	•	•	•	•	•	•
A/duck/Cambodia/b0120501/2017	H7N3	•	•	R	•	•	V	K	•	•	•	•	•	D	G
A/duck/Hokkaido/W19/2013	H7N2	•	•	R	•	•	V	•	•	•	•	•	•	•	•
A/duck/Hokkaido/Vac-2/2004	H7N7	•	•	•	•	•	•	•	•	•	E	•	•	•	•
A/Anhui/1/2013	H7N9	•	•	•	S	•	V	•	•	•	•	•	L	•	•
A/chicken/North Korea/7916/2005	H7N7	•	•	•	•	•	•	•	•	•	•	•	•	•	•
A/duck/Japan/AQ-HE29-22/2017	H7N9	•	•	E	S	•	V	•	•	•	•	•	•	•	•
A/turkey/Italy/4580/1999	H7N1	•	•	•	•	•	•	•	•	•	•	E	•	•	•
A/duck/Taiwan/Ya103/1993	H7N7	I	K	R	E	•	V	K	L	•	•	•	•	D	•
A/chicken/New South Wales/327/1997	H7N4	•	•	N	E	•	V	•	•	•	•	•	•	•	•
A/seal/Massachusetts/1/1980	H7N7	T	P	N	K	•	V	K	•	T	•	•	•	D	T

Table 3 *(cont)***.** Amino acid sequence of antigenic sites for H7 viruses

Virus	Subtypes	Antigenic region[§]					
		E					
		57	**59**	**83**	**94**	**260**	**265**
A/Netherlands/219/2003	H7N7	R	V	S	S	L	M
A/duck/Vietnam/HU10-64/2018	H7N7	•	•	•	•	•	•
A/duck/Vietnam/HU10-48/2018	H7N7	•	•	•	•	•	•
A/duck/Vietnam/NCVD-197/2009	H7N3	•	•	•	N	•	•
A/duck/Vietnam/OIE-0178/2012	H7N1	•	•	•	N	•	•
A/duck/Cambodia/b0120501/2017	H7N3	•	•	•	•	•	•
A/duck/Hokkaido/W19/2013	H7N2	•	•	•	•	•	•
A/duck/Hokkaido/Vac-2/2004	H7N7	•	•	•	•	•	•
A/Anhui/1/2013	H7N9	•	•	•	•	•	•
A/chicken/North Korea/7916/2005	H7N7	•	•	•	•	•	•
A/duck/Japan/AQ-HE29-22/2017	H7N9	•	•	•	•	•	•
A/turkey/Italy/4580/1999	H7N1	•	•	•	•	•	•
A/duck/Taiwan/Ya103/1993	H7N7	•	I	•	N	•	•
A/chicken/New South Wales/327/1997	H7N4	K	I	T	N	F	T
A/seal/Massachusetts/1/1980	H7N7	•	T	•	N	F	L

Phylogenetic analysis of the H6 LPAIVs

Based on the HA genes, H6 viruses were phylogenetically classified into two lineages: Eurasian and North American. Furthermore, the Eurasian lineage was divided into five distinct sublineages: Group I, Group II, Group III, W312, and Early as described in the previous study [20]. While the major H6 viruses isolated in Vietnam from 2014 to 2018 were classified into Group II, three viruses were clustered into Group III with the viruses isolated in Vietnam from 2010 to 2012 (Figure 2).

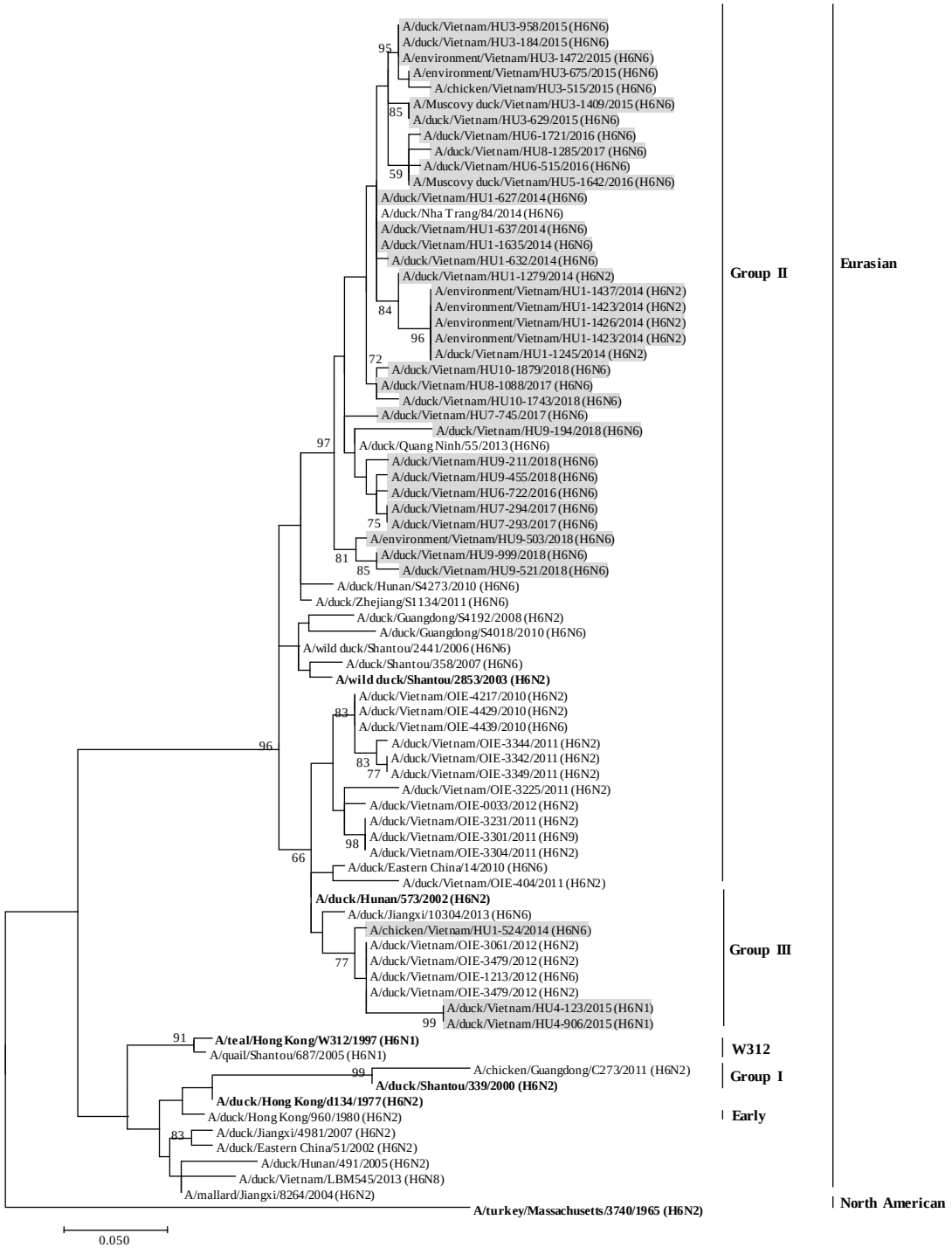


Figure 2. Phylogenetic tree of the HA gene segment of H6 avian influenza viruses. The HA gene segment of the H6 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H6 viruses in this study are highlighted in gray, the representative of each sublineage is indicated in bold.

Phylogenetic analysis of the H7N7 LPAIVs

To investigate the phylogenetic relationships between the Vietnamese H7N7 LPAIVs and other H7 AIVs, the full-length nucleotide sequences of their eight gene segments were analyzed using the ML method. In a phylogenetic tree based on the HA gene, H7 HA genes were phylogenetically divided into five lineages (i.e., Eurasian, Historical European, Australian, North American, and Equine; Figure 3), as previously reported [48]. The Eurasian lineage comprised three sublineages: Old–Eurasian, Far–Eastern, and European–Asian. The present H7N7 viruses both belonged to the European–Asian sublineage and showed a close relationship with an HA gene segment of H7N3 viruses that were recently isolated in Cambodia in 2017. It should be noted that the H7 HA genes of the recent H7N7 viruses are genetically distinct from those of Chinese H7N9 viruses and two H7 viruses previously detected in Vietnam, in 2009 and 2012.

In the NA phylogenetic tree, similarly to the HA-based phylogenetic trees, both A/duck/Vietnam/HU10-48/2018 (H7N7) and A/duck/Vietnam/HU10-64/2018 (H7N7) were grouped within the European–Asian sublineage of the Eurasian lineage, although they branched from the ancestor of the European–Asian sublineage together with an H10N7 virus previously isolated in Vietnam in 2012 (Figure 4). The H7N9 China genetic group was newly proposed to make 11 genetic groups in this study because the sequence of internal genes was phylogenetically divided into 10 genetic groups in the previous study [20]. Among the internal genes, the M-gene-based phylogenetic tree showed a different topology compared with the other phylogenetic trees (Figure 4). The M gene segment of A/duck/Vietnam/HU10-48/2018 (H7N7) clustered separately from the A/duck/Vietnam/HU10-64/2018 (H7N7) virus, suggesting different origins for these two M gene segment RNAs. All of the other internal genes were seen to be closely related to those of viruses isolated from domestic poultry in the same area as well as from poultry and wild birds in China and other East and Southeast Asian countries (Figure 4). However, the relationships with Cambodian H7N3 viruses differed among segments; the M gene of the A/duck/Vietnam/HU10-64/2018 (H7N7) virus and polymerase basic 1 (PB1) gene segments of both viruses was closely related to the Cambodian viruses, as the HA gene, while the M gene of A/duck/Vietnam/HU10-48/2018 (H7N7) virus, PB2, polymerase acidic (PA), nucleoprotein (NP), and nonstructural (NS) gene segments of both viruses were distinct from them.

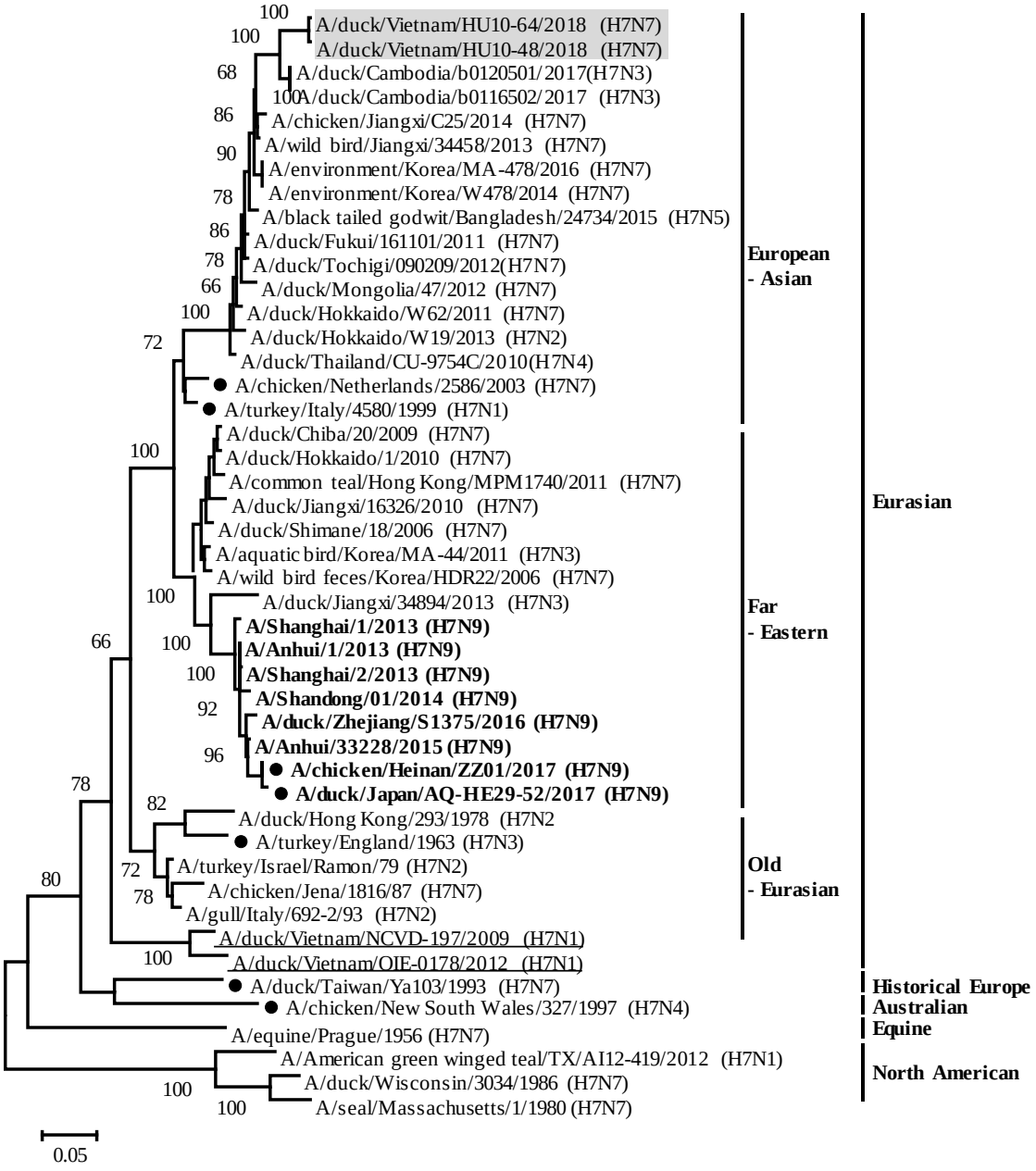


Figure 3. Phylogenetic tree of the HA gene segment of H7 avian influenza viruses. The full-lengths of the HA gene segment of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

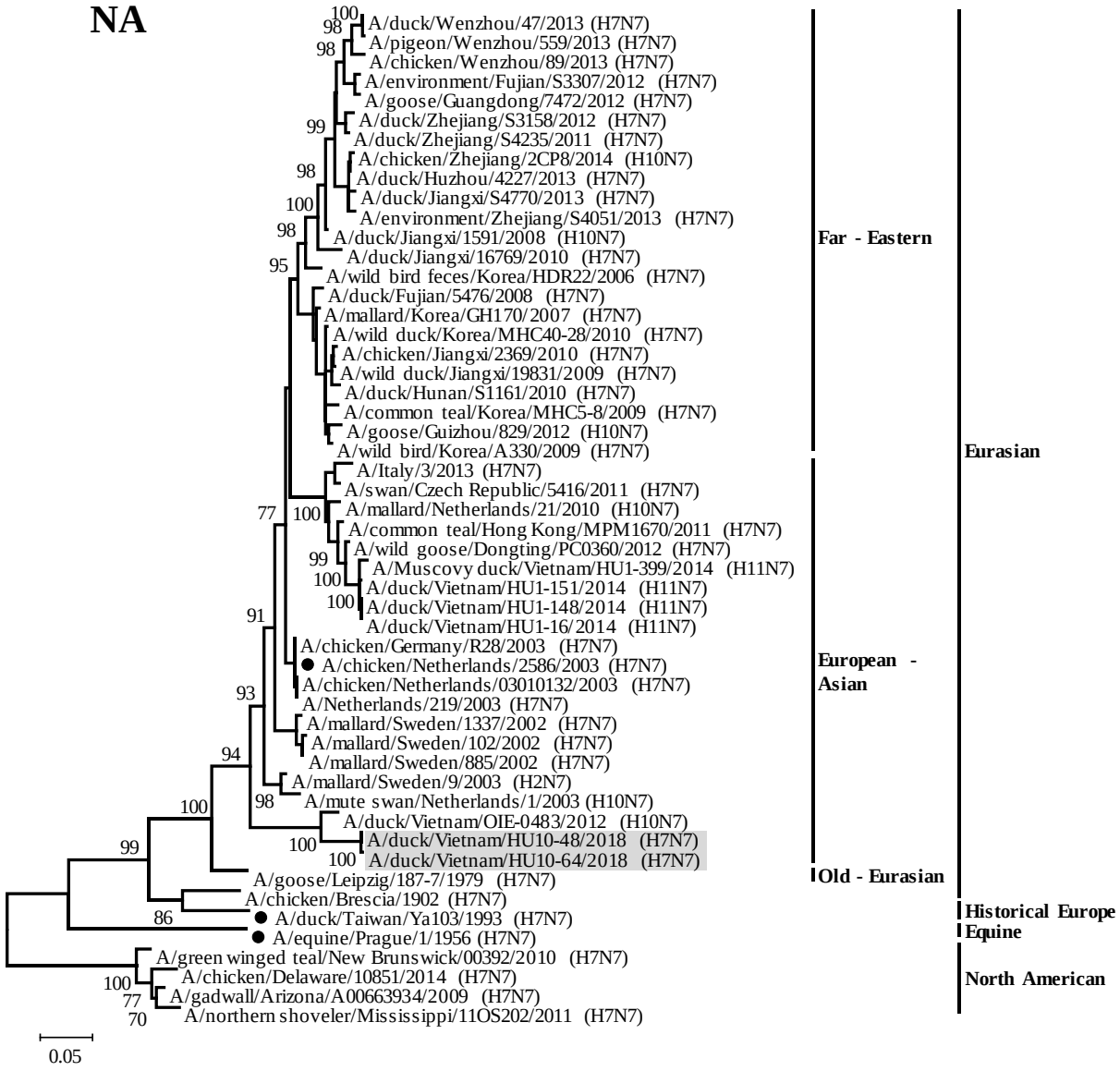


Figure 4. Phylogenetic tree of seven gene segments of H7 avian influenza viruses. The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

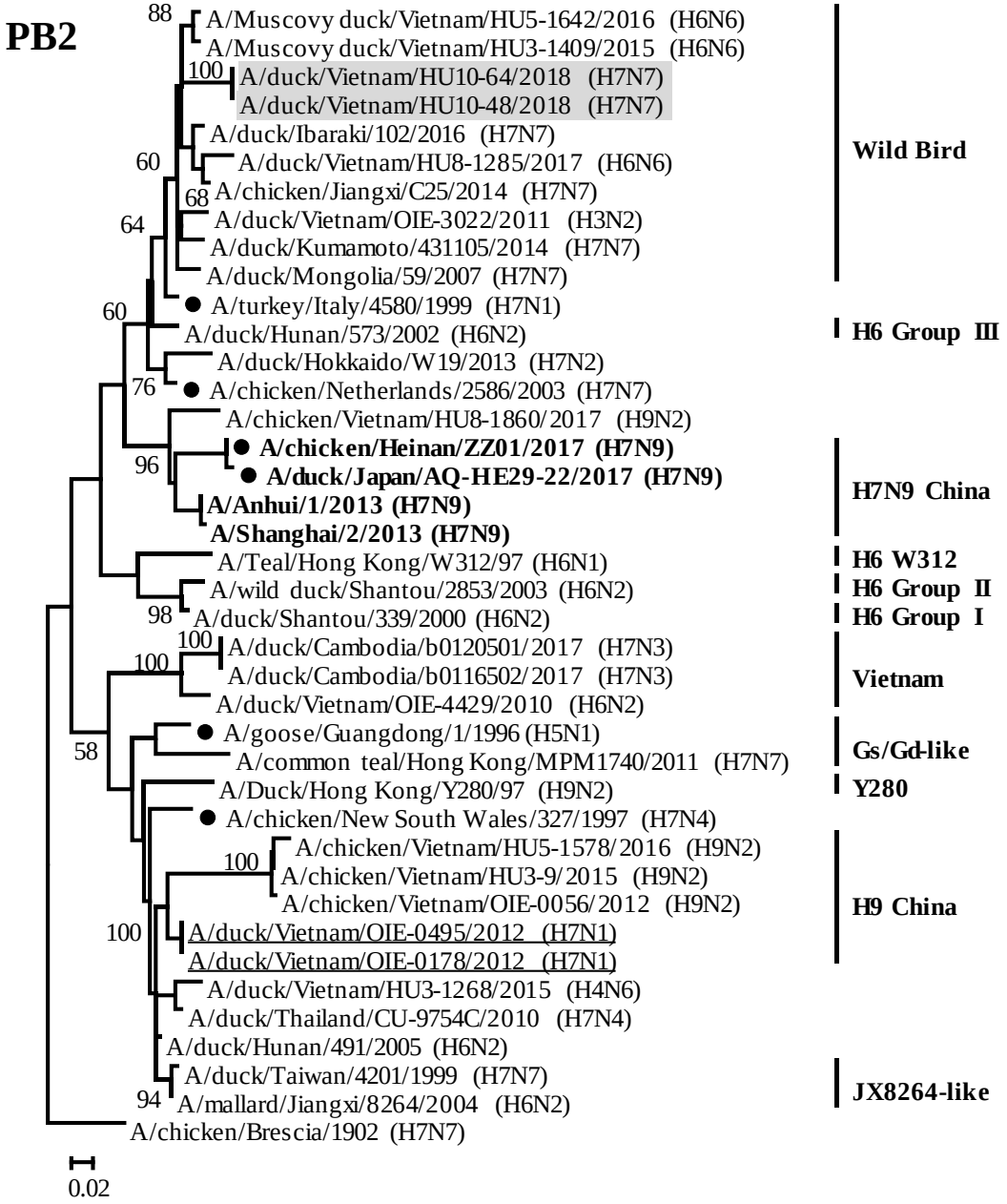


Figure 4 *(cont)***. Phylogenetic tree of seven gene segments of H7 avian influenza viruses.** The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

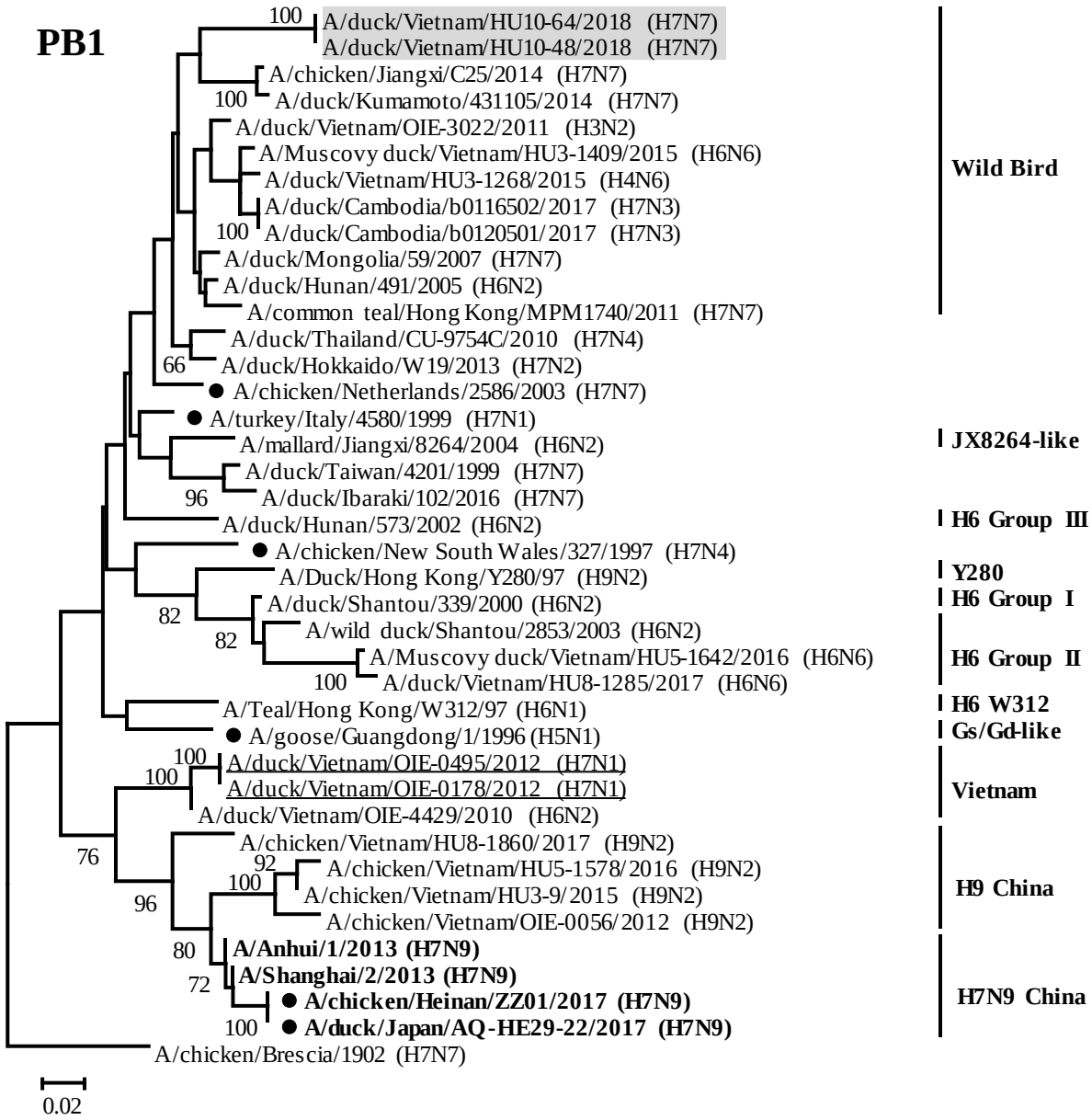


Figure 4 *(cont)***. Phylogenetic tree of seven gene segments of H7 avian influenza viruses.** The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

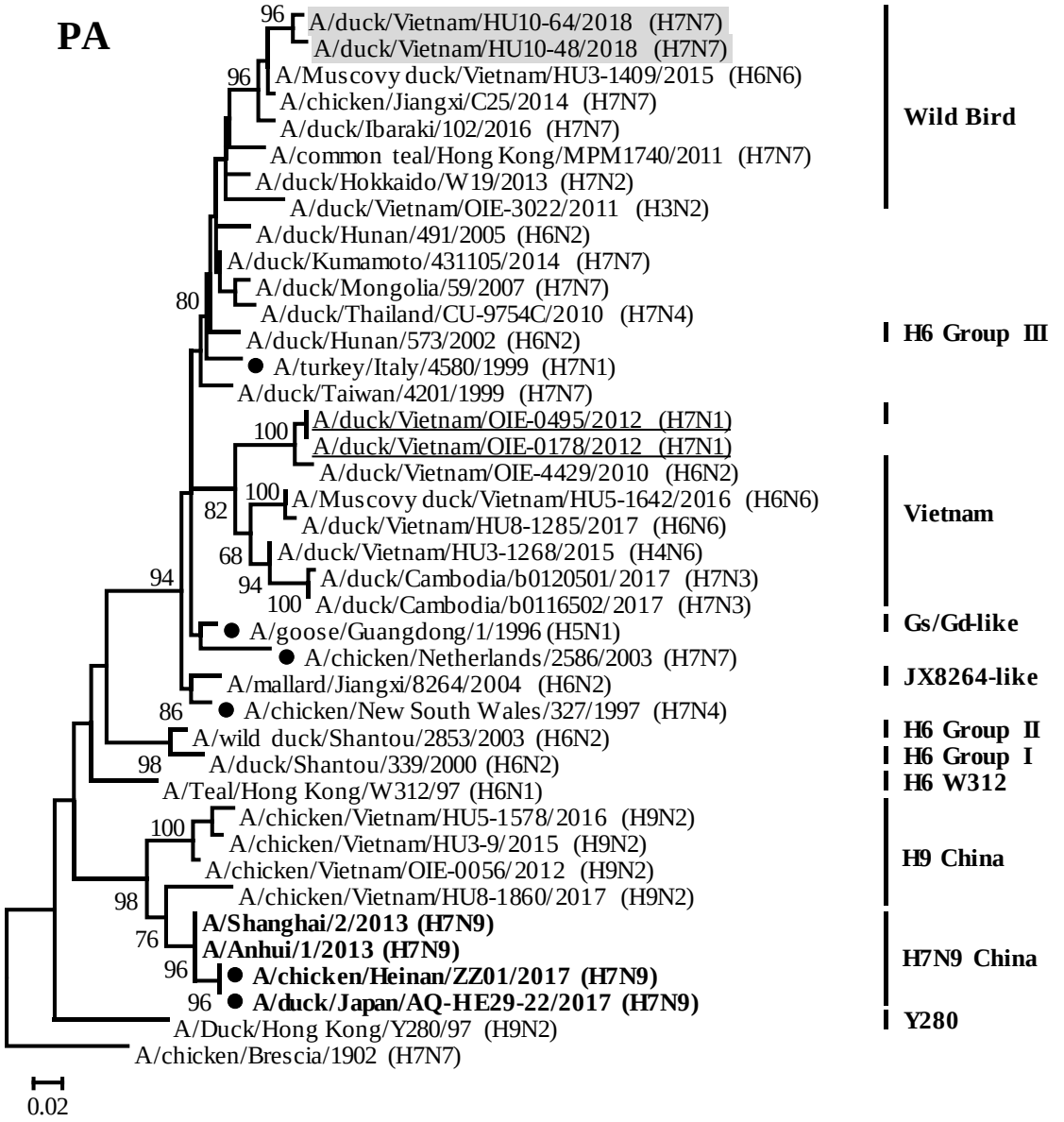


Figure 4 *(cont)***. Phylogenetic tree of seven gene segments of H7 avian influenza viruses.** The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

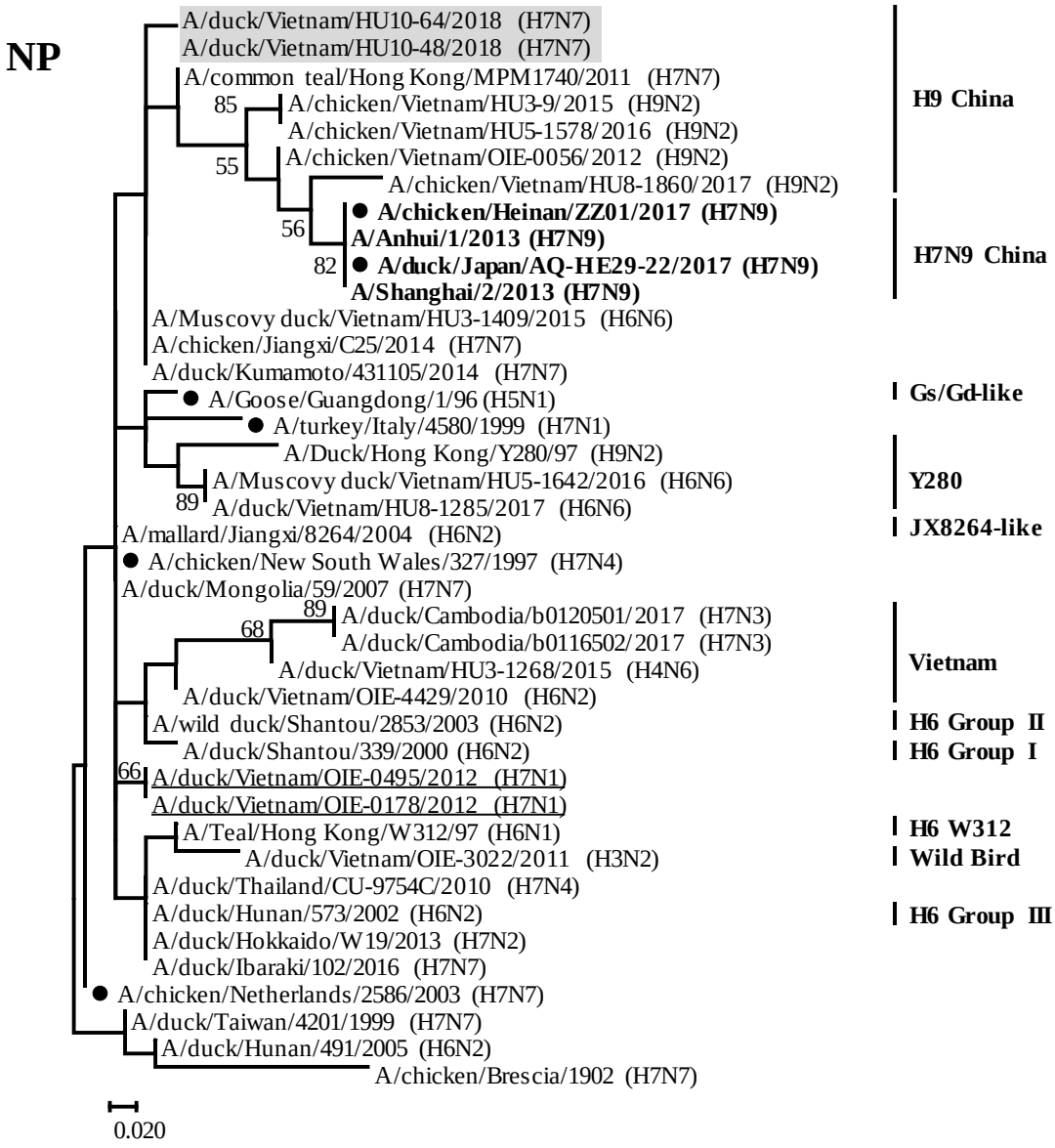


Figure 4 *(cont)***. Phylogenetic tree of seven gene segments of H7 avian influenza viruses.** The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

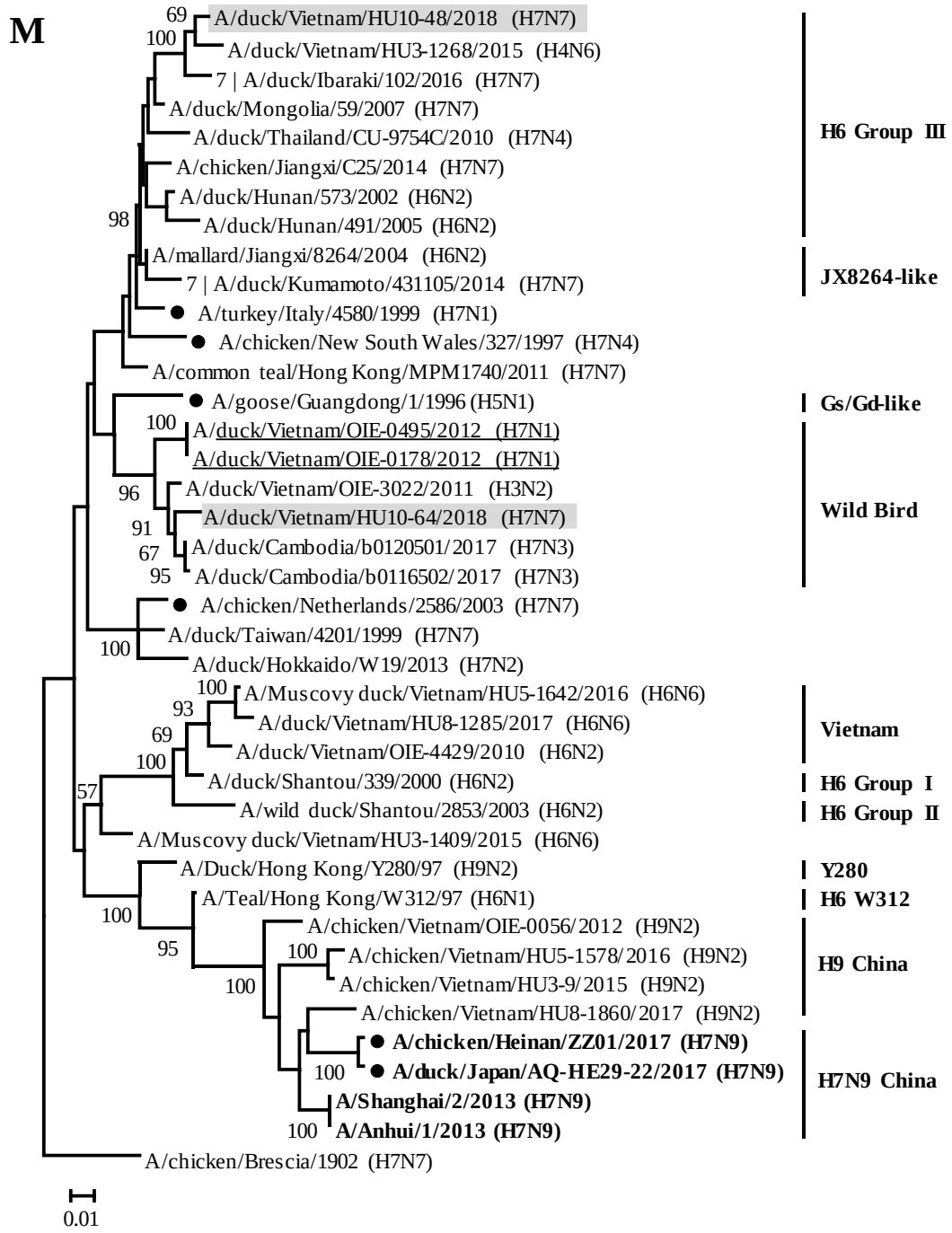


Figure 4 *(cont)***. Phylogenetic tree of seven gene segments of H7 avian influenza viruses.** The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

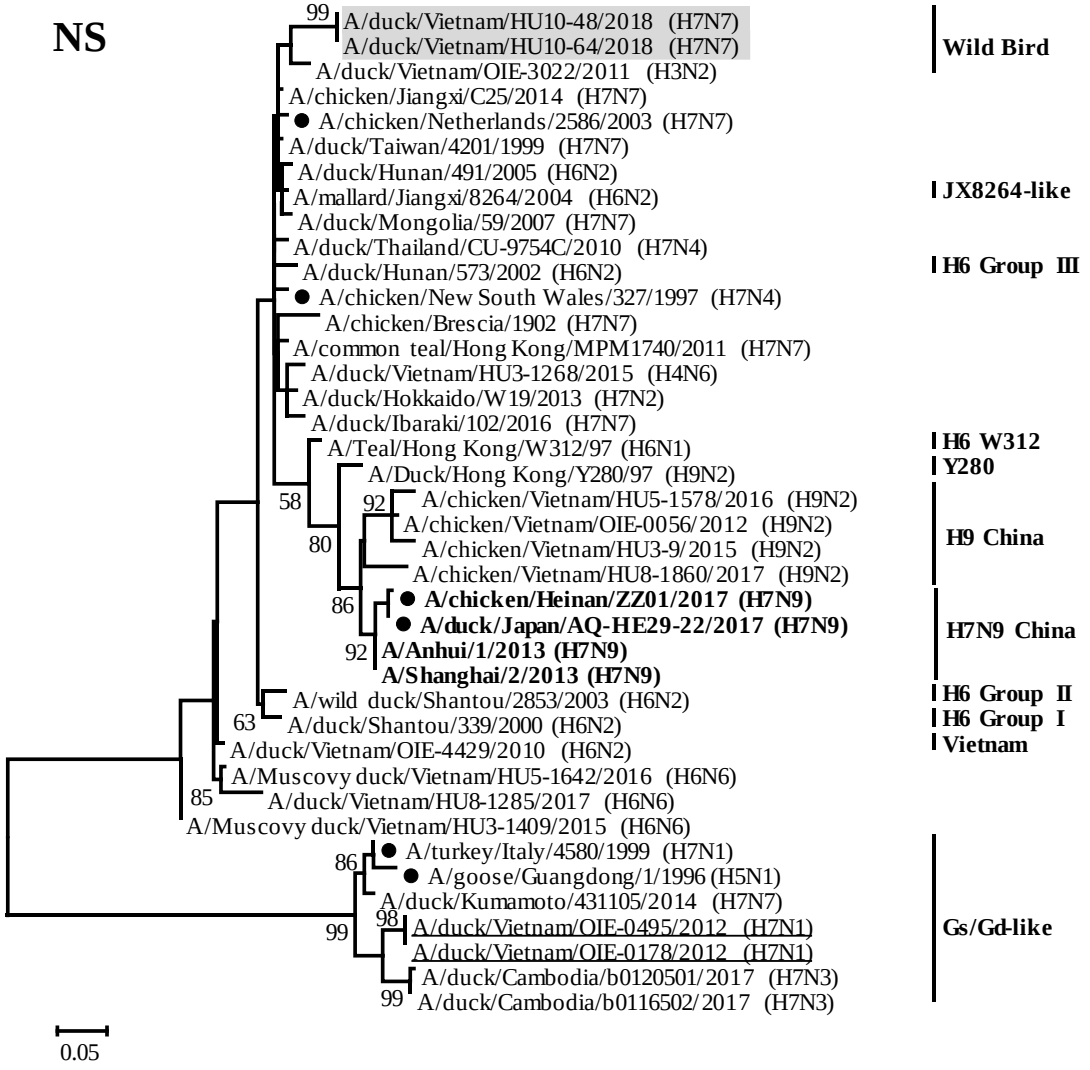


Figure 4 *(cont).* **Phylogenetic tree of seven gene segments of H7 avian influenza viruses.** The full-lengths of the NA, PB2, PB1, PA, M, NP, and NS gene segments of the H7 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H7 viruses isolated in this study are highlighted in gray, HPAIVs are indicated by black circles, Chinese H7N9 viruses are indicated in bold, and viruses previously isolated in Vietnam are underlined.

Phylogenetic analysis of the H9 LPAIVs

The H9 HA genes were phylogenetically classified into two lineages: Eurasian and North American lineages. The H9 viruses isolated in this surveillance from 2014 to 2018 were clustered into the Clade 15 of Y280/BJ94 sublineage in the Eurasian lineage. These viruses were genetically derived from the virus isolated previously in North Vietnam in 2012 and those isolated from poultry in China between 1997 and 2012. Although the virus belonging to the Y439 sublineage was isolated previously in Vietnam, there was no further detection (Figure 5).

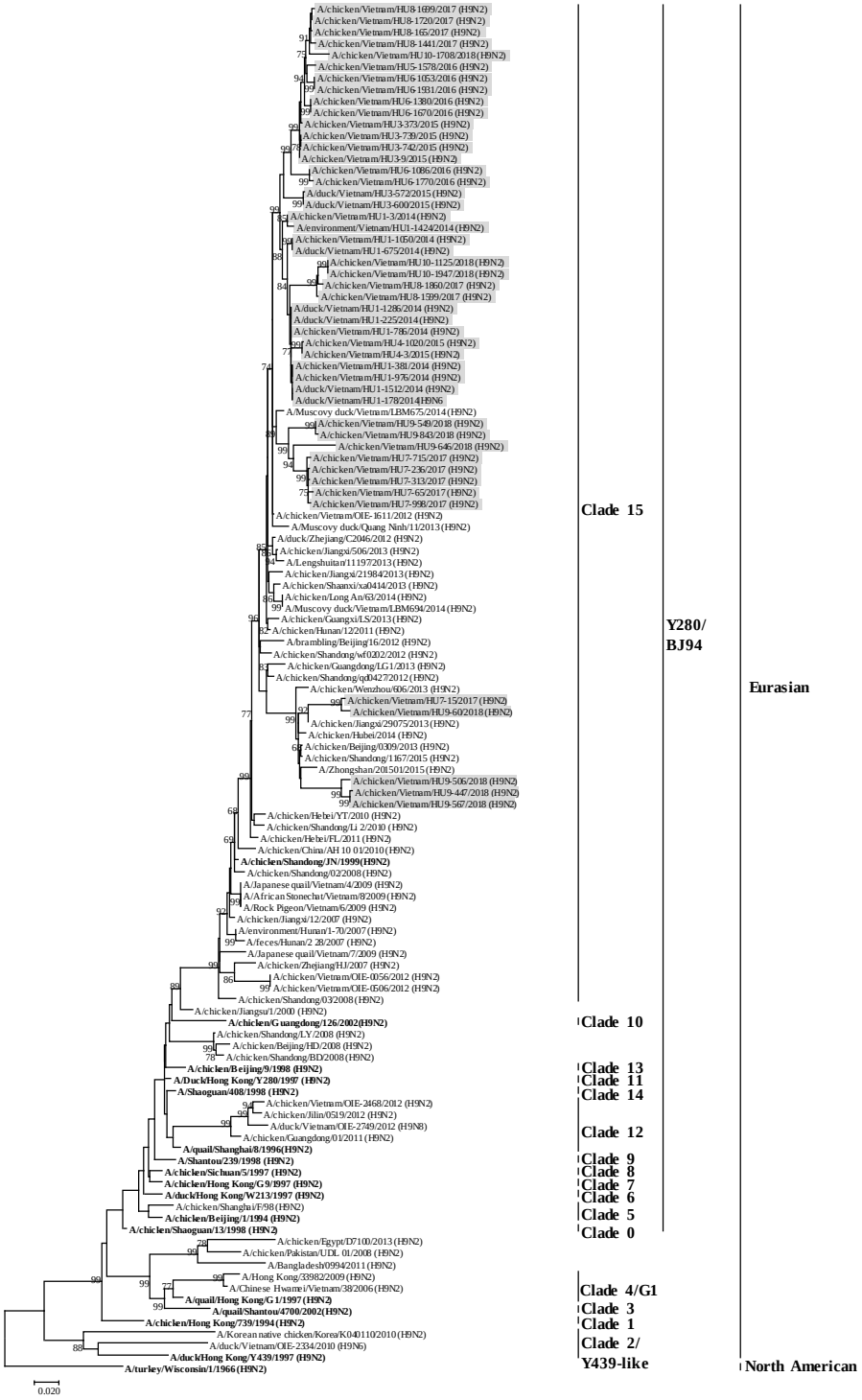


Figure 5. Phylogenetic tree of the HA gene segment of H9 avian influenza viruses. The HA gene segment of the H9 subtype viruses along with those of reference strains were analyzed by the ML method using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The H9 viruses in this study are highlighted in gray, the representative of each sublineage is indicated in bold.

The NA genes of H6 and H9 AIVs isolated in this study

The NA gene segments were classified based on previous studies [20,53]. While the N2 NA genes of the H9 AIVs were phylogenetically classified into group Y280/BJ94, N2 NA genes of the H6 AIVs classified into Group II. All of the N6 NA genes of the H6 viruses belonged to Group II (Figure 6).

Figure 6. Phylogenetic tree of the NA gene segments of H6 and H9 avian influenza viruses. The N2 and N6 genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

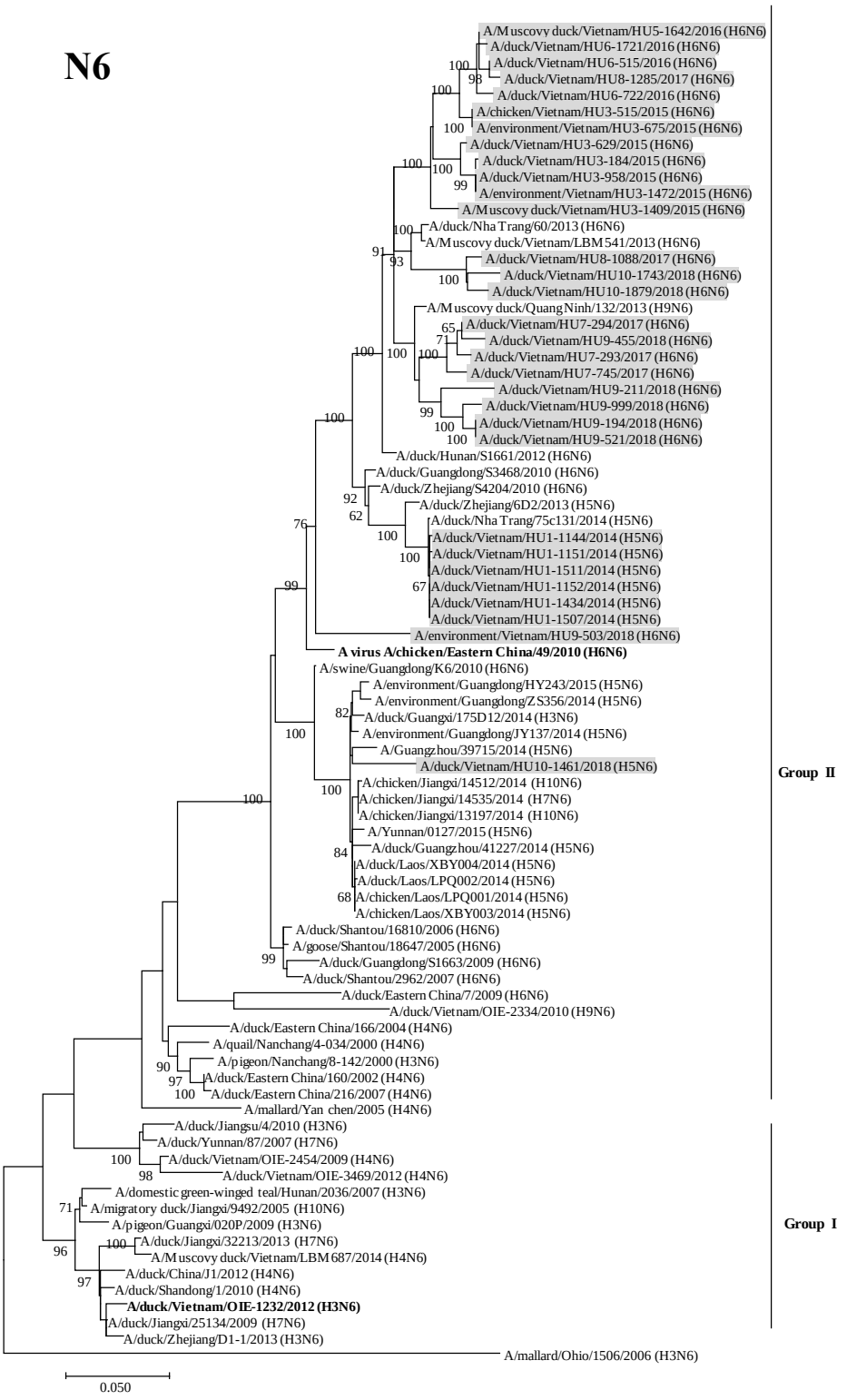


Figure 6 *(cont)***. Phylogenetic tree of the NA gene segments of H6 and H9 avian influenza viruses.** The N2 and N6 genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

Genotyping of H6 and H9 AIVs isolated in this study

The phylogenetic analysis was applied for 6 internal gene segments of the AIVs isolated in Vietnam to investigate the genetic diversity of AIVs circulating recently (Figure 7). The names of genetic groups for each internal gene were defined in previous studies [20,53]. The six internal gene segments were classified into H6 Group I, Group II, Group III, W312, JX8264-like, Vietnam, Hunan491-like, Y280/BJ94, H9 China, Wild bird, and Gs/Gd-like. Among Vietnamese representative H9 viruses, 6 genotypes were identified in which a genotype was identified in around 61% of representative H9 viruses. A total of 19 genotypes were identified among representative H6 viruses and none of the genotypes was dominated (Figure 8). These results indicated that the genetic diversification of Vietnamese H6 viruses seems to be higher than H9 viruses.

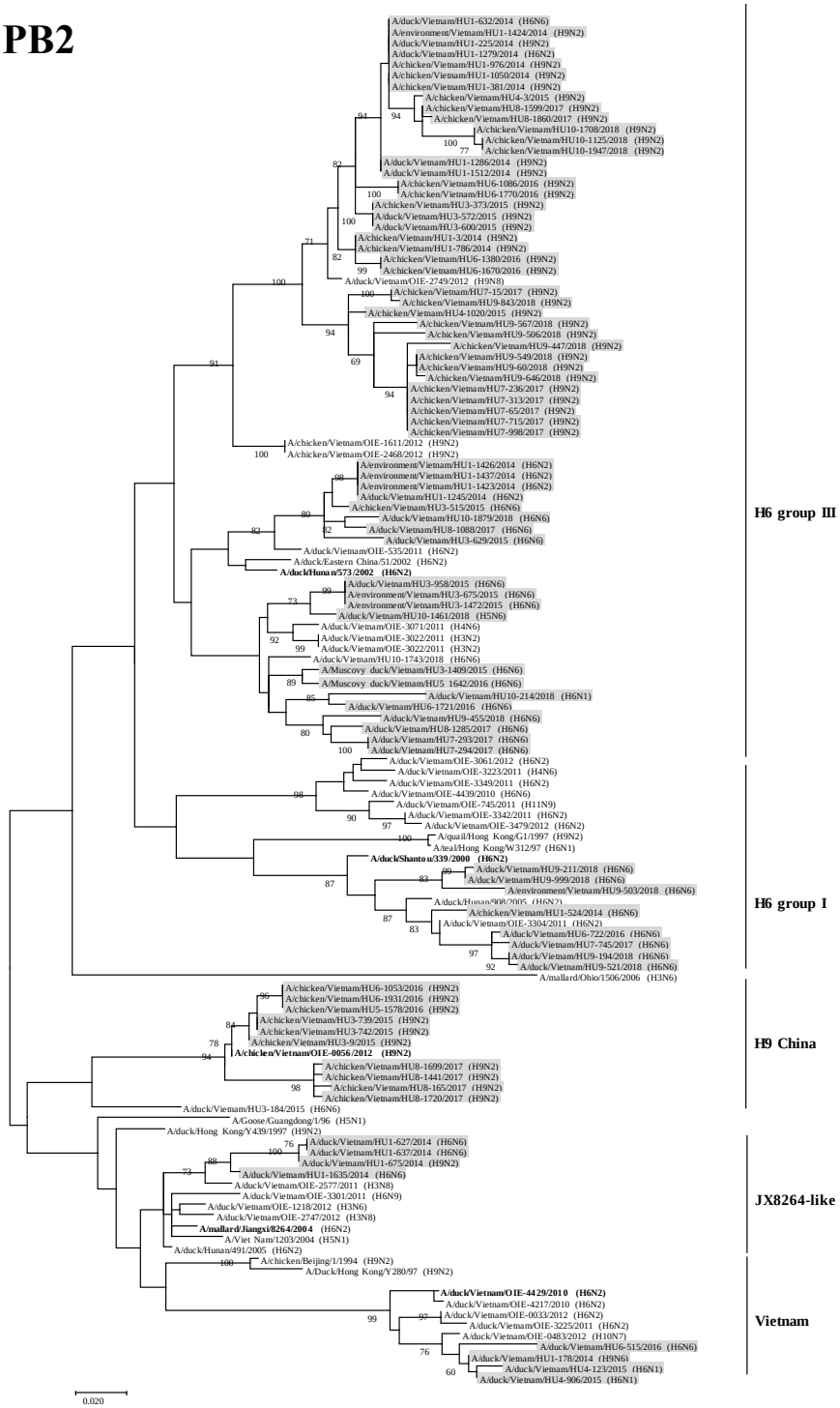


Figure 7. Phylogenetic tree of internal gene segments of H6 and H9 avian influenza viruses. The PB2, PB1, PA, NP, M, and NS genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

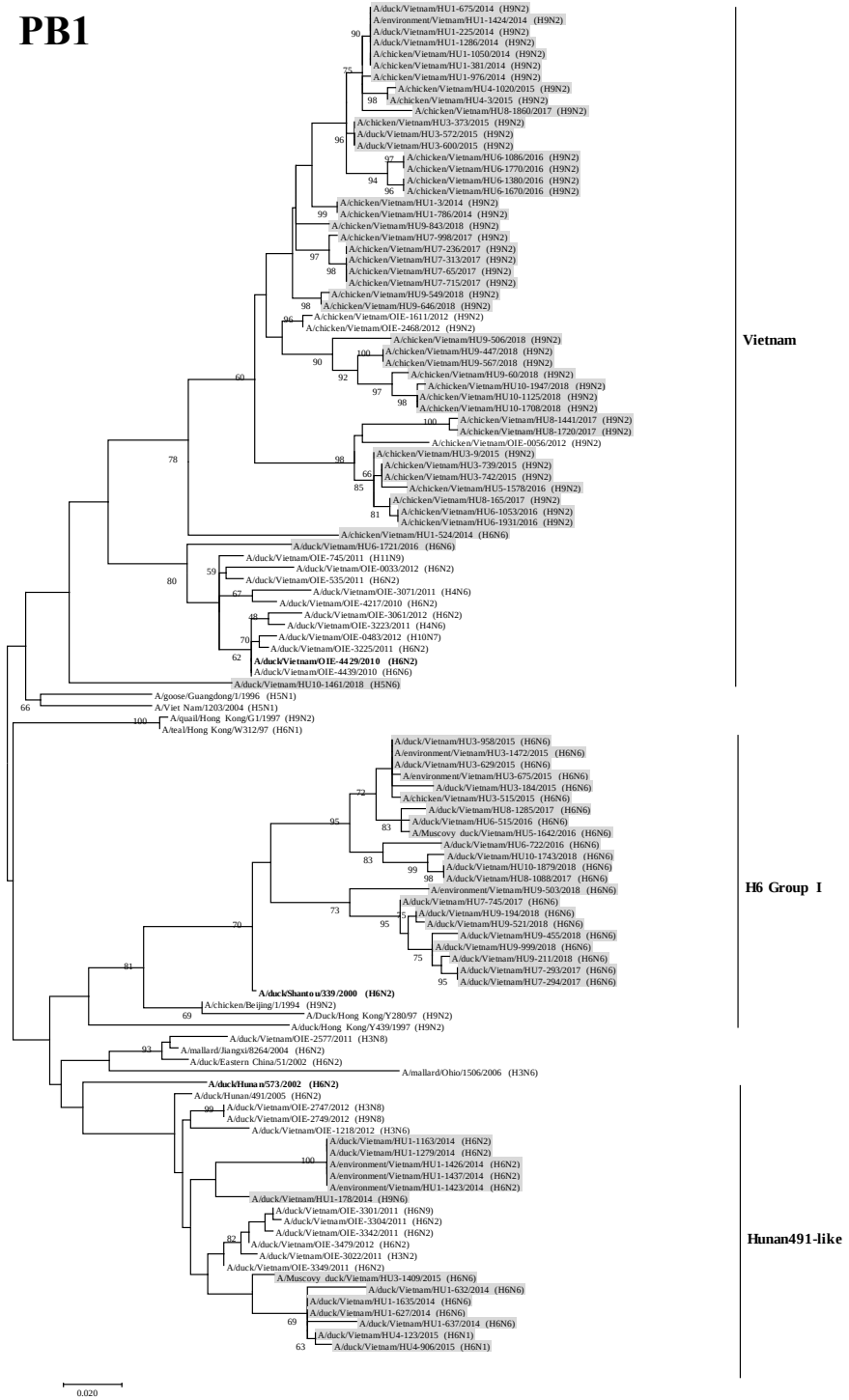


Figure 7 *(cont)***. Phylogenetic tree of internal gene segments of H6 and H9 avian influenza viruses.** The PB2, PB1, PA, NP, M, and NS genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

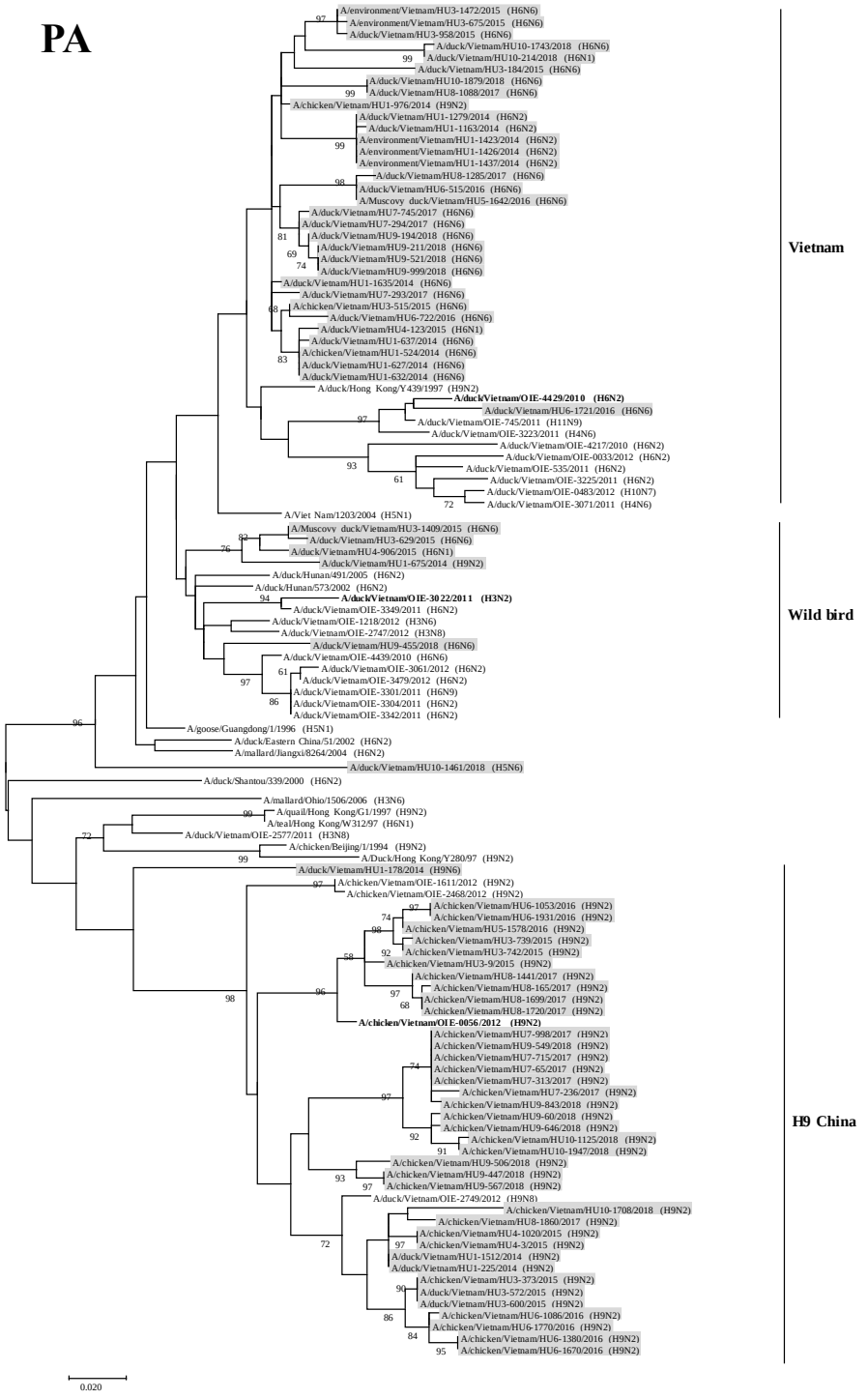


Figure 7 *(cont)***. Phylogenetic tree of internal gene segments of H6 and H9 avian influenza viruses.** The PB2, PB1, PA, NP, M, and NS genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

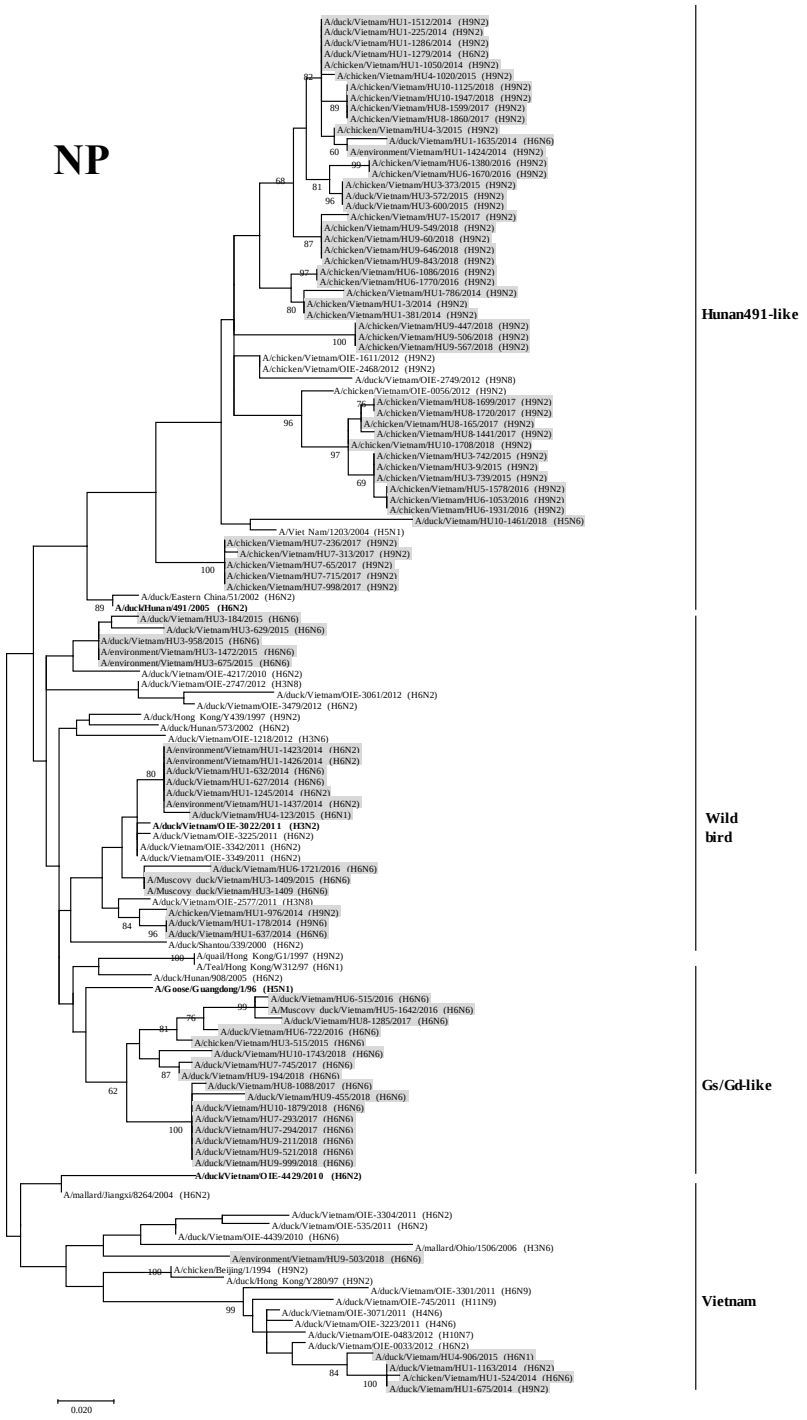


Figure 7 *(cont)***. Phylogenetic tree of internal gene segments of H6 and H9 avian influenza viruses.** The PB2, PB1, PA, NP, M, and NS genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

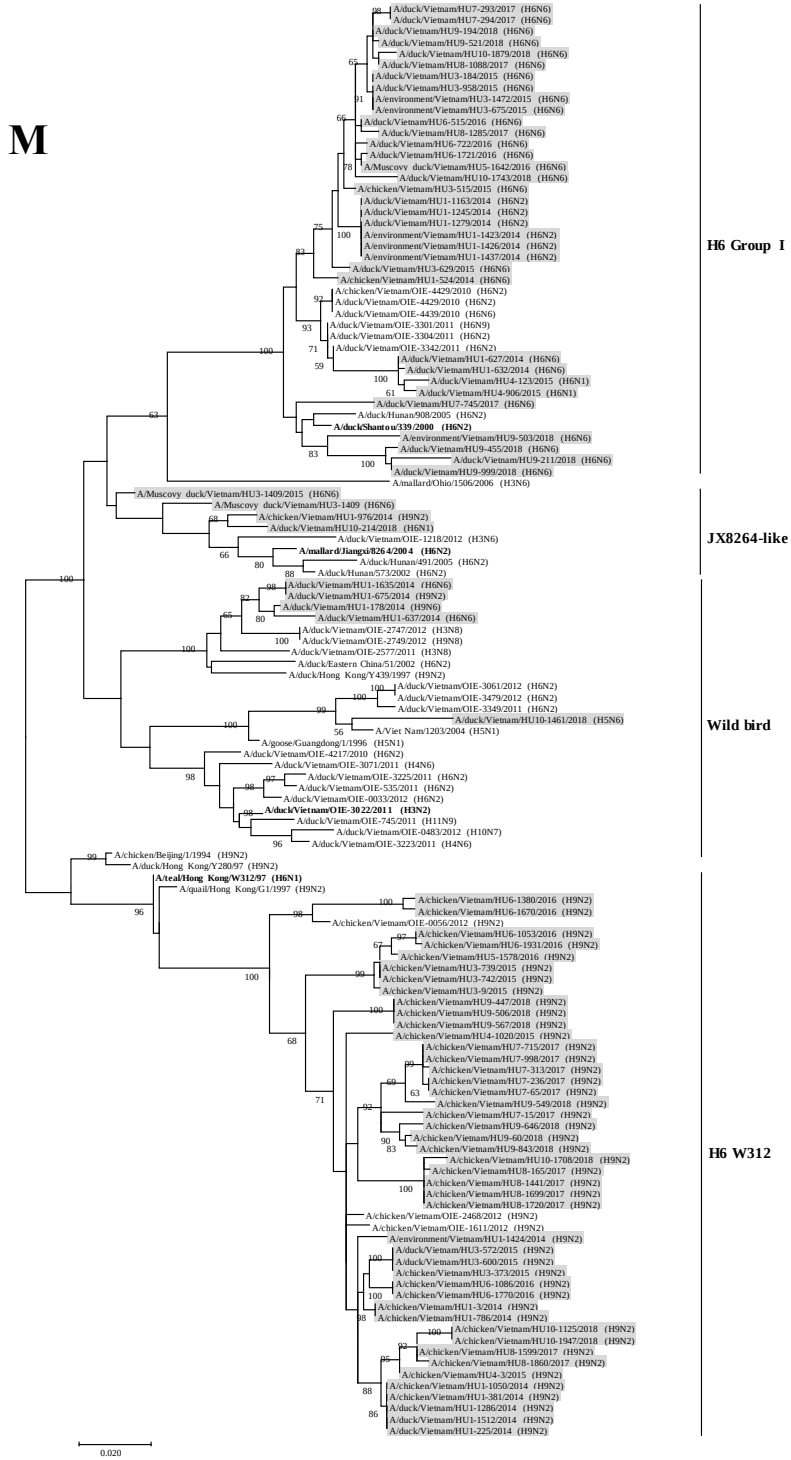


Figure 7 *(cont)***. Phylogenetic tree of internal gene segments of H6 and H9 avian influenza viruses.** The PB2, PB1, PA, NP, M, and NS genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

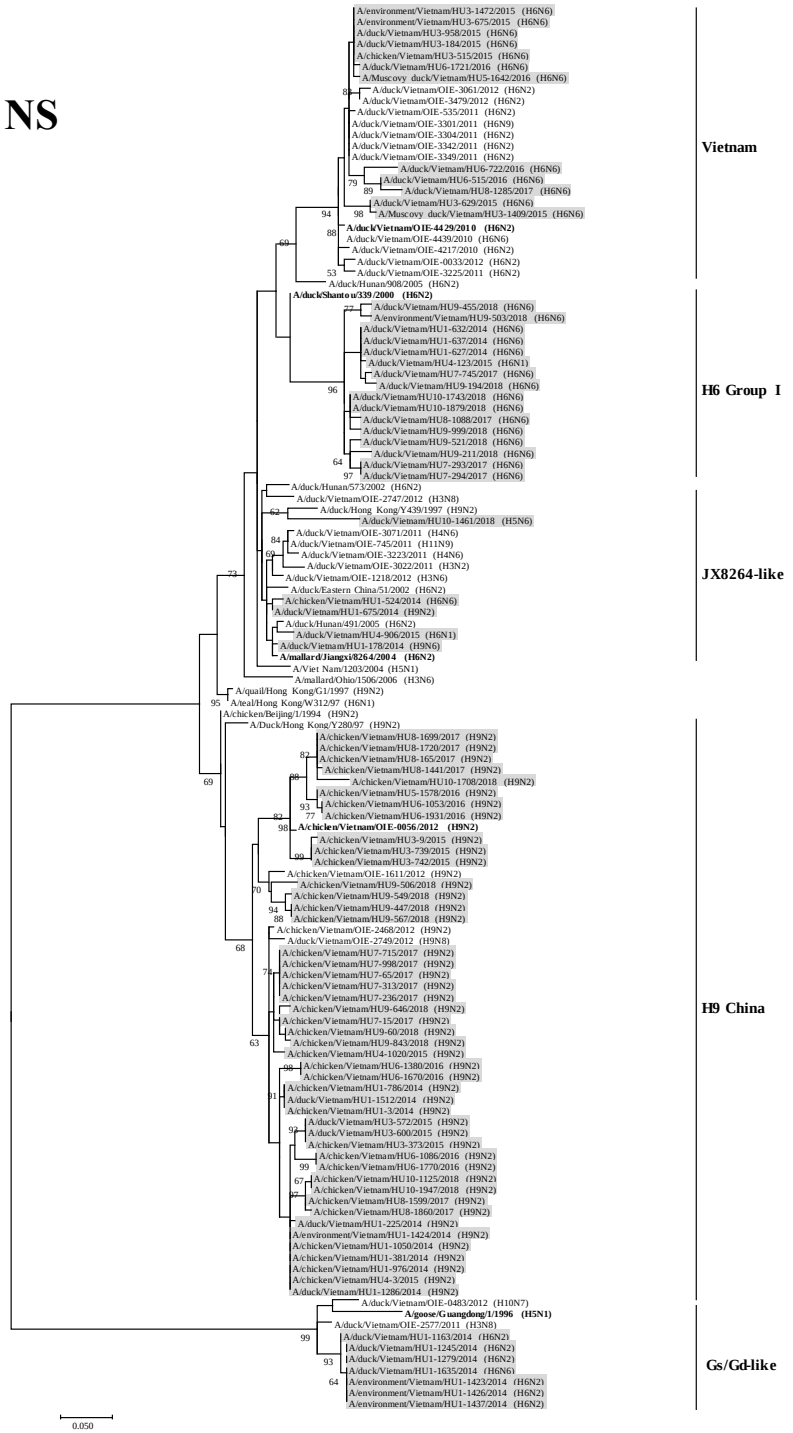


Figure 7 *(cont)***. Phylogenetic tree of internal gene segments of H6 and H9 avian influenza viruses.** The PB2, PB1, PA, NP, M, and NS genes were used for ML phylogenetic analysis using MEGA 7.0 software. Digits at the nodes indicate the probability of the confidence levels in a bootstrap analysis with 1,000 replications. The viruses in this study are highlighted in gray and the representative of each sublineage is indicated in bold.

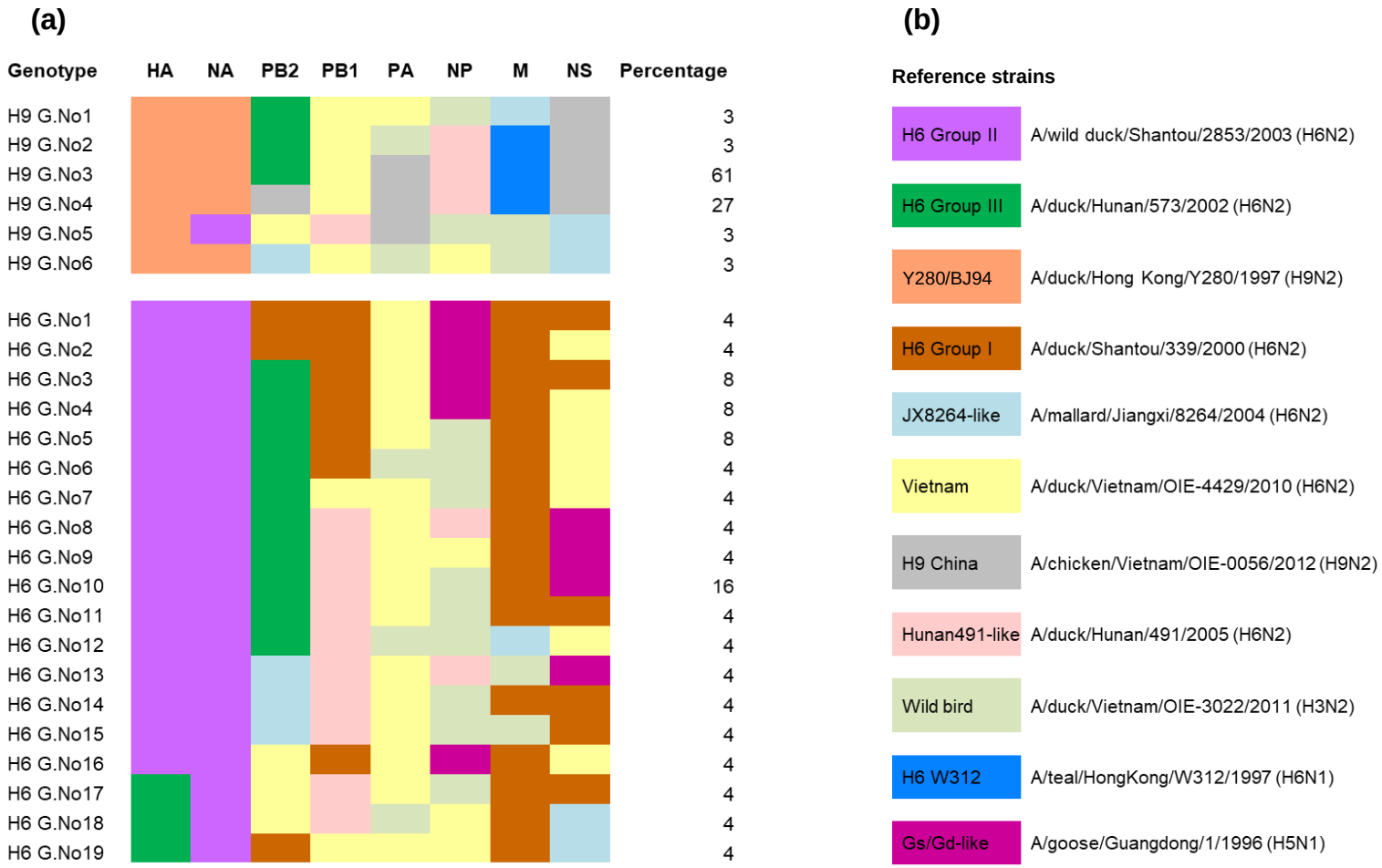


Figure 8. The gene constellations of H6 and H9 avian influenza viruses isolated from poultry in Vietnam. (a) The different colors indicate segments whose sequences fall into different major clades clustered. (b) the representative strains of phylogenetic analysis.

Antigenic analysis of HA of the H6 LPAIV isolates

Ten representative H6 strains were selected for antigenic analysis by cross-HI test using a panel of chicken antisera against eight viruses of different sublineages (Table 4). Most of the representative H6 viruses show a weak reaction with A/duck/Vietnam/OIE-4429/2010 (H6N2) antiserum, a virus belonging to Group II sublineage. The results of the cross-HI test were used to produce the antigenic cartography. The antigenic map indicated that representative H6 viruses form into two antigenic groups, and diverted from the wild bird antigenic group (Figure 9). These results implied that the H6 viruses isolated in this study have antigenically differed from viruses isolated in wild birds and those isolated in Vietnam previously.

Table 4. Antigenic analyses of H6 influenza viruses by cross-HI test

Lineage	Sub lineage	Virus	HI titers of the antiserum							
			Eurasian							North American
			Early	W312	Group II			Group III		
			HK/960 /1980	HK/W312/1997	VN/OIE-4429 /2010	VN/HU1-637 /2014	VN/HU8-1088 /2017	Hok/262/ 2004	Aus/1 /1972	Mass/3740 /1965
Eurasia	Early	A/duck/Hong Kong/960/1980 (H6N2)	5,120	160	160	320	1,280	2,560	640	160
	W312	A/teal/Hong Kong/W312/1997 (H6N1)	2,560	1,280	40	320	640	320	160	160
	Group II	A/duck/Vietnam/OIE-4429/2010 (H6N2)	2,560	80	5,120	1,280	2,560	160	80	160
		A/duck/Vietnam/HU1-1245/2014 (H6N2)	640	80	640	20,480	20,480	320	80	80
		A/duck/Vietnam/HU1-637/2014 (H6N6)	640	80	320	20,480	10,240	160	40	20
		A/duck/Vietnam/HU3-629/2015 (H6N6)	160	20	160	2,560	5,120	160	20	20
		A/duck/Vietnam/HU6-1721/2016 (H6N6)	320	40	160	10,240	10,240	80	40	<20
		A/duck/Vietnam/HU7-745/2017 (H6N6)	80	20	160	1,280	1,280	40	<20	<20
		A/duck/Vietnam/HU8-1088/2017 (H6N6)	640	20	80	5,120	10,240	80	20	<20
		A/duck/Vietnam/HU9-455/2018 (H6N6)	640	20	80	5,120	10,240	80	20	<20
		A/duck/Vietnam/HU10-1879/2018 (H6N6)	160	20	160	5,120	10,240	80	<20	<20
	Group III	A/duck/Hokkaido/262/2004 (H6N1)	5,120	160	160	160	2,560	2,560	1,280	160
		A/duck/Vietnam/HU4-906/2015 (H6N6)	2,560	160	5,120	640	2,560	320	80	80
	-	A/shearwater/Australia/1/1972 (H6N5)	2,560	320	160	160	640	1,280	1,280	320
North American		A/turkey/Massachusetts/3740/1965 (H6N2)	2,560	320	160	160	2,560	640	1,280	640

Viruses isolated in this study are highlighted in bold.
Homologous titers are underlined.
Dk duck, Ck chicken, Ty Turkey, Tl Teal, Sh Shearwater, Hok Hokkaido, HK Hong Kong, Aus Australia, Mass Massachusetts.

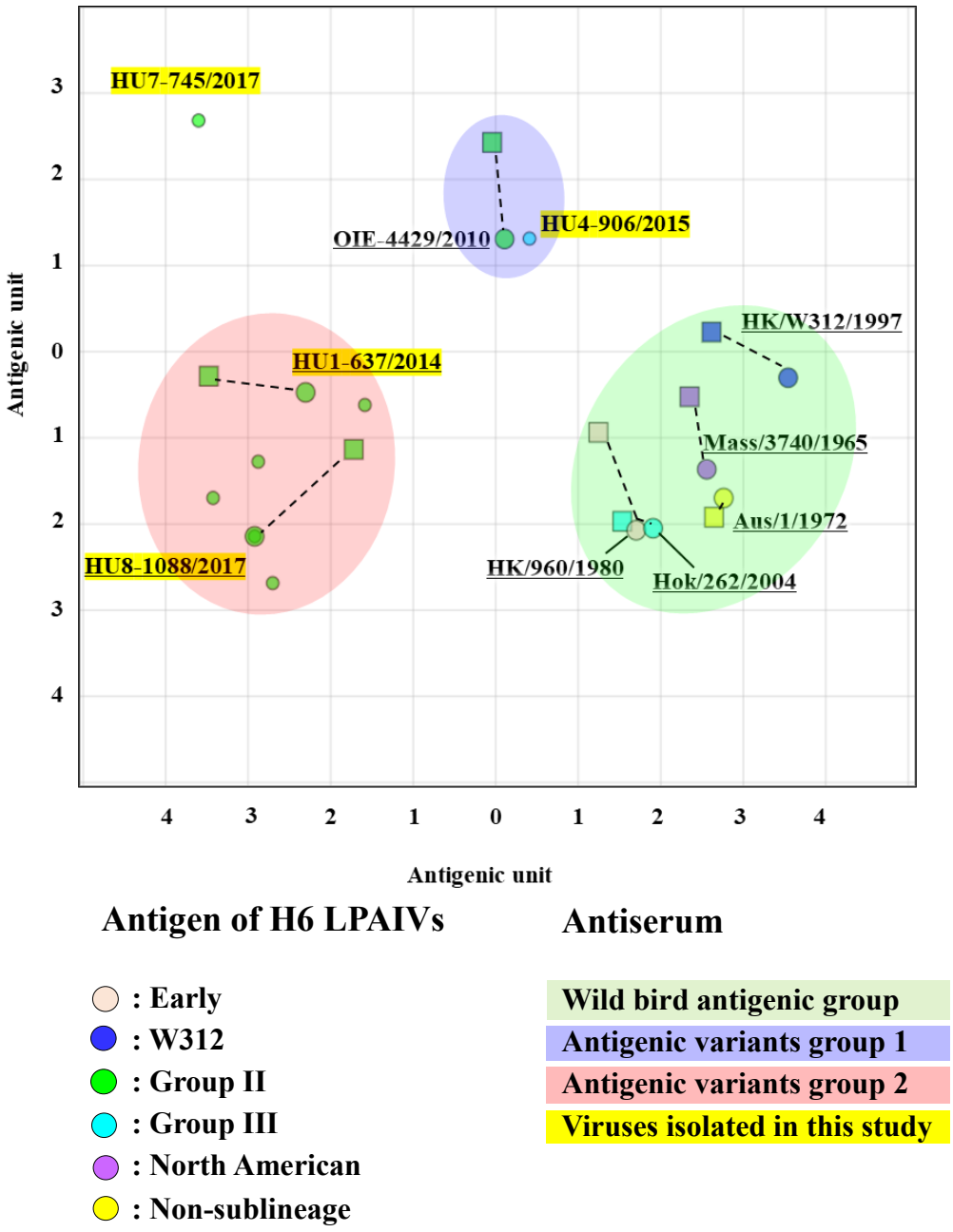


Figure 9. An antigenic map of H6 viruses based on the cross-HI tests on viruses and sera of different lineages. In an antigenic map, both vertical and horizontal axes represent antigenic distance. The spacing between grid lines represents a distance of 1 antigenic-unit distance, corresponding to a 2-fold dilution in the HI assay (e.g. 2 units correspond to a 4-fold dilution, 3 units correspond to an 8-fold dilution etc.). Different antigenic groups are indicated by different colors (green, blue, and red). Sera are indicated by a square symbol and antigens are indicated by a round symbol. Dot line indicates homologous combination.

Antigenic analysis of HA of the H7N7 LPAIV isolates

The antigenicity of the H7N7 viruses isolated during the present study was analyzed by an HI test using a chicken hyper-antisera panel (Table 5). The results of the cross-HI test were used to produce the antigenic cartography necessary for projecting the dataset into 2D cartography (Figure 10). The HI titers of A/duck/Vietnam/HU10-48/2018 (H7N7) and A/duck/Vietnam/HU10-64/2018 (H7N7) to each of the antisera against viruses in the Eurasian lineage were different by approximately 1 to 2 antigenic units, respectively. In addition, the two H7N7 viruses did not react with most of the antisera at lower concentrations compared with the homologous strain, indicating that these two strains are antigenically different from the majority of strains that belong to the Eurasian lineage. Based on the antigenic cartography, the H7Nx viruses examined in the present study were likely to form three distinct antigenic groups. The major antigenic group (green) comprised the majority of viruses, which belonged to the Eurasian, Australian, and North American lineages. The Historical European lineage was likely to form a single antigenic group (blue). The present H7N7 viruses formed another antigenic group (red), together with A/turkey/Italy/4580/1999 (H7N1) and A/duck/Japan/AQ-HE29-22/2017 (H7N9). Furthermore, the antigenic drift of the A/duck/Japan/AQ-HE29-22/2017 (H7N9) from that of A/Anhui/1/2013 (H7N9) and A/duck/Hokkaido/W19/2013 (H7N7) was reported in our previous study [54]. The mass vaccination for H7 subtype was applied in China might generate the immune escape viral mutants, which was identified as the main cause of antigenic drift of AIVs [75]. A/duck/Vietnam/HU10-48/2018 (H7N7) and A/duck/Vietnam/HU10-64/2018 (H7N7) were the most antigenically distant from the viruses of the major antigenic group [more than approximately 1 and 2 antigenic units from A/duck/Hokkaido/W19/2013 (H7N7) to A/duck/Vietnam/HU10-48/2018 (H7N7) and A/duck/Vietnam/HU10-64/2018 (H7N7), respectively]. This result suggested that there were important antigenic differences between the newly isolated H7N7 viruses in Vietnam and the majority of H7 LPAIVs isolated thus far elsewhere in the world.

Table 5. Antigenic analyses of H7 influenza viruses by cross-HI test

Lineage	Sub lineage	Virus	HI titers of the antiserum								
			Eurasian							Australian	North American
			Far -Eastern				European - Asian		Historical Europe		
			Dk/Hok/Vac-2/04	Anhui/1/13	Ck/NK/7916/05	Dk/JP/AQ-29-22/17	Dk/Hok/W19/13	Ty/Italy/4580/99	Dk/TW/Ya103/93	Ck/NSW/327/97	Sl/Mass/1/80
Eurasian	Far - Eastern	A/duck/Hokkaido/Vac-2/2004 (H7N7)	20,480	5,120	10,240	10,240	20,480	5,120	2,560	10,240	5,120
		A/Anhui/1/2013 (H7N9)	10,240	5,120	5,120	20,480	10,240	5,120	1,280	5,120	1,280
		A/chicken/North Korea/7916/2005 (H7N7)	2,560	2,560	5,120	10,240	5,120	256	640	5,120	320
		A/duck/Japan/AQ-HE29-22/2017 (H7N9)	640	640	640	5,120	1,280	640	160	2,560	80
	European - Asian	**A/duck/Vietnam/HU10-48/2018 (H7N7)**	1,280	640	1,280	5,120	2,560	640	640	2,560	640
		A/duck/Vietnam/HU10-64/2018 (H7N7)	640	160	640	2,560	1,280	320	160	1,280	320
		A/duck/Vietnam/OIE-0178/2012 (H7N1)	5,120	2,560	5,120	10,240	10,240	2,560	1,280	5,120	1,280
		A/duck/Hokkaido/W19/2013 (H7N2)	5,120	2,560	2,560	10,240	5,120	2,560	640	5,120	1,280
		A/turkey/Italy/4580/1999 (H7N1)	320	160	640	2,560	640	1,280	320	1,280	160
	Historical Europe	*A/duck/Taiwan/Ya103/1993 (H7N7)*	160	320	640	1,280	640	160	5,120	320	80
Australian		*A/chicken/New South Wales/327/1997 (H7N2)*	5,120	2,560	5,120	20,480	10,240	2,560	1,280	10,240	640
North American		A/seal/Massachusetts/1/1980 (H7N7)	20,480	5,120	10,240	10,240	10,240	5,120	640	10,240	5,120

Viruses isolated in this study are highlighted in bold.
HPAIVs are shown in italic.
Homologous titers are underlined.
Dk duck, Ck chicken, Ty Turkey, Sl Seal, Hok Hokkaido, JP Japan, NK North Korea, TW Taiwan, NSW New South Wales, Mass Massachusetts.

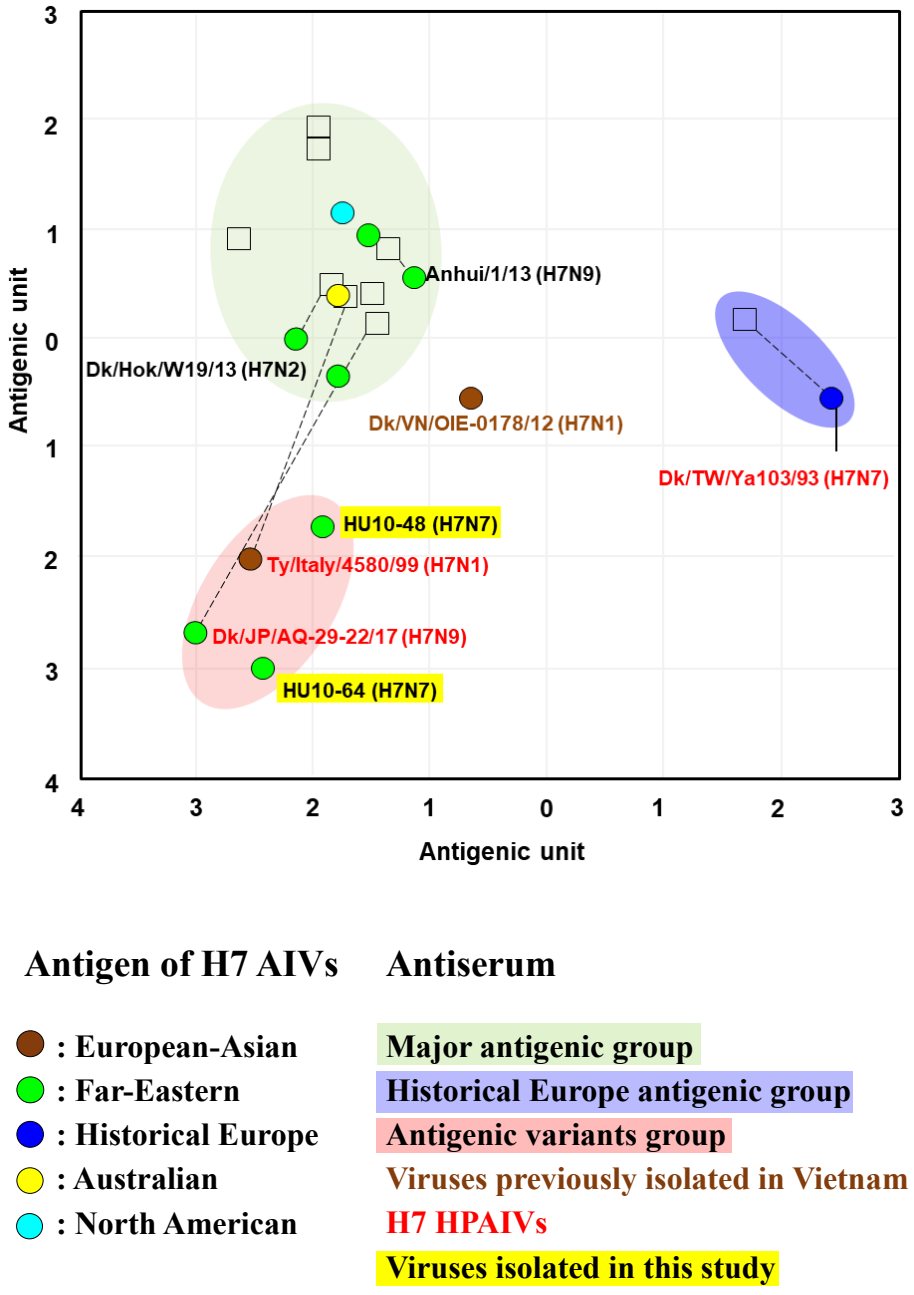


Figure 10. An antigenic map of H7 viruses based on the cross-HI tests on viruses and sera of different lineages. In an antigenic map, both vertical and horizontal axes represent antigenic distance. The spacing between grid lines represents a distance of 1 antigenic-unit distance, corresponding to a 2-fold dilution in the HI assay (e.g. 2 units correspond to a 4-fold dilution, 3 units correspond to an 8-fold dilution etc.). Different antigenic groups are indicated by different colors (green, blue, and red). Sera are indicated by a square symbol and antigens are indicated by a round symbol. Dot line indicates homologous combination.

Antigenic analysis of HA of the H9 LPAIV isolates

A total of seven representative H9 viruses were antigenically analyzed by cross-HI test (Table 6). All the Vietnam isolates in this study show the strong reaction with the antiserum of A/duck/Hong Kong/Y280/1997 (H9N2) virus and newly prepared antiserum of A/chicken/Vietnam/HU8-1860/2017 (H9N2) belonging to the Y280/BJ94 sublineage. The moderate reaction with antisera of Y349 sublineage virus and weak reaction with antisera of either G1 sublineage virus or North American lineages were observed in all the Vietnam isolates. The results of the cross-HI test were used to produce the antigenic cartography. The antigenic map indicated that Vietnam representative H9 viruses are distant from the wild bird antigenic group but formed same cluster with the domestic bird antigenic groups (Figure 11). These results suggested that the antigenicity of the Vietnamese H9 viruses was stable during the circulation in the poultry population.

Table 6. Antigenic analyses of H9 influenza viruses by cross-HI test

Lineage	Sub lineage	Viruses	HI titers of the antisera				
			Eurasian				North American
			Y280/BJ94		G1	Y439	
			HK/Y280/97	VN/1860/17	HK/G1/97	Hok/49/98	Wis/1/66
Eurasian	Y280/ BJ94	A/Duck/Hong Kong/Y280/1997 (H9N2)	20,480	10,240	1,280	5,120	640
		A/chicken/Vietnam/OIE 1611/2012 (H9N2)	10,240	10,240	1,280	2,560	320
		A/chicken/Vietnam/HU1 786/2014 (H9N2)	10,240	10,240	1,280	5,120	320
		A/chicken/Vietnam/HU3-742/2015 (H9N2)	10,240	10,240	1,280	5,120	640
		A/chicken/Vietnam/HU7-236/2017 (H9N2)	20,480	10,240	1,280	5,120	320
		A/chicken/Vietnam/HU8-1860/2017 (H9N2)	20,480	10,240	2,560	10,240	1,280
	G1	A/quail/Hong Kong/G1/1997 (H9N2)	2,560	640	10,240	1,280	640
		A/duck/Vietnam/OIE-2592/2009 (H9N2)	10,240	1,280	5,120	5,120	640
	Y439	A/duck/Hokkaido/49/1998 (H9N2)	320	80	80	2,560	320
		A/duck/Vietnam/OIE 2334/2010 (H9N6)	160	40	160	1,280	320
North American	-	A/turkey/Wisconsin/1/1966 (H9N2)	80	40	40	1,280	2,560

Viruses isolated in this study are highlighted in bold.

Homologous titers are underlined.

Dk duck, Ck chicken, Ty Turkey, Hok Hokkaido, HK Hong Kong, Wis Wisconsin.

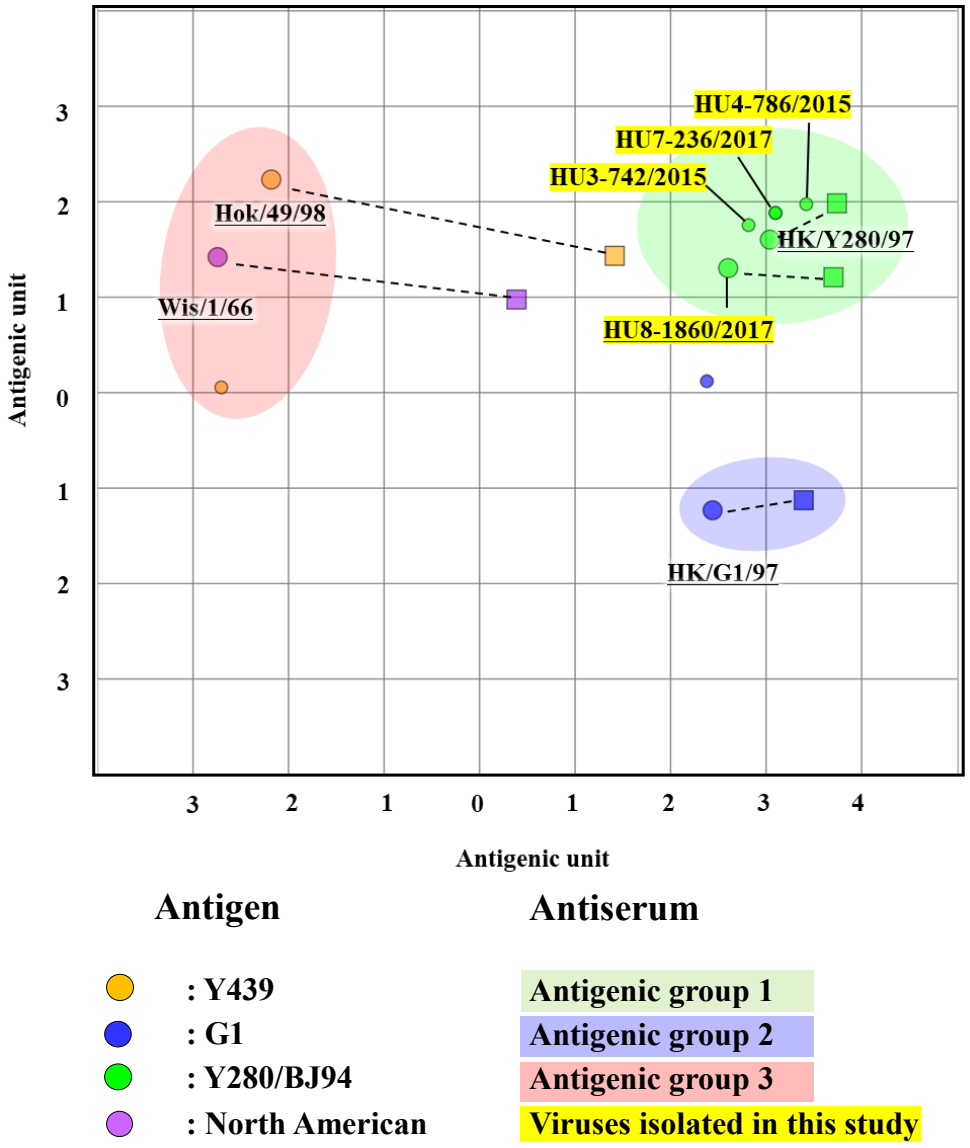


Figure 11. An antigenic map of H9 viruses based on the cross-HI tests on viruses and sera of different lineages. In an antigenic map, both vertical and horizontal axes represent antigenic distance. The spacing between grid lines represents a distance of 1 antigenic-unit distance, corresponding to a 2-fold dilution in the HI assay (e.g. 2 units correspond to a 4-fold dilution, 3 units correspond to an 8-fold dilution etc.). Different antigenic groups are indicated by different colors (green, blue, and red). Sera are indicated by a square symbol and antigens are indicated by a round symbol. Dot line indicates homologous combination.

Discussion

Among AIV subtypes, H6 and H9 AIVs were the dominant subtypes and detected widely in poultry and wild birds in Asia [33,34]. In which, H9N2 AIVs were the most prevalent subtype in China [76] and in Vietnam [20,21]. All the H6 and H9 viruses isolated in this study were phylogenetically close to viruses previously isolated in poultry in Vietnam [20-22] and those isolated in China in 2008, indicating that these viruses circulated and were predominant in poultry population after introducing into Vietnam. During the circulation, the reassortment of H6 and H9 LPAIVs seems to be high-frequent events not only in Vietnam but also in neighboring countries [77,78]. The antigenicity of representative H6 viruses was divided into different groups, indicating that the antigenicity of Vietnamese H6 viruses has undergone a diversification similar to those in China [77]. Most of the Vietnamese H6 viruses were isolated from ducks, implying that Vietnamese H6 viruses seem to be adapted in duck rather than chicken. Moreover, the free-grazing duck was a common farming model in Vietnam and the repetitive infections of the duck population might be continuous events during farming practice. The antibodies induced by the natural infection might promote the antigenic drift of Vietnamese H6 viruses. However, further studies are necessary to prove antigenic drift of H6 viruses in the duck population in Vietnam. In contrast, the antigenicity of Vietnamese H9 viruses was almost close and formed into a single antigenic group. Conserved antigenicity of H9 isolates suggested that the viruses were maintained in immunologically naïve poultry population in Vietnam even in high prevalence of H9 viruses.

H7N7 LPAIVs have been detected in several countries on the East Asian–Australasian Flyway (including Mongolia, Japan [48], China [79], and South Korea [53]), indicating the circulation of these viruses in migratory waterfowl. In the present study, two H7N7 LPAIV were isolated from domestic ducks in Vietnam; this is the first report of H7N7 LPAIV detection in the country. As previously reported [53], H7N7 LPAIVs circulate in wild birds and are likely to be introduced into domestic ducks. The genetic analysis of two new isolates revealed possible ongoing reassortment of H7 LPAIVs and antigenic diversification. In the present phylogenetic analyses, possible transmission routes of the H7N7 viruses have been identified; the viruses carrying the H7 HA gene

belonging to the European–Asian sublineage circulate in East Asia and were introduced first into Cambodia as an H7N3 virus and then into Vietnam. The virus carrying the N7 NA gene of the European–Asian sublineage has circulated in Vietnam since at least 2012 as an H10N7 virus, and likely shares this N7 gene with the newly isolated H7N7 viruses. The internal genes of viruses reported for the first time in this study, may be shared with other AIVs circulating in Vietnam and other viruses that have been isolated in East Asian–Australasian flyway countries; HA, PB1, and M of A/duck/Vietnam/HU10-64/2018 (H7N7) seem to have been introduced into Vietnam from Cambodia. The M gene of A/duck/Vietnam/HU10-48/2018 (H7N7), as well as its PB2, PA, NP, NA, and NS genes are likely to be shared with other viruses in the same area and have been a result of spillover from wild birds and are now maintained in the poultry population in Vietnam. The distinct M gene segments identified on the single farm in this study suggest an ongoing reassortment event, although it's conceivable that genetic reassortment occurred during the virus isolation in the laboratory. In addition, a previous field epidemiological study indicated that poultry movement across areas that border Cambodia might lead to proliferation of the H7 LPAIVs circulating in both Vietnam and Cambodia [80]. Taken together, these results suggest that Vietnam and Cambodia have a close relationship in terms of AIV ecology and further studies should focus on this relationship.

Interestingly, the two H7 AIVs in this study showed similar antigenicity to HPAIVs of two distinct sublineages: European–Asian [A/turkey/Italy/4580/1999 (H7N1)] and Far Eastern [A/duck/Japan/AQ-HE29-22/2017 (H7N9)], despite differences in their amino acid sequences (94% and 91% homology, respectively). The single mutation at A138T in the 130-loop may play an important role in the antigenic differences between A/duck/Vietnam/HU10-64/2018 (H7N7) and A/duck/Vietnam/HU10-48/2018 (H7N7), which are indicated in Figure 10. In general, non-pathogenic AIVs circulate among wild ducks under the relatively low selective pressure of antibodies; thus, they are antigenically stable [81]. However, the long-term circulation of H7 viruses in the poultry population may lead to selective pressure from antibodies induced by natural infections in poultry, accelerating antigenic variation. The H7 vaccine has not been used in Vietnam thus far, so the antigenic variance of the two H7 AIVs in this study suggests antigenic diversification occurred in domestic ducks following natural infections. Furthermore, the similarities in antigenic properties of the present H7N7 LPAIVs with Chinese H7N9

HPAIVs suggest that the multi-direction of antigenic diversity of AIVs in poultry population and these newly isolated H7N7 viruses would be considered as potential candidates for vaccine strain. The antigenic similarity despite the differences in amino acid sequence suggests the synonymous mutation on Vietnamese H7N7 viruses.

H7N9 AIVs of the Chinese group have not been detected during our influenza surveillance of poultry. However, some H7 LPAIV strains are capable of undergoing systemic replication and efficient transmission in chickens [82], and the circulation of H7 LPAIVs in a poultry population can increase their pathogenicity [61]. In addition, domestic birds in Vietnam are mainly raised in households in a free-range manner, and poultry can come into direct contact with wild animals. Therefore, the monitoring system should pay more attention to LPAIVs. The detection of H7 LPAIVs in the south of Vietnam at different time periods, as well as in Cambodia in recent years [59,60], has acted as a warning of the silent circulation of AIVs in the southern border area.

A previous study indicated that the transmission of AIVs occurs through a combination of local and long-distance spreading [83]. Taking the above results together, combining a number of countermeasures such as stamping out LPAIVs completely, better hygiene practices, and improved biosecurity is key to controlling LPAIVs in Vietnam. Of these, stamping out LPAIVs of H5 and H7 subtypes is highly recommended so as to remove them from the poultry population before they mutate into HPAIVs and become antigenically divergent viruses [84]. Further studies would be necessary to monitor the circulation and analyze the epidemiology of LPAIVs in Vietnam, giving a more comprehensive data of the economic impact and human health risk of the viruses [85]. Thus, active surveillance should be conducted continuously in the high-risk areas to monitor the circulation of AIVs together with the specific countermeasures will be an appropriate combination to control not only HPAIVs but also LPAIVs.

Brief summary

A total of 1,361 AIVs of various subtypes were isolated in the surveillance from 2014 to 2018, in which H6 and H9 were the dominant subtypes and H7N7 was initially detected. The phylogenetic analysis of the HA genes revealed that Vietnamese H6 and H9 LPAIVs were classified into Group II and Y280/BJ94 sub-lineages, respectively, and clustered together with previous isolates in Vietnam and neighboring countries. The H7 LPAIVs were clustered together with Cambodian isolates but not the H7 LPAIVs previously isolated in Vietnam or Chinese H7N9 HPAIVs. The silent spread of Vietnamese H7N7 viruses in chickens may lead to acquire high pathogenicity in chickens although the zoonotic potential of the viruses seems to be low since these viruses retain typical avian-specific motifs in the receptor-binding site in the HA. The antigenicity among Vietnamese H6 and H7 viruses showed a slight diverse and formed into different antigenic groups from preexisting viruses, meanwhile H9 viruses isolated in the study period were almost identical. Conserved antigenicity of H9 isolates from poultry suggested that the viruses were maintained in immunologically naïve poultry population in Vietnam even in high prevalence of H9 viruses. Although H9 viruses were classified as LPAIV, they could acquire high pathogenicity due to the coinfection with the other pathogens in the field. Thus, these results highlight the need for intensive surveillance and control measure in Vietnam, targeting not only HPAIVs but also LPAIVs.

Chapter II

A systematic approach to illuminate a new hot spot of avian influenza virus circulation in South Vietnam, 2016-2017

Introduction

AIV circulation has been reported in many countries, including Vietnam [15]. Particularly, since 1996, outbreaks of HPAI have occurred in poultry throughout Asia despite large-scale vaccination campaigns and stamping-out programs in a number of countries [86,87]. Although the number of HPAI outbreaks in Vietnam due to infection with H5N1 subtype viruses has markedly decreased since 2004 [19], substantial losses in the domestic poultry sector continue to occur. A number of studies have improved our understanding of the epidemiology of AI by identifying drivers of virus spread [20-23,27,29]. As part of their efforts to reduce AIV infection risk, the Vietnamese government has developed both active and passive surveillance programs. One of the advantages of active surveillance programs is that they can detect the introduction of new virus strains into a population or detect the evolution of virus strains relatively quickly. In contrast, passive surveillance programs rely on prompt reporting by poultry farmers for timely disease event detection.

The results of data collected by active surveillance programs that have been operational in Asia, Europe, and North America since 2014, show that diversification of AIV subtypes has increased [88,89]. Despite some AIVs being categorized as LPAIVs, they can cause substantial poultry production losses such as high rates of mortality, reductions in egg production [31], and pose a concern for global health security arising from the risk of zoonotic infection. Due to variations in the pathogenicity of AIVs dependent on subtype, it is essential to monitor virus subtypes circulating in the field [25].

In a number of previous studies, the movement of live birds arising from trade has shown to be an important determinant of AIV spread [90-92]. In addition, LBMs play an important role in AIV circulation [30,93-96]. During an outbreak of H7N9 AIV in China in 2013 which was the cause of up to 45 human deaths, the closure of LBMs was remarkably effective in reducing human infection rates by up to 99% [97]. Although LBM closures break the viral amplification cycle, AIVs are often re-introduced once they are re-opened [98]. A previous Vietnamese study investigating the effectiveness of virus control measures in LBMs showed no differences in AIV prevalence between LBMs with and without biosecurity interventions [23]. One interpretation of these

findings is that the introduction of AIV into LBMs occurs continuously. The absence of differences in AIV prevalence between intervention and non-intervention LBMs supports the hypothesis that the source of AIV in the value chain of poultry products in Vietnam has not yet been fully identified and controlled.

As a result of active surveillance programs for AI that have been operational in Vietnam since 2015, it was shown that PDSs play a role connecting poultry farms, LBMs and poultry slaughterhouses (Figure 12). Backyard farms are characterized by their small-scale, the mixing of poultry species and relatively low levels of biosecurity whereas commercial farms routinely practice several AIV control measures such as separating poultry species, routinely disinfecting those entering and leaving premises and limiting contact between poultry and wildlife. LBMs tend to receive poultry from nearby backyard and semi-commercial poultry enterprises [99]. In contrast, PDSs are private businesses which usually receive birds from much larger catchment areas (up to 100 km) and mix several species of poultry under relatively poor biosecurity conditions.

A cross-sectional study of AI was conducted to assess the biosecurity practices among four poultry enterprise groups (backyard farms, commercial farms, LBMs and PDSs) in Vinh Long province, Vietnam in 2016 and 2017. The specific aims were to: (1) estimate the individual bird-level prevalence of AIV in each of the four enterprise groups; and (2) identify characteristics of those responsible for the management of birds that were associated with AIV infection positivity. Identifying poultry flock manager characteristics that increase the risk of AIV positivity across different industry players is a necessary step towards the design of effective, evidence-based measures to reduce the risk of AIV infection through the supply chain of poultry products in Vietnam.

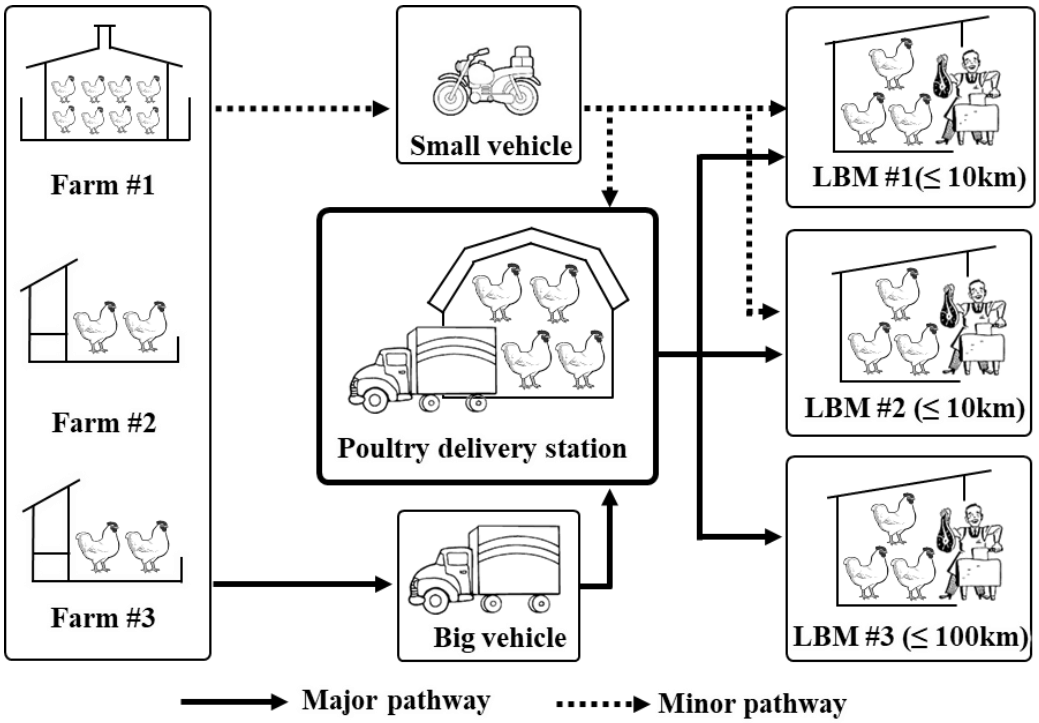


Figure 12. Flowchart of the role of PDS in the poultry value chain.

Materials and Methods

Study design and study area

This was a cross-sectional study of owners of backyard poultry farms, managers of commercial poultry farms, poultry sellers at LBMs and PDS traders in four of the eight districts of Vinh Long province, Vietnam (Figure 13). Data were collected over two sampling rounds: the first in December 2016 and the second in August 2017. From a sampling frame of enterprises provided by local DAH officials those eligible for the study were selected at random from each of the four poultry enterprise groups. The key decision maker of each selected enterprise was contacted and asked if they consented to take part in the study. A total of 228 decision-makers agreed to take part representing 101 backyard farms, 50 commercial poultry farms, 58 sellers at LBMs and 19 traders at PDSs. For the purpose of this study, enterprises that had not applied any prevention measures following local authority guidelines such as keeping poultry in a separate place, vaccination, and disinfection were defined as backyard farms. Enterprises, where at least more than one of several control measures (such as keeping poultry in a separate place, the use of routine vaccination and disinfection) were applied, were defined as commercial poultry farms. Up to two LBMs from each of the four study districts of Vinh Long were selected at each of the two sampling rounds, leading to a total of 12 individual LBMs included in the study. Similarly, up to two PDSs per study district were selected at each sampling round, returning 13 individual PDSs included in the study. In each of the two sampling rounds, the average number of birds sampled was 10 for backyard farms (minimum of 5, maximum of 20), 26 for commercial poultry farms (minimum of 10, maximum of 50), 11 for LBM sellers (minimum of 10, maximum of 52) and 40 for PDS traders (minimum of 19, maximum of 52). At the time of bird sampling, key decision makers (referred to as ‘respondents’ in the remainder of this thesis) from selected backyard farms, commercial poultry farms, LBM traders and PDSs were interviewed with the support of staff from the Sub-Department of Animal Health (SDAH) staff of Vinh Long province for the purpose of questionnaire administration.

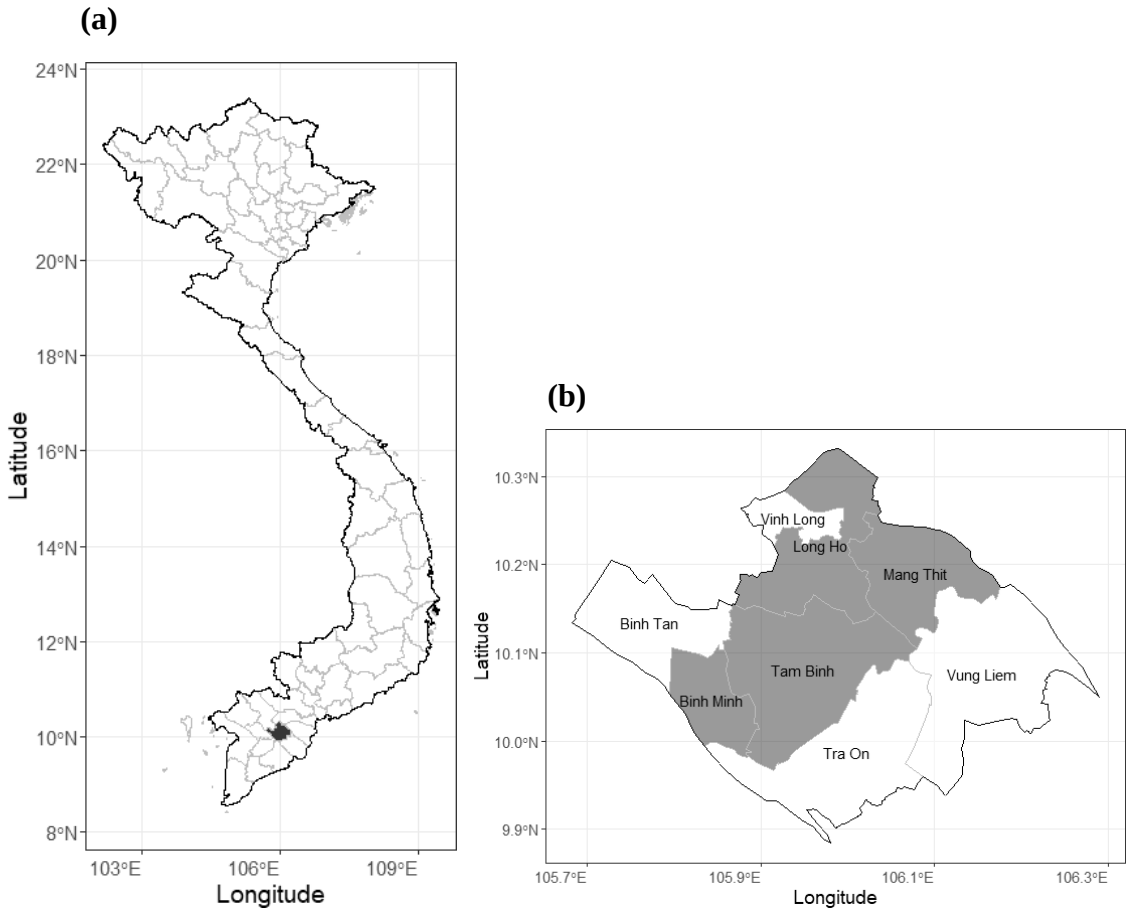


Figure 13. (a) Map of Vietnam showing the location of Vinh Long province; (b) map showing the district boundaries in Vinh Long and the location of the four districts in which sampling was carried out (gray).

Laboratory procedures

Oropharyngeal swabs, cloacal swabs and fecal samples were collected from chickens, ducks, and Muscovy ducks from each participant enterprise at each sampling round. The oropharyngeal and cloacal swabs from the same poultry were kept in one sterile tube containing transport medium, as described in Chapter I [100]. Samples were transported to the Regional Animal Health Office No. 7 (RAHO7), Can Tho, Vietnam. Under ISO 17025:2017 certification for the diagnostic procedure in RAHO7, the aliquot of ten samples collected from the same enterprise were pooled to test for the presence of influenza type A virus using real-time reverse transcription-PCR (RT-PCR) targeting the M gene with the primer design and thermal cycle [101] following methods described by the OIE [56]. All samples were then transferred to the Laboratory of Microbiology in the Faculty of Veterinary Medicine, Hokkaido University, Japan for virus isolation.

Virus isolation

The virus isolation by using ten-day-old chicken embryonated eggs then subtyping by using HI and NI tests were conducted by applying the same method described in Chapter I.

Questionnaire and interview

By referring to previous survey documents developed by the DAH, Ha Noi, a questionnaire to collect details of knowledge, attitudes, and practices regarding AIV was developed in partnership with SDAH staff. This questionnaire was then modified to suit the specific conditions for respondents from backyard farms and commercial poultry farms (Appendix 1), LBM sellers (Appendix 2) and PDS traders (Appendix 3). In detail, the questionnaires comprised of 87, 82 and 118 questions were established for farms, LBM and PDS, respectively. All three questionnaires asked respondents to provide details on: (1) their demographic status; (2) the source, type and numbers of poultry present on their enterprise on the day of interview; (3) their general knowledge regarding AIV; (4) their attitudes about AI control measures; and (5) AI biosecurity measures routinely used.

At the start of the first sampling round SDAH staff from Vinh Long (n=8) who were recruited for data collection received instruction on questionnaire administration.

Questionnaire surveys were administered by SDAH staff to each respondent. A total of 228 face-to-face interviews were carried out during the two sampling rounds in the four districts. In each sampling round, birds were sampled and questionnaires administered to respondents on each of the participant backyard farms and commercial farms in the early stage. Immediately after the early stage was finished, the same procedure was then applied in LBMs and PDSs at the later stage. The sampling schedule was announced to respondents and local veterinarians as well in advance and, for both rounds, samples were collected and questionnaires administered over a period of 8 days.

Data management

Each of the respondents enrolled into the study were assigned a unique identification code. Questionnaire responses at each sampling round and the results of AIV isolation from sampled poultry were recorded in two tables in a relational database with the respondent identification code providing the link between each table.

The diagnostic sensitivity and specificity of both the RT-PCR and virus isolation were assumed to be both 100% [101,102].

Multiple correspondence analysis

Multiple correspondence analysis (MCA) was used to produce a graphic representation of the relationships between responses provided in each of the four sections of the questionnaire: demographic details, AIV knowledge, AIV attitude and AIV practice [103].

MCA is a generalization of principle component analysis suitable for categorical variables. In an MCA, the rows and columns of an $I \times J$ indicator matrix (where I is the set of i individual responses to a given question and J is the set of j categories of responses for each question) are assumed to be points in a high-dimensional Euclidean space. The method aims to redefine the dimensions of the space so that the principal dimensions ('components') capture the most variance. The results of the MCA are presented as a scatterplot for the first and second principle components – that is, the dimensions that capture most of the variability in the data. In an MCA scatterplot, questionnaire responses that are similar in distribution across respondents are positioned close on the plot. MCA scatterplots were produced using responses to each of the four

sections of the questionnaire and, for each plot, cluster analysis using hierarchical clustering on principal components (HCPC) was carried out using Ward's method. This allowed to aggregate respondents into relatively homogeneous subgroups ('clusters') for each section of the questionnaire. These assigned clusters were then used as explanatory variables in a multivariable logistic regression model of bird-level AIV infection risk. MCA analyses were performed using the contributed FactoMineR package [104] in R version 4.0.5 [105].

Mixed-effects logistic regression

A mixed-effects logistic regression model was developed to quantify the association between respondent-level explanatory variables and the risk of a bird being AIV positive at the time of sampling. Unconditional associations between each of the explanatory variables and the outcome variable (AIV status) were expressed as the odds ratio (OR). Explanatory variables associated with the outcome at $P \leq 0.2$ (two-sided) at the unconditional level were selected for multivariable modeling.

For multivariable model, the probability that a bird was AIV positive p_i was parameterized as a function of the candidate cluster variables (as described above) in addition to a single categorical variable comprised of four levels defining respondent enterprise type (backyard farm, commercial poultry farm, LBM seller and PDS trader). If Y_i defines AIV positivity status for the ith bird, this model takes the following form under the assumption of $p_i = P(Y_i = 1)$ and that Y_i are mutually independent:

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

In Equation 1, β_0 represents the intercept term and $\beta_1, \ldots, \beta_m$ the regression coefficients for each of the m explanatory variables in the model.

To account for the lack of independence arising from the hierarchical structure of the data, that is, individual birds clustered within respondents, Equation 1 was extended to a mixed-effects model as follows:

$$log\left(\frac{p_{ij}}{1 - p_{ij}}\right) = \beta_0 + \beta_1 x_{1ij} + \cdots + \beta_m x_{mij} + P_j + \varepsilon_{ij} \qquad \text{Equation 2}$$

In Equation 2, p_{ij} represents the probability of the ith bird from the jth respondent being AIV positive. Variable P_j is a zero mean random effect term with variance σ_P^2

indicating the effect of the jth respondent on AIV positivity. The term P_j was included in the model to account for unexplained extrabinomial variation arising from unmeasured respondent-level influences on AIV positivity.

A backward stepwise approach was used for explanatory variable selection. Each of the explanatory variables unconditionally associated with the outcome at $P \leq 0.2$ were included in the fixed-effects model (Equation 1). Explanatory variables were removed from the model, one at a time, starting with the least significant until all variables that remained were associated with the outcome at $\alpha < 0.05$. Explanatory variables that were excluded in univariable analyses were tested for inclusion in the final model and were retained if their inclusion changed any of the estimated regression coefficients by more than 20%. Biologically plausible two-way interactions between explanatory variables were assessed; none were found to be significant at $\alpha = 0.05$. The model was then extended to include the random effect term P_i (Equation 2). Explanatory variables were retained in the mixed-effects model, regardless of their statistical significance.

The assumptions of normality and homogeneity of variance were investigated by constructing histograms of residuals from the multilevel model and scatterplots of the residuals as a function of the predicted values, respectively. Estimates of the variance attributable to the three levels of the data (respondent, bird) were calculated assuming the level 1 (bird) variance on the logit scale was $\frac{\pi^2}{3}$ where $\pi = 3.1416$ [103].

A Receiver Operating Characteristic (ROC) curve was constructed on the basis of the bird-level AIV positivity status predicted by the model. The area under the ROC curve (AUC), which ranges from zero to one, provided a measure of the model's ability to discriminate between AIV-positive and AIV-negative birds. The greater the AUC is the better the model's discriminatory power is.

The unconditional measures of association analyses were carried out using the contributed epiR package in R [106]. The mixed-effects logistic regression model was developed using the contributed lme4 package in R [107].

Ethics statements

The handling process of the chicken embryo for virus isolation was carried out with guidelines by the Hokkaido University, Faculty of Veterinary Medicine. Fieldworks were

conducted based on the volunteer of participants and the process was approved by the Department of Animal Health, Vietnam.

Results

Descriptive statistics and unconditional associations

Details of the number of birds sampled, the number of AIV positive samples and the prevalence of AIV positivity stratified by enterprise type, species, sampling round and district are shown in Table 7. A total of 3,597 birds were sampled; 1,056 from 101 backyard farms, 1,200 from 50 commercial poultry farms, 660 from 58 sellers at 12 LBMs and 681 from 19 traders at 13 PDSs. Two hundred and seventy-four of 3,597 birds (7.6%; 95% CI: 6.8%–8.5%) were AIV positive. In total, 13 H3N2, 21 H5N1, 127 H6N6, 105 H9N2, 2 H10N3, 5 H11N9, and 1 H12N5 AIVs were identified from collected samples (Table 8). Isolation rates for AIV varied by poultry enterprise type with the highest prevalence among birds sampled from PDSs (21.0%; 95% CI: 18.0%–24.0%), followed by LBMs (14.0%; 95% CI: 12.0%–17.0%), backyard farms (3.0%; 95% CI: 2.1%–4.3%) and commercial poultry farms (0.6%; 95% CI: 0.2%–1.2%) (Figure 14).

The numbers of chickens and ducks sampled were 1,801 (50%) and 1,575 (44%), respectively. Because the total number of Muscovy ducks, geese and environment samples was 221 (6.1%), only AIV positivity for chicken and duck samples were compared. The prevalence of AIV positivity for ducks (10.0%; 95% CI: 8.5%–12.0%) was significantly higher than the prevalence of AIV positivity for chickens (5.6%; 95% CI: 4.5%–6.7%; $P<0.01$). This result reflects the field situation that the environment in which ducks are typically kept facilitates AIV survival, much more than that of the environment in which chickens are kept. The prevalence of AIV positivity differed across the two sampling rounds with a lower prevalence in 2016 (5.9%; 95% CI: 4.9%–7.1%) compared with 2017 (9.4%; 95% CI: 8.1%–11.0%).

Table 7. Numbers of birds sampled, numbers of samples AIV positive and AIV positivity prevalence, expressed as the number of AIV-positive birds per 100 birds at risk by enterprise type, species, sampling round and district

Variable	No. of samples	AIV positive	Prevalence (%) (95% CI) [a]	*P*-value
Enterprise type				
Commercial	1,200	7	0.6 (0.2–1.2)	Ref
Backyard farm	1,056	32	3.0 (2.1–4.3)	<0.01
LBM	660	94	14.0 (12.0–17.0)	<0.01
PDS	681	141	21.0 (18.0–24.0)	<0.01
Species				
Chicken	1,801	100	5.6 (4.5–6.7)	Ref
Duck	1,575	157	10.0 (8.5–12.0)	<0.01
Muscovy duck	189	16	8.5 (4.9–13.0)	0.11
Environment	18	0	0.0 (0.0–18.0)	0.97
Goose	14	1	7.1 (0.2–34.0)	0.27
Sampling round				
1 (2016)	1,814	107	5.9 (4.9–7.1)	Ref
2 (2017)	1,783	167	9.4 (8.1–11.0)	<0.01
District				
Binh Minh	910	61	6.7 (5.2–8.5)	Ref
Long Ho	909	61	6.7 (5.2–8.5)	0.84
Mang Thit	867	53	6.1 (4.6–7.9)	0.61
Tam Binh	911	99	10.9 (8.9–13.0)	<0.01

[a] Number of AIV positive birds per 100 birds at risk.
Ref reference.

Table 8. Numbers of birds sampled, numbers of samples AIV positive and AIV positivity prevalence (expressed as the number of AIV-positive birds per 100 birds at risk) and details of AIV subtypes isolated by enterprise type and species from 2016 to 2017

Enterprise type	Species	No. of samples	AIV positive	Prevalence (%) (95% CI)	Subtype (no. of isolates)
Backyard farm	Chicken	419	10	2.4 (1.2–4.3)	H9N2 (10)
	Duck	612	22	3.6 (2.3–5.4)	H3N2 (1), H6N6 (19), H10N3 (2)
	Muscovy duck	25	0	0.0	
Commercial	Chicken	638	0	0.0	
	Duck	560	7	1.3 (0.5–2.6)	H3N2 (4), H11N9 (3)
	Muscovy duck	2	0	0.0	
LBM	Chicken	347	52	15.0 (11.4–19.2)	H3N2 (1), H9N2 (51)
	Duck	215	25	11.6 (7.7–16.7)	**H5N1 (8)**, H6N6 (14), H9N2 (2), H12N5 (1)
	Geese	3	1	33.3 (0.8–90.6)	**H5N1 (1)**
	Muscovy duck	95	16	16.8 (9.9–25.9)	H3N2 (1), **H5N1 (10),** H6N6 (2), H9N2 (1), H11N9 (2)
PDS	Chicken	398	38	9.5 (6.8–12.9)	H9N2 (38)
	Duck	195	103	52.8 (45.6–60.0)	H3N2 (6), **H5N1 (2)**, H6N6 (92), H9N2 (3)
	Environment	18	0	0.0	
	Geese	3	0	0.0	
	Muscovy duck	67	0	0.0	
Total		3,597	274	7.6 (6.8–8.5)	

High pathogenicity avian influenza viruses are highlighted in bold.

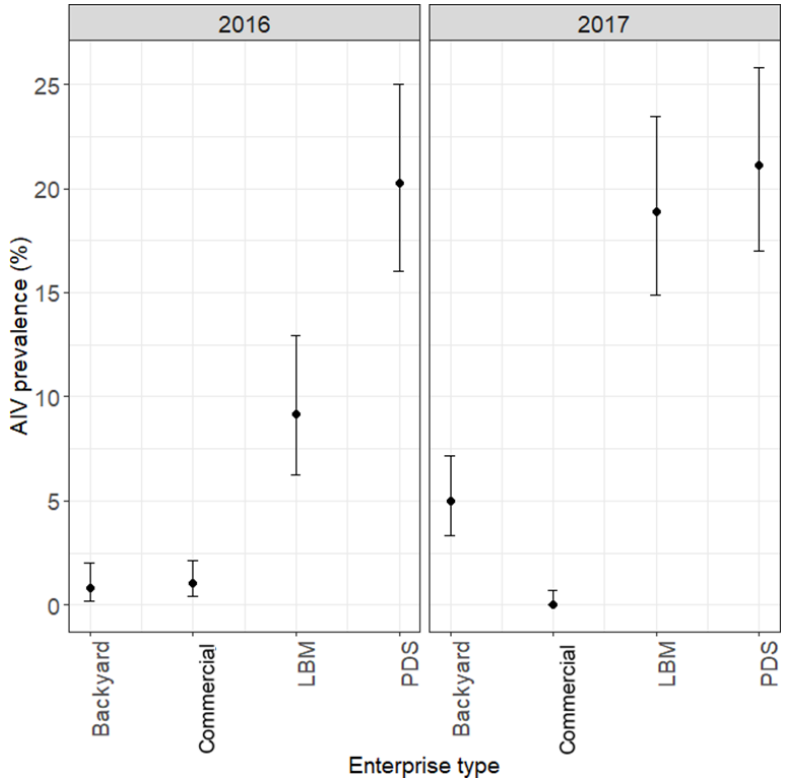


Figure 14. Error bar plot showing AIV prevalence and its 95% CI for backyard farms, commercial farms, LBM, and PDS by sampling round (2016 and 2017).

MCA

MCA scatterplots developed from responses to each of the four sections of the questionnaire (demographic details, AIV knowledge, AIV attitude and AIV practice) are shown in Figures 15a to 18a. Accompanying each scatterplot is an error bar plot showing the prevalence of AIV positivity as a function of the identified cluster group, stratified by enterprise type (Figures 15b to 18b).

In an MCA scatterplot, the relationships among categories of questionnaire responses are reflected by the distance between pairs of marks with questionnaire responses further from the origin more discriminating in the data. Superimposed on each MCA scatterplot (Figures 15a to 18a) are ellipses delineating the clusters identified using the HCPC method.

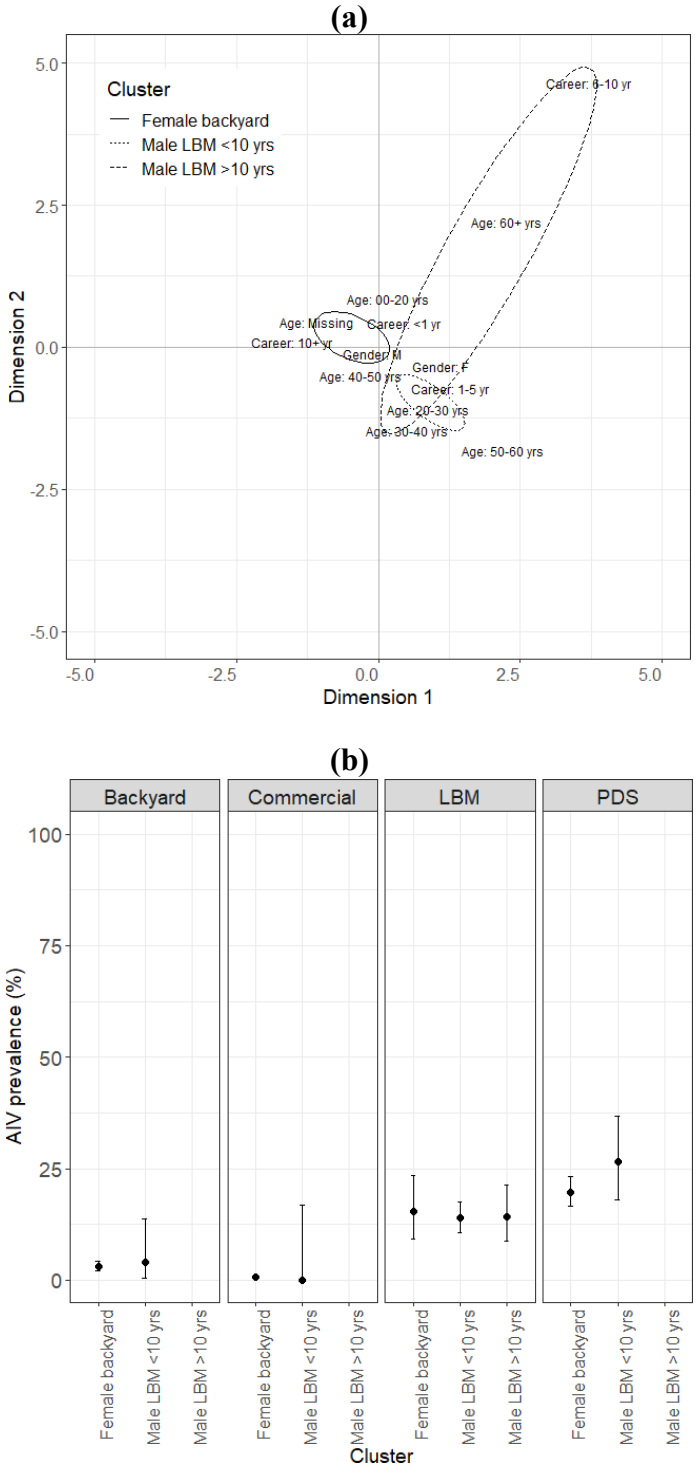


Figure 15. (a) MCA biplot showing questionnaire responses related to respondent demographics; (b) error bar plot showing AIV prevalence (and its 95% CI) for the three clusters shown in (a) by enterprise type.

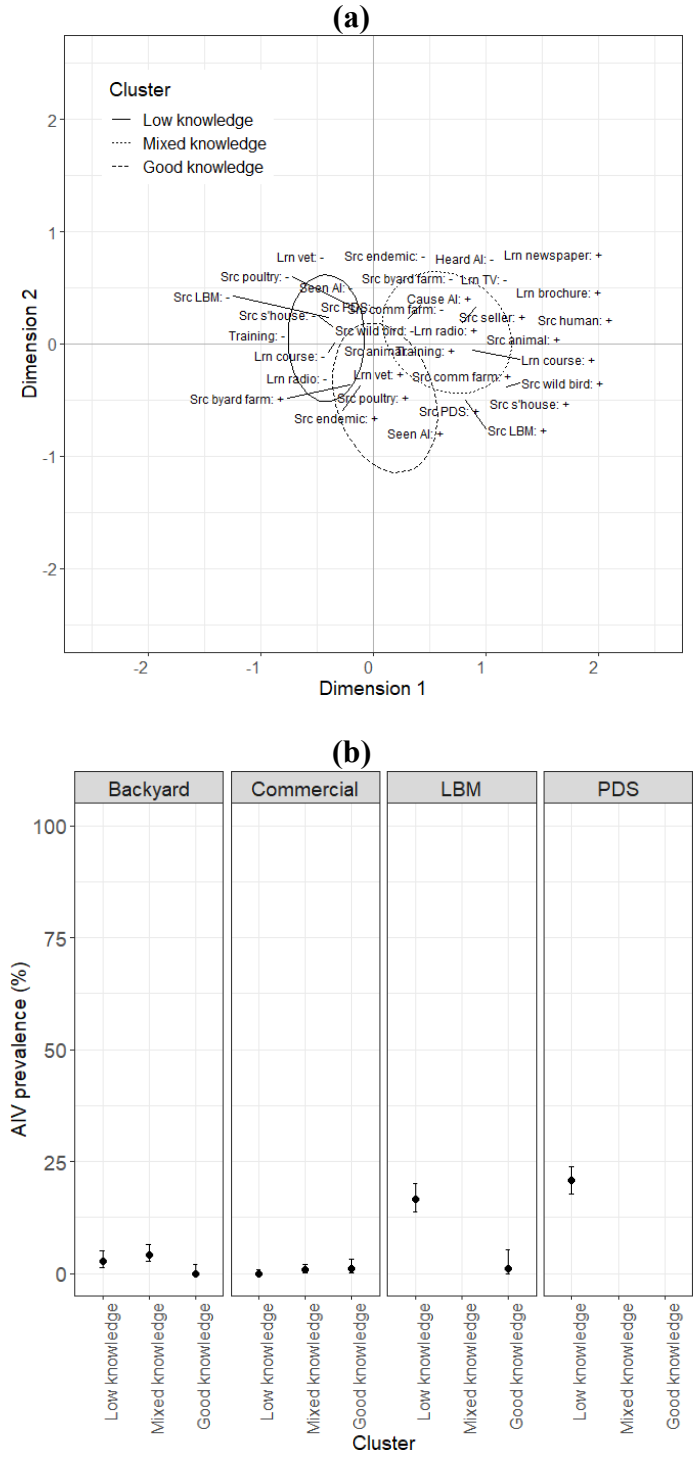


Figure 16. (a) MCA biplot showing questionnaire responses related to respondent AI knowledge; (b) error bar plot showing AIV prevalence (and its 95% CI) for the three clusters shown in (a) by enterprise type.

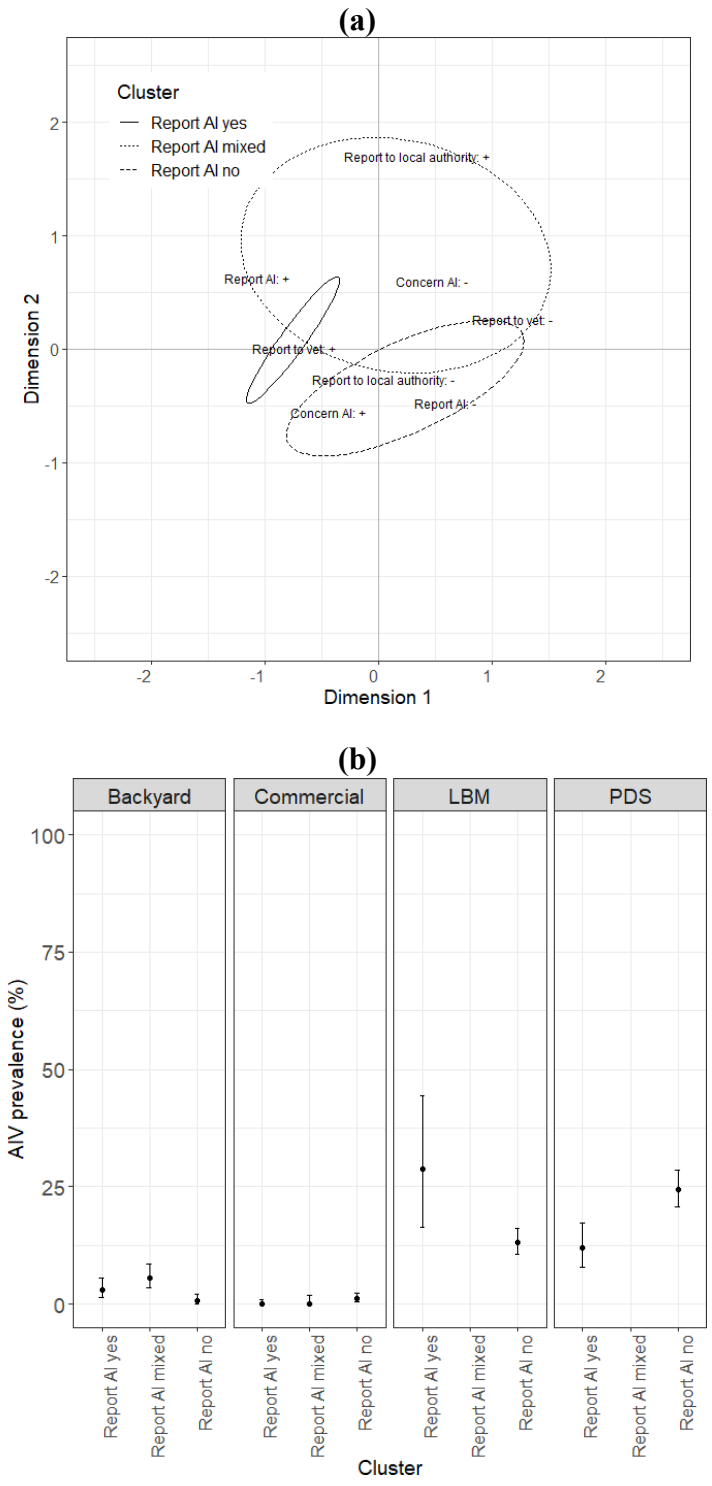


Figure 17. (a) MCA biplot showing questionnaire responses related to respondent AI attitude; (b) error bar plot showing AIV prevalence (and its 95% CI) for the three clusters shown in (a) by enterprise type.

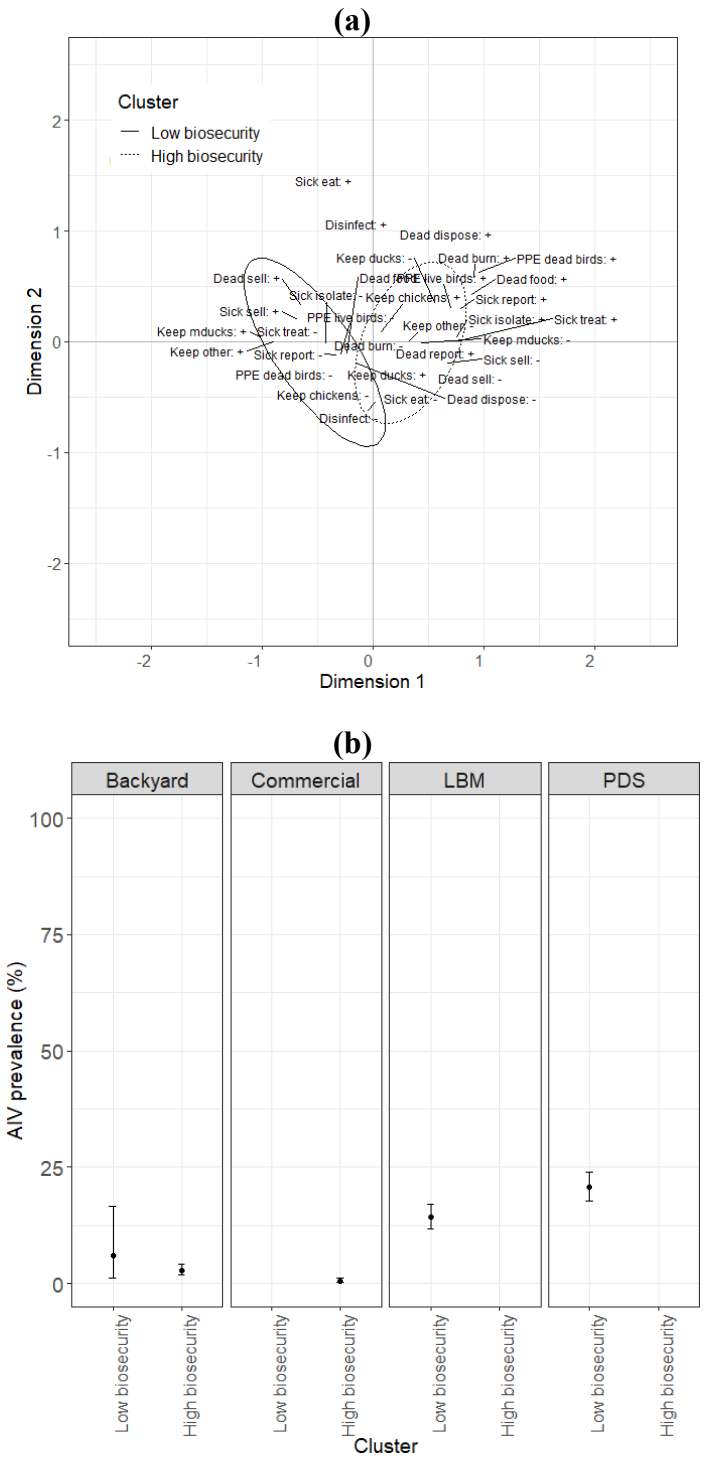


Figure 18. (a) MCA biplot showing questionnaire responses related to respondent AI practice; (b) error bar plot showing AIV prevalence (and its 95% CI) for the two clusters shown in (a) by enterprise type.

Details of the questionnaire responses for each identified cluster are provided in Tables 9 to 12. In effect, the above tables are interpreted as the 'profiles' for questionnaire responses of respondents through demographic details, AIV knowledge, AIV attitude, and AIV practice section.

In the demographic section of the questionnaire, three clusters were identified (Table 9 and Figure 15). The first (n=158) was comprised predominantly of female respondents from backyard farms working with poultry for up to 10 years. The second (n=46 respondents) were mostly males from LBMs working with poultry for a shorter period of time, up to five years. The third, smaller cluster (n=13 respondents) was similar to the second with the exception that a greater proportion working with poultry for more than 10 years. In Table 9 and Figure 15, the first, second and third clusters are labelled 'Female backyard', 'Male LBM≤10 yrs' and 'Male LBM>10 yrs', respectively.

For AIV knowledge three clusters were identified (Table 10 and Figure 16). The first cluster (n=29 respondents) was comprised predominantly of those that had heard about AI and knew that infected birds were a source of infection, primarily domestic poultry and interactions with those from backyard farms, commercial farms, LBMs and PDSs. Most in this cluster obtained their information about AI from the television and local veterinarians; 59% had seen AI before, and most had received training on AI control and prevention. Respondents in the second cluster (n=67) were evenly divided in terms of having heard about AI. Questions regarding the way how AIV can be spread (by domestic poultry, wild birds, domestic animals) were similarly evenly split. Most in this cluster obtained information about AI from the radio and local veterinarians. Interestingly, 94% of those in this cluster had attended training on AI control and prevention. The third cluster (n=121 respondents) was comprised predominantly of those that had heard about AI but were not so sure which was the cause of AI. While those in this cluster were generally not of the belief that AI could be spread by domestic poultry, wild birds, domestic animals (apart from poultry) and interactions with other poultry farmers, poultry traders and LBMs at a reasonably high proportion were of the belief that AIV could be spread by interactions with those from backyard poultry farms. Most in this cluster obtained information about AI from the television and less than 50% receiving information from their local veterinarian. Most in this cluster (70%) had not seen AI and had not received formal training on AI control and prevention (76%). In Table 10 and

Figure 16, the first, second and third clusters are labelled ‘Good knowledge’, ‘Mixed knowledge’ and ‘Low knowledge’, respectively.

For AIV attitudes, three clusters were identified (Table 11 and Figure 17). For the first, all respondents (n=55) were willing to report an AI outbreak if detected, mostly to local veterinarians (87%) but not to SDAH officials. For the second cluster (n=41), there was relatively even split between willingness to report an AI outbreak if detected (44% yes; 56% no). If an outbreak was to be reported, it would be to SDAH officials. For the third cluster (comprised of n=121 respondents), all declared that they would not be willing to report an AI outbreak if detected. If an outbreak was to be reported, 55% of them stated that they would report to local veterinarians and 100% stated that they would not report the outbreak to SDAH officials. In Table 11 and Figure 17, clusters 1, 2 and 3 are labelled ‘Report AI yes’, ‘Report AI mixed’ and ‘Report AI no’, respectively.

Finally, for AIV practice two clusters were identified (Table 12 and Figure 18). Respondents that comprised the first cluster (n=135) mostly kept chickens (90%) and around 40% of them used personal protective equipment (PPE) when handling live or dead birds. This group disposed of dead birds using usual methods for garbage disposal and were less likely to manage sick birds by selling them. Respondents that comprised the second cluster (n=82) kept a mix of poultry species (chickens, ducks and Muscovy ducks), did not generally use PPE when handling live or dead birds, disposed of dead birds using usual methods for garbage disposal and sold sick birds. In Table 12 and Figure 18, clusters 1 and 2 are labelled ‘High biosecurity’ and ‘Low biosecurity’, respectively.

Table 9. Numbers of respondents in each identified of the three respondent demographic cluster groups (n=217) and percentages of responses for each question type

Variable	**'Female backyard'** **(n = 158)**	**'Male LBM ≤10 yrs'** **(n = 46)**	**'Male LBM >10 yrs'** **(n = 13)**
Enterprise type			
Backyard farm	60.8	8.7	0.0
Commercial	24.7	2.2	0.0
LBM	4.4	82.6	100.0
PDS	10.1	6.5	0.0
Gender			
Female	70.9	21.7	30.8
Male	29.1	78.3	69.2
Length of career			
Less than 1 year	40.5	4.3	15.4
1 to 5 years	7.0	95.7	46.2
6 to 10 years	52.5	0.0	0.0
More than 10 years	0.0	0.0	38.5

Table 10. Numbers of respondents in each identified of the three respondent AI knowledge cluster groups (n=217) and percentages of responses for each question type

Variable	'Good knowledge' (n = 29)	'Mixed knowledge' (n = 67)	'Low knowledge' (n = 121)
Heard of AI			
Yes	89.7	52.2	92.6
No	10.3	47.8	7.4
Know the cause of AI			
Yes	55.2	55.2	31.4
No	44.8	44.8	68.6
Know that the source of AIV is an infected bird			
Yes	75.9	52.2	70.2
No	24.1	47.8	29.8
Know that AIV can be spread by domestic poultry			
Yes	75.9	47.8	23.1
No	24.1	52.2	76.9
Know that AIV can be spread by wild birds			
Yes	27.6	41.8	1.7
No	72.4	58.2	98.3
Believe that AIV can be spread by domestic animals (excluding poultry)			
Yes	20.7	53.7	3.3
No	79.3	46.3	96.7
Believe that AIV can be spread by interactions with other poultry farmers			
Yes	3.4	55.2	0.8
No	96.6	44.8	99.2
Believe that AIV can be spread by interactions with poultry traders			
Yes	10.3	64.2	0.8
No	89.7	35.8	99.2
Believe that AIV can be spread by interactions with those from backyard farms			
Yes	69.0	40.3	62.8
No	31.0	59.7	37.2

Table 10 *(cont).* Numbers of respondents in each identified of the three respondent AI knowledge cluster groups (n=217) and percentages of responses for each question type

Variable	'Good knowledge' (*n* = 29)	'Mixed knowledge' (*n* = 67)	'Low knowledge' (*n* = 121)
Believe that AIV can be spread by interactions with those from commercial farms			
Yes	65.5	53.7	18.2
No	34.5	46.3	81.8
Believe that LBMs are a source of AI			
Yes	96.6	55.2	4.1
No	3.4	44.8	95.9
Believe that PDSs are a source of AI			
Yes	96.6	49.3	7.4
No	3.4	50.7	92.6
Believe that slaughterhouses are a source of AI			
Yes	65.5	52.2	1.7
No	34.5	47.8	98.3
Obtain information about AI from the television			
Yes	86.2	43.3	94.2
No	13.8	56.7	5.8
Obtain information about AI from printed material			
Yes	3.4	55.2	0.0
No	96.6	44.8	100.0
Obtain information about AI by attending training courses			
Yes	13.8	61.2	13.2
No	86.2	38.8	86.8
Obtain information about AI from the radio			
Yes	20.7	85.1	14.9
No	79.3	14.9	85.1
Obtain information about AI from the newspaper			
Yes	0.0	53.7	2.5
No	100.0	46.3	97.5
Obtain information about AI from their local veterinarian			
Yes	86.2	83.6	48.8
No	13.8	16.4	51.2

Table 10 *(cont)***.** Numbers of respondents in each identified of the three respondent AI knowledge cluster groups (n=217) and percentages of responses for each question type

Variable	'Good knowledge' (*n* = 29)	'Mixed knowledge' (*n* = 67)	'Low knowledge' (*n* = 121)
Have seen AI and are familiar with the clinical signs of AI			
Yes	58.6	40.3	29.8
No	41.4	59.7	70.2
Have attended training on AI control and prevention			
Yes	62.1	94.0	24.0
No	37.9	6.0	76.0

Table 11. Numbers of respondents in each identified of the three respondent AI attitude cluster groups (n=217) and percentages of responses for each question type

Variable	'Report AI yes' (n = 55)	'Report AI mixed' (n = 41)	'Report AI no' (n = 121)
Concerned about AI			
Yes	58.2	48.8	50.4
No	41.8	51.2	49.6
Willing to report an AI outbreak			
Yes	100.0	43.9	0.0
No	0.0	56.1	100.0
Would report an AI outbreak to local veterinarians			
Yes	87.3	51.2	55.4
No	12.7	48.8	44.6
Would report an AI outbreak to SDAH officials			
Yes	0.0	100.0	0.0
No	100.0	0.0	100.0

Table 12. Numbers of respondents in each identified of the three respondent AI practice cluster groups (n=217) and percentages of responses for each question type

Variable	'High biosecurity' (n = 135)	'Low biosecurity' (n = 82)
Keep chickens		
Yes	90.4	69.5
No	9.6	30.5
Keep ducks		
Yes	54.8	84.1
No	45.2	15.9
Keep Muscovy ducks		
Yes	13.3	56.1
No	86.7	43.9
Keep other domestic species		
Yes	14.1	46.3
No	85.9	53.7
Use PPE when handling live birds		
Yes	40.7	12.2
No	59.3	87.8
Use PPE when handling dead birds		
Yes	40.0	0.0
No	60.0	100.0
Routinely disinfect their vehicle after transporting poultry		
Yes	37.0	34.1
No	63.0	65.9
Dispose of dead birds using usual methods for garbage disposal		
Yes	100.0	76.8
No	0.0	23.2
Dispose of dead birds by cremation		
Yes	31.9	0.0
No	68.1	100.0
Dispose of dead birds by selling		
Yes	22.2	82.9
No	77.8	17.1
Dispose of dead birds by feeding them to livestock		
Yes	34.1	0.0
No	65.9	100.0

Table 12 *(cont).* Numbers of respondents in each identified of the three respondent AI practice cluster groups (n=217) and percentages of responses for each question type

Variable	'High biosecurity' (n = 135)	'Low biosecurity' (n = 82)
Dispose of dead birds by composting		
Yes	28.1	1.2
No	71.9	98.8
Isolate sick birds		
Yes	58.5	0.0
No	41.5	100.0
Sell sick birds		
Yes	22.2	93.9
No	77.8	6.1
Treat sick birds		
Yes	71.1	0.0
No	28.9	100.0
Feed sick birds to livestock		
Yes	17.8	31.7
No	82.2	68.3

Multivariable logistic regression analyses

Estimated regression coefficients for enterprise type, knowledge cluster and attitude cluster and estimates of the variability of the farm and bird-level random effect terms from the mixed-effects logistic regression model are shown in Table 13. The marked difference in AIV prevalence by enterprise type, the odds of a bird being AIV positive if it was from an LBM or PDS was 45.0 (95% CI: 3.4–590.0) and 25.0 (95% CI: 1.4–460.0), respectively, times higher to the odds of a bird from a commercial poultry farm being AIV positive. Although cluster 1 ('Good knowledge') in the AI knowledge section and cluster 1 ('Report AI yes') in the AI attitude section showed the difference in the odds of birds being AIV positive, the significant difference was not recorded.

After adjusting for the fixed effects included in the model, the proportions of unexplained variance at the enterprise and bird level was $10.37 \div \left(10.37 + \frac{\pi^2}{3}\right) = 0.76$ and $\frac{\pi^2}{3} \div \left(10.37 + \frac{\pi^2}{3}\right) = 0.24$, respectively. The AUC for the fixed-effects model was 0.81, indicating a satisfactory to good ability to discriminate between AIV-positive and AIV-negative birds. The AUC for the mixed-effects model was 0.98.

Table 13. Regression coefficients and their standard errors from a mixed-effects logistic regression model quantifying the association between enterprise type, cluster membership and AIV positivity

Explanatory variable	Samples	AIV positive	Coefficient (SE)	*z*	*P*-value	OR (95% CI)
Intercept	3,597	274	-7.8884 (1.6737)			
Enterprise type						
Commercial	1,200	7	Ref	Ref	Ref	Ref
Backyard farm	1,056	32	0.9482 (1.2031)	0.788	0.43	2.6 (0.2–27.0) [a]
LBM	660	94	3.8104 (1.3164)	2.895	<0.01	45.0 (3.4–590.0)
PDS	681	141	3.2215 (1.4823)	2.173	0.03	25.0 (1.4–460.0)
Knowledge						
Good knowledge	547	4	Ref	Ref	Ref	Ref
Mixed knowledge	1014	25	1.6018 (1.6809)	0.953	0.34	5.0 (0.2–130.0)
Low knowledge	2036	245	1.2422 (1.4750)	0.842	0.40	3.5 (0.2–62.0)
Attitude						
Report AI yes	1,000	48	Ref	Ref	Ref	Ref
Report AI mixed	527	19	0.4036 (1.4656)	0.275	0.78	1.5 (0.1–26.0)
Report AI no	2,070	207	0.0831 (0.9282)	0.090	0.93	1.1 (0.2–6.7)
Random effects	Variance	SE				
Enterprise	10.37	3.221				

[a] Interpretation: After adjusting for the effect of respondent knowledge category, attitude category and unmeasured enterprise-level effects the odds of a bird being AIV positive if it was from a backyard farm was 2.6 (95% CI: 0.2–27.0) times higher than the odds of a bird from a commercial poultry farm being AIV positive. Ref reference.

Discussion

This cross-sectional study quantified the prevalence of AIV positivity among poultry from backyard farms, commercial poultry farms, LBMs, and PDSs in Vinh Long province over two sampling rounds in 2016 and 2017. In Vietnam, control measures for AI have been applied to backyard poultry farms, commercial poultry farms and LBMs since the first outbreaks of AI were reported in 2003. The current finding indicated that one in five poultry sampled from PDSs were AIV positive (21%; 95% CI: 18%–24%, Table 7), demonstrates a relatively high prevalence of AIV in poultry in this sector and indicates that PDSs should receive emphasis for interventions in AI control programs. Unlike LBMs, where control measures for AI are supervised by local veterinarians and supported by local government authorities, AI control measures in PDSs are primarily implemented by PDS traders themselves mainly because PDSs are not recognized as official areas. The inevitable variability in the application and effectiveness of sanitary measures that occurs as a result makes the relatively high prevalence of AIV positivity a not unexpected finding. The current results support the proposal that PDSs receive AI control measure oversight similar to that applied to LBMs [108]. These findings are consistent with the cross-sectional study of Soares Magalhães, *et al.* [109] which identified wholesale markets as hot spots for AIV circulation in Ha Noi in 2006 and 2007 and the study of Meyer *et al.* [110] which found that PDSs and PDS-like enterprises (such as wholesale markets and duck yards) often lacked regular disinfection procedures, routinely kept poultry from different sources in the same cage and received a low level of oversight from local veterinary authorities.

A previous study carried out in the south of Vietnam under similar conditions identified a slightly lower prevalence of AIV (5.3%) among farms and LBMs [20] compared to the 7.6% identified in this study. Furthermore, the prevalence of AIV positivity among LBMs in this study (14%) was higher than the AIV positivity prevalence of 6.9% among LBMs in the center of Vietnam identified by Chu *et al.* [23] and 5.8% in the north of Vietnam identified by Thuy *et al.* [111]. Assuming these differences in prevalence are real and not due to, for example, seasonal and yearly fluctuations in the incidence of AI, current results imply that LBMs in southern Vietnam play a more dominant role in maintaining AIV circulation in the poultry population compared to other

areas of the country. Similar to PDSs, the higher prevalence of AIV positivity among poultry sampled from LBMs is likely to be due to the routine gathering of large numbers of birds from different sources [112] and generally lower levels of biosecurity compared with both backyard and commercial poultry farms.

The questionnaire designed for this study was comprehensive and sought to solicit respondent demographic information and details of their knowledge, attitude and practice with respect to AI. The questionnaire comprised a total of 46 questions which presented difficulties when developing a parsimonious regression model to identify risk factors for AIV positivity. To address this issue, MCA analyses were carried out using responses from each of the four sections of the questionnaire (demography, AI knowledge, AI attitude and AI practice). Clusters of responses for each section were identified and used as explanatory variables for multivariable logistic regression model. In effect, these clusters can be interpreted as respondent 'profiles' for demographics, AIV knowledge, AIV attitude and AIV practice. This allowed to develop a model indicative of broad trends in the questionnaire data as opposed to developing a model starting with 46 candidate explanatory variables and attempting to identify responses to single, highly specific questions that were predictive of AIV positivity. This 'profile-based' approach provided results allowing to identify of broad trends in the data sufficient to guide policy development.

For the fixed-effects logistic regression model, the explanatory variable representing the three cluster categories of AI knowledge (good knowledge, mixed knowledge and low knowledge) and the explanatory variable representing the three cluster categories of AI attitude (report AI yes, report AI mixed and report AI no) were significantly associated with bird level AIV positivity status. After accounting for unmeasured, individual enterprise level effects through inclusion of enterprise identifier as a random effect term, the sign and magnitude of the point estimates of the regression coefficients were similar to that of the fixed effects regression model but both explanatory variables were no longer significantly associated with AIV positivity status. Respondents with a level of knowledge about AI classified as 'mixed' (i.e., where some facts regarding AI transmission and spread were correctly recalled and others were not) and respondents where their level of knowledge about AI was classified as 'low' had a 5.0 (95% CI: 0.2–130.0) and 3.5 (95% CI: 0.2–62.0) fold increase in the odds of their birds being AIV

positive compared with respondents classified as having a good knowledge of AIV transmission and spread. Similar trends were noted for AI attitude. Respondents that provided inconsistent responses in terms of their likelihood to report an outbreak of AI to authorities ('Report AI mixed') and those that were unlikely to report an outbreak of AI to authorities ('Report AI no') had a 1.5 (95% CI: 0.1–26.0) and 1.1 (95% CI: 0.2–6.7) fold increase in the odds of their birds being AIV positive compared with respondents classified as being likely to report an outbreak of AI to authorities ('Report AI yes'). The substantial increase in the uncertainty around each of these measures of association after inclusion of the enterprise level random effect term reflect what is believed to be substantial individual enterprise-level influence on these associations.

Traders in PDSs and sellers at LBMs usually run their business dependent on market demand [110], which means that they tend to leave the industry if a sufficient financial return is not achieved. For this reason, there is a relatively high population turnover of PDS traders and LBM sellers with those that are new to the industry often lacking knowledge about AI and its control. The knowledge and practice of participants from LBMs and PDSs are likely to be important in a given area because these industry players directly influence AIV circulation risk in a given market catchment area. In contrast, backyard and commercial poultry farmers run their businesses based on their ability and resources, meaning that they strive to obtain more knowledge and adopt better practices to generate more income [113]. This explanation is indirectly supported by the findings from this study: AIV positivity among birds from backyard farms and commercial farms was relatively low. An analysis to assess the interaction between enterprise type and AI knowledge, attitude and practice cluster assignment on AIV positivity risk was planned to investigate this hypothesis further. Zero counts of AIV positive birds in some strata combinations made this analysis not possible.

In conclusion, consistent with previous studies, current survey identified a higher prevalence of AIV positivity among poultry sampled from LBMs and PDSs compared with poultry sampled from backyard and commercial poultry farms, which means that LBMs and PDSs should receive specific emphasis in AI control programs. These findings provide evidence to support the hypothesis that incomplete respondent knowledge of AI and how it is spread was associated with an increased risk of AIV positivity. Delivery of education programs specifically designed for each industry sector (backyard farms,

commercial farms, LBMs and PDSs) are likely to assist in this regard. The timing and frequency of delivery of education programs is likely to be important if the turnover of those working in LBMs and PDSs is high. Furthermore, the previous studies in Mekong Delta suggested that the farming practice of the farmers and trading system in this region was similar among the provinces. Implying that the result in this study might be applied for AI control in the other provinces of the Mekong Delta.

Brief summary

In south Vietnam, LBMs are key in the value chain of poultry products and spread of AIV, although they may not be the sole determinant of AIV prevalence. For this reason, a risk analysis of AIV prevalence was conducted accounting for all value chain factors. A cross-sectional study of enterprise managers and poultry on backyard farms, commercial (high biosecurity) farms, LBMs, and PDSs in four districts of Vinh Long province was conducted between December 2016 and August 2017. A total of 3,597 swab samples were collected from birds from 101 backyard farms, 50 commercial farms, 58 sellers in LBMs, and 19 traders in PDSs. Swab samples were submitted for AIV isolation. At the same time a questionnaire was administered to flock managers asking them to provide details of their knowledge, attitude and practices related to AI. MCA and a mixed-effects multivariable logistic regression model were developed to identify enterprise and flock manager characteristics that increased the risk of AIV positivity. A total of 274 birds were positive for AIV isolation, returning an estimated true prevalence of 7.6% (95% CI: 6.8%–8.5%). The odds of a bird being AIV positive if it was from an LBM or PDS were 45.0 (95% CI: 3.4–590.0) and 25.0 (95% CI: 1.4–460.0), respectively, times higher than the odds of a bird from a commercial poultry farm being AIV positive. The odds of birds being AIV positive for respondents with a mixed (uncertain or inconsistent) level and a low level of knowledge about AI were 5.0 (95% CI: 0.2–130.0) and 3.5 (95% CI: 0.2–62.0), respectively, times higher than the odd of birds being positive for respondents with a good knowledge of AI. LBMs and PDSs should receive specific emphasis in AI control programs in Vietnam. The findings of this study provide evidence to support the hypothesis that incomplete respondent knowledge of AI and AIV spread mechanism were associated with an increased risk of AIV positivity. Delivery of education programs specifically designed for those in each enterprise will assist in this regard. The timing and frequency of delivery of education programs are likely to be important if the turnover of those working in LBMs and PDSs is high.

Chapter III

Risk profile of low pathogenicity avian influenza virus infections in farms in southern Vietnam

Introduction

AI caused by an infection of AIV, is one of the contagious diseases worldwide in poultry. Based on the antigenic characteristics of glycoproteins on the surface of the virus particles, AIVs are categorized into 16 HA and 9 NA subtypes [114]. Based on their pathogenicity in chickens, AIVs are classified into HPAIVs and LPAIVs. Unlike HPAIV, which causes extremely high mortality of up to 100%, LPAIV usually causes mild or low pathogenicity with less severe or no clinical signs in chicken [115]. The natural reservoir of AIVs is wild waterfowl, which could harbor all AIV subtypes but rarely show any clinical signs [87]. Originally, natural reservoirs, such as ducks, are not infected with HPAIV. However, HPAIVs recently circulating worldwide cause mild or high pathogenicity, resulting in disease spread via migration of wild waterfowl [4,116]. Outbreaks of HPAIV infection in poultry, following wild birds, due to the spillover of contagious pathogens are frequently reported [15]. Compared to high mortality in chickens, HPAIVs' infection in ducks with minor or atypical clinical signs is hardly detected in the flock, leading to huge damage in the poultry industry [29]. However, the substantial poultry production losses caused by LPAIV infection could be recognized through increased mortality and decreased egg production [31].

The Vietnamese government has officially developed systemic documents to support the strategy in an effort of AI control, particularly targeting HPAI. Active and passive surveillance are important activities of the AI control strategy in poultry because the numbers of H5N1 HPAI outbreaks in Vietnam have markedly decreased through maintaining these activities since 2004 [15]. Many studies were established based on the output of active surveillance to improve knowledge on AIV ecology in birds [20-23,29]. Despite remarkable improvements in AI control, especially in reducing HPAIV outbreaks, substantial economic losses in the poultry industry probably due to AIV infection are continuously suspected. The current diagnosis system in Vietnam has been focused on HPAI only, therefore, there are still remaining concerns about how the circulation of LPAIV in the flock would influence the ecology of AI in poultry and economic loss due to it, although LPAIV infection does not cause any apparent clinical signs in host birds. However, there are very few reports regarding the potential damage of LPAIV infection and circulation in Vietnam's poultry population.

Long-term surveillance has been conducted in poultry to characterize AIV ecology through different geographic regions in Vietnam. From 2009 to 2019, a total of 26,347 samples were collected from poultry and environments: 6,262 samples from two provinces in northern, 5,085 samples from one province in central, 15,000 samples from five provinces in southern Vietnam (Figure 19 and Table 14) [20-23,29]. In Vietnam, AIVs were maintained in the poultry population and spread by farming or trading activities. The introduction of new AIV in northern Vietnam was supposedly related to the cross-border transmission whereas the southern tends to maintain the predominant AIVs in Vietnam [33]. Based on the surveillance outcomes, a cross-sectional study of AI focusing on LPAIV was conducted in Vinh Long province to identify the characteristics of farmers associated with LPAIV infection in poultry. The direct multivariable analysis might identify the individual risk factors contributing to LPAIV infection but the field situation might not change dramatically due to the existence of single factors. Therefore, the group of factors that tend to co-occurrence might provide more detail about the relationship between the farmer's response tendency and LPAIV infection.

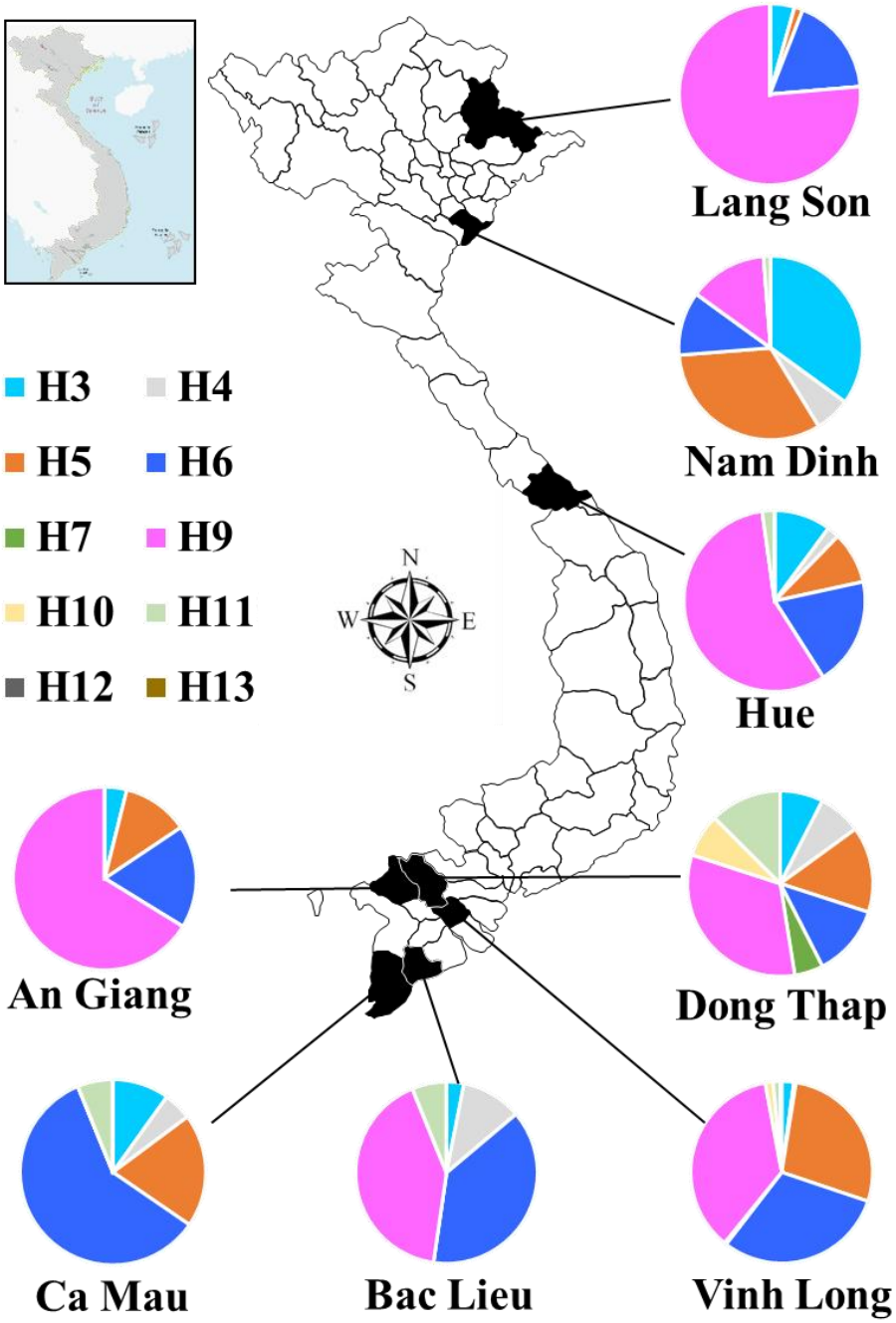


Figure 19. Diversity of AIV isolates in each sampling area, Vietnam. The geographical location of Vietnam in Southeast Asia is indicated by the small rectangle. The map of Vietnam shows the location of eight provinces where sampling was indicated (black), and the proportions of HA subtypes of AIV in each location are indicated in the pie charts.

Table 14. Summary of avian influenza virus surveillance in Vietnam from 2009 to 2019

Year	Region	Province	No. of samples	AIV positive	Prevalence (%) (95% CI)	Subtype (no. of isolates)	Reference
2009	North	Nam Dinh	700	0	0.0		
	South	Bac Lieu	758	39	5.1 (3.7–7.0)	H3N2 (1), H3N8 (1), H4N6 (7), H9N2 (26), H11N3 (3), (H11N9 (1)	Nomura *et al.* 2012
2010	North	Nam Dinh	761	0	0.0		
	South	Bac Lieu	1,327	26	2.0 (1.3–2.9)	H6N2 (24), H6N6 (1), H9N6 (1)	Nomura *et al.* 2012
2011	North	Nam Dinh	600	6	1.0 (0.4–2.2)	H3N8 (1), H4N2 (1), **H5N1 (1)**, H6N6 (3)	Okamatsu *et al.* 2013
	South	Ca Mau	1,511	81	5.4 (4.3–6.6)	H3N6 (1), H3N8 (7), H4N6 (4), **H5N1 (16)**, H6N2 (46), H6N9 (2), H11N5 (3), H11N9 (2)	Okamatsu *et al.* 2013
2012	North	Nam Dinh	1,201	74	6.2 (4.9–7.7)	H3N2 (11), H3N6 (9), H3N8 (10), H4N6 (6), **H5N1 (26)**, H5N2 (1), H6N2 (4), H6N6 (6), H9N2 (10), H9N8 (1), H11N9 (4)	Okamatsu *et al.* 2013
	South	Dong Thap	1,224	40	3.3 (2.3–4.4)	H4N6 (1), **H5N1 (4)**, H6N2 (1), H7N1 (2), H9N2 (13), H10N7 (3), H11N3 (2)	Okamatsu *et al.* 2013
2014	Central	Hue	3,045	178	5.8 (5.0–6.7)	H3N2 (18), H3N6 (1), H4N6 (2), **H5N6 (8)**, H6N2 (14), H6N6 (16), H9N2 (109), H9N6 (5), H11N6 (1), H11N7 (4)	Chu *et al.* 2016 Chu *et al.* 2017

High pathogenicity avian influenza viruses are highlighted in bold.

Table 14 *(cont).* Summary of avian influenza virus surveillance in Vietnam from 2009 to 2019

Year	Region	Province	No. of samples	AIV positive	Prevalence (%) (95% CI)	Subtype (no. of isolates)	Reference
2015	Central	Hue	2,040	49	2.4 (1.8–3.2)	H3N1 (1), H3N8 (3), H4N2 (3), **H5N1 (4)**, **H5N6 (9)**, H6N1 (14), H9N2 (15)	Nguyen *et al.* 2019
	South	Vinh Long	1,400	243	17.4 (15.4–19.4)	H3N2 (1), H4N6 (1), **H5N1 (130)**, H6N6 (24), H9N2 (86), H11N9 (1)	Nguyen *et al.* 2019
2016	South	Vinh Long	3,300	131	4.0 (3.3–4.7)	H3N2 (11), H3N8 (2), **H5N1 (5)**, H6N6 (69), H9N2 (31), H10N6 (7), H11N9 (5), H12N5 (1)	Le *et al.* 2021
2017	North	Lang Son	1,000	148	14.8 (12.7–17.2)	**H5N6 (6)**, H6N6 (3), H9N2 (139)	
	South	Vinh Long	1,800	167	9.3 (8.0–10.7)	H3N2 (2), **H5N1 (21)**, H6N6 (63), H9N2 (79), H10N3 (2)	Le *et al.* 2021
2018	North	Lang Son	1,000	306	30.6 (27.8–33.6)	H3N2 (29), **H5N6 (2)**, H6N6 (89), H9N2 (186)	
	South	Vinh Long	1,846	139	7.5 (6.4–8.8)	H3N2 (1), H4N6 (2), **H5N1 (17)**, **H5N6 (11)**, H6N6 (52), H7N7 (3), H9N2 (47), H9N6 (1), H11N1 (1), H11N9 (3), H13N9 (1)	Le *et al.* 2020
2019	North	Lang Son	1,000	206	20.6 (18.1–23.2)	**H5N6 (2)**, H6N6 (25), H9N2 (179)	
	South	Vinh Long	1,634	109	6.7 (5.5–8.0)	**H5N1 (12)**, **H5N6 (22)**, H6N6 (31), H9N2 (42), H10N3 (2)	
	South	An Giang	200	77	38.5 (31.7–45.6)	H3N2 (3), **H5N6 (9)**, H6N6 (14), H9N2 (51)	
Total			26,347	2,019	7.7 (7.3–7.9)		

Materials and Methods

Study design and area

A questionnaire survey was conducted to collect epidemiological data from the participants in August 2019. Based on the evaluation of the DAH, the provinces at risk for AI were selected over the years. The eligible participants were farmers of the backyard and high biosecurity farms which were randomly selected from a sampling frame provided by the SDAH. All participants were asked if they consented to take part in the study before collecting samples from birds and implementing direct interviews with the support of staffs from the SDAH of Vinh Long province. The backyard farms were likely to lack prevention measures following local authority guidelines, such as disinfection, vaccination, keeping poultry in a separate place, and raising poultry for self-consumption. Farms applying at least two prevention measures and raising poultry mainly for trading were defined as high biosecurity farms in the present study.

Swab sample collection and laboratory procedures

A total of 1,634 swab samples were collected for AIV isolation from birds in backyard farms, high biosecurity farms, LBMs, and PDSs. Swab samples (oropharyngeal, cloacal, and fecal) collected from chickens, ducks, Muscovy ducks, and the environment swab from the sampling sites were stocked in the transport medium used in the previous study [100]. The samples were transported to either the National Centre for Veterinary Diagnostics (Ha Noi, Vietnam) or the RAHO7. Aliquots of up to 10 samples collected from the same flock were pooled to investigate the presence of influenza type A virus using real-time RT-PCR targeting the M gene [101]. After finishing the screening by real-time RT-PCR in Vietnam, all samples were transferred to Japan for virus isolation at the Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University.

Virus isolation

The virus isolation by using ten-day-old chicken embryonated eggs then subtyping by using HI and NI tests were conducted by applying the same method described in Chapter I.

Questionnaire study and data management

The adapted questionnaire was developed by referring to previous studies conducted by the DAH, with specific questions related to LPAIV [23]. In detail, the questionnaire, which comprised 150 questions (Appendix 4) regarding knowledge, attitudes, practices, and economic losses associated with LPAIV infection or prevention in farms, was developed in partnership with the provincial DAH staff to collect details on (1) demographic characteristics, (2) general knowledge regarding LPAIV, (3) attitudes on the control measures of LPAI and AI, (4) routine biosecurity measures applied, and (5) suspected economic losses. A total of 62 face-to-face interviews were carried out by the SDAH staff of Vinh Long Province after training. The sampling schedule was also announced to participants and local veterinarians in advance. Questionnaire responses from the participants and the AIV isolation results were recorded in two tables. A unique identification code was assigned for each respondent enrolled in this study to link between tables for making a relational database.

Univariable analysis

The LPAIV prevalence at the bird level was defined as the proportion of the number of individual birds with LPAIV-positive samples per the total number of birds sampled. Unconditional associations between the responses on the questionnaire (explanatory variables) and the laboratory results (numbers of the presence or absence of LPAIV per bird) were expressed as the OR. Analysis of variance (ANOVA) was performed to determine the effects of independent variables on the dependent variable in a regression study to determine the potential factors related to LPAIV infection. Any explanatory variables with $P \leq 0.2$ (two-sided) of unconditional association were applied in the multilevel model.

MCA

In total, 61 farms were retained in the final dataset because one H5 HPAI-positive farm did not satisfy the enrollment criteria for risk factor analysis of LPAIV infection. The relationships between responses in each of the four sections of the questionnaire (demographic details, knowledge, attitude, and practice) were graphically represented using MCA. MCA is a generalization of principal component analysis suitable for

categorical variables and was performed according to the protocol in the previous study and Chapter II [117].

Mixed-effects logistic regression

The probability of LPAIV infection in a bird was parameterized as a function of m explanatory variables in a fixed-effects multiple logistic regression model according to the previous study [117]. The model was then extended by including the effect of herd-level (farm-level) and explanatory variables were retained in the mixed-effects model regardless of their statistical significance according to the previous study [117]. The assumptions of normality and homogeneity of variance were investigated by constructing the histograms of the residuals in the multilevel model and plots between the residuals and the predicted values, respectively. In the multilevel model, extrabinomial variation was not included in the individual bird variance. Estimates of variance at each of the two levels (farms and birds) were regarded as the lowest level of variance on the logit scale of $\frac{\pi^2}{3}$, where π=3.1416 [103]. The detail of the analysis method was described in Chapter II.

The LPAIV positivity status at the bird level predicted by the model was evaluated to construct the ROC curve. The ability to discriminate between LPAIV-positive and LPAIV-negative birds in the model was assessed by the AUC. The unconditional measures of association analyses in the data were carried out using the epiR package [106]. The mixed-effects logistic regression model was developed under the contribution of the lme4 package [107]. All analyses were conducted in R (version 4.0.5).

Results

Descriptive statistics

Based on the outcomes of the active surveillance since 2009, Vinh Long Province was selected to conduct a cross-sectional study in 2019 to assess the risk factors associated with LPAIV infection. Because Vinh Long province had the highest number of samples among the eight provinces, the poultry value chains among PDS, LBM, backyard farms, and high biosecurity farms in the province were assessed [117]. A total of 1,634 samples were collected from 45 backyard farms (10 samples per farm; range 9–20; median=10; total 459 samples), 17 high biosecurity farms (29 samples per farm; range 20–30; median=30; total 500 samples), 31 sellers in 10 LBMs (14 samples per seller; range 1–36; median=10; total 420 samples), and 7 PDSs (36 samples per PDS; range 13–61; median=40; total 255 samples) (Table 15). Because the impact of LPAIV should be assessed only in farms as the cumulative losses in a long time, backyard and high biosecurity farms were selected for further analysis. AIVs, including LPAIVs, were isolated only in backyard farms with a prevalence of 3.9% (95% CI: 2.3%–6.1%) in which the HPAIVs were isolated in a single farm, returning in LPAIV prevalence to 2.4% (95% CI: 1.2%–4.2%). The LPAIVs were isolated in 4 out of 62 farms, returning in LPAIV prevalence at farm level to 6.5% (95% CI: 1.8%–15.7%).

Table 15. Avian influenza viruses isolated in Vinh Long province in Vietnam in 2019

Model	Species	No. of samples	AIV positive	Prevalence (%) (95% CI)	Subtype (no. of isolates)
Backyard	Chicken	220	17		**H5N1 (7)**, H9N2 (10)
	Duck	189	1	3.9 (2.3–6.1)	H10N3 (1)
	Muscovy duck	50	0		
Biosecurity	Chicken	330	0		
	Duck	140	0	0.0	
	Muscovy duck	30	0		
LBM	Chicken	212	16		H9N2 (16)
	Duck	140	28	10.7 (7.9–14.1)	**H5N1 (2)**, **H5N6 (20)**, H6N6 (4), H9N2 (2)
	Muscovy duck	68	1		H10N3 (1)
PDS	Chicken	106	14		H9N2 (14)
	Duck	117	32	18.0 (23.5–23.3)	**H5N1 (3)**, **H5N6 (2)**, H6N6 (27)
	Muscovy duck	32	0		
Total		1,634	109	6.7 (5.5–8.0)	

High pathogenicity avian influenza viruses are highlighted in bold.

Univariable analysis

There were 21 explanatory variables that show the association with virus isolation positivity at $P \leq 0.2$ in the ANOVA analysis (Table 16). Most of the birds were raised by male (708 of 928; 76%) and the odds of birds raised by male being AIV positive was 0.03 (95% CI: 0.00–0.16) times higher than that of birds raised by female. Besides that, the odds of birds being AIV positive in farms with Muscovy ducks during the poultry farming practice was 19 (95% CI: 5.08–131.44) times higher than farms without Muscovy duck. The odds of birds being AIV positive in farms bought the hatchlings in the same commune was 0.18 (95% CI: 0.03–0.69) times higher than that in farms imported the hatchlings from other communes. Moreover, buying the hatchlings in cheap price was 11 (95% CI: 1.49–92.19) times at higher risk in birds with AIV positive than that due to the accessibility. The farmers who are unwilling to report an AI event was 27 (95% CI: 7.79–127.45) times at higher risk of possessing AIV positive birds than those who are reporting an AI event. Interestingly, report to the local officers, veterinarians, or local government could reduce the risk of birds with AIV positive [OR=0.04 (95% CI: 0.01–0.13)] compared with farmers who did not report the event to anywhere.

Table 16. Unconditional associations between the outcome variable (virus isolation positive) and the 21 explanatory variables

Variable	LPAIV positive	Birds	OR (95%CI)	*P*-value
Sampling species				
Chicken	10	530	1.0	Ref
Duck	1	329	0.2 (0.0–0.8)	0.08
Muscovy duck	0	80	NA	0.99
Age				
Under 20 year-old	1	60	1.0	Ref
20 - 30 year-old	10	290	2.1 (0.4–39.0)	0.48
31 - 40 year-old	0	280	NA	0.99
41 - 50 year-old	0	279	NA	0.99
Upper 50 year-old	0	30	NA	1.00
Gender				
Female	10	230	1.0	Ref
Male	1	709	0.0 (0.0–0.2)	<0.01
Education				
Primary	2	320	1.00	Ref
High school	9	419	3.5 (0.8–16.3)	0.99
College	0	60	NA	1.00
University	0	10	NA	1.00
No	0	130	NA	0.99
Experience				
Under 1 year	1	50	1.0	Ref
1 - 5 years	9	440	1.0 (0.2–19.1)	0.98
6 - 10 years	1	289	0.2 (0.0–4.4)	0.21
More 10 years	0	160	NA	0.99
Keep duck				
No	10	560	1.00	Ref
Yes	1	379	0.2 (0.0–0.8)	0.07
Keep Muscovy duck				
No	2	759	1.0	Ref
Yes	9	180	19.9 (5.1–131.4)	<0.01
Buy the hatchlings from the same commune				
No	9	420	1.0	Ref
Yes	2	519	0.2 (0.0–0.7)	0.03
Reason to by the hatchlings				
Cheap	10	299	1.0	Ref
Convenience	1	340	0.1 (0.0–0.5)	0.02
Conversant	0	300	NA	0.99

NA: not assessable, Ref: reference.

Table 16 *(cont)***.** Unconditional associations between the outcome variable (virus isolation positive) and the 21 explanatory variables

Variable	LPAIV positive	Birds	OR (95%CI)	*P*-value
AI can spread by contact with the contaminated equipment				
No	10	649	1.0	Ref
Yes	1	290	0.2 (0.0–1.2)	0.15
AI clinical sign can be observe in duck				
No	10	530	1.0	Ref
Yes	1	409	0.1 (0.0–0.7)	0.05
Willing to report if recognize an AI event around				
Yes	3	849	1.0	Ref
Not sure	8	90	27.5 (7.8–127.5)	<0.01
Report AI event to the local officer				
No	8	90	1.0	Ref
Yes	3	849	0.0 (0.0–0.1)	<0.01
Report AI event to the local vet				
No	8	90	1.0	Ref
Yes	3	849	0.0 (0.0–0.1)	<0.01
Report AI event to the local government				
No	8	90	1.0	Ref
Yes	3	849	0.0 (0.0–0.1)	<0.01
Do you think AI situation in your area become more severe?				
No	10	179	1.0	Ref
Yes	1	760	44.9 (8.5–827.0)	<0.01
Brochure is an effective way to collect the AI information				
No	10	580	1.0	Ref
Yes	1	359	0.2 (0.0–0.8)	0.08
Newpaper is an effective way to collect the AI information				
No	10	609	1.0	Ref
Yes	1	330	0.2 (0.0–1.0)	0.11
Share vehicle				
Always	9	140	1.0	Ref
Some time	2	299	0.1 (0.0–0.4)	<0.01
Never	0	500	NA	0.99

Table 16 *(cont)***.** Unconditional associations between the outcome variable (virus isolation positive) and the 21 explanatory variables

Variable	LPAIV positive	Birds	OR (95%CI)	*P*-value
Report the dead bird to the local authority if found it around				
No	8	90	1.0	Ref
Yes	3	849	0.0 (0.0–0.1)	<0.01
Report the sick bird to the local authority if found inside farm				
No	9	50	1.0	Ref
Yes	2	889	0.0 (0.0–0.0)	<0.01

Multivariable logistic regression analysis for individual variables

The mixed-effects logistic regression model with estimated regression coefficients of the five variables is retained in the final model (Table 17). Keeping the Muscovy ducks could significantly exacerbate the risk of LPAIV infection at 208 times more compared to not-raising the Muscovy duck. In the current model, 66 (44%) variables were excluded due to the zero count of LPAIV in the exposure or non-exposure group, meaning that the effect of these variables would not be fully included regardless of their potential contribution to the LPAIV positivity. Although a significant difference was observed, the standard error of coefficient in this model was high (1,643), suggesting that the other variables might contribute to the risk of LPAIV infection.

Table 17. A mixed-effects logistic regression model quantifying the association between factors and LPAIV positivity

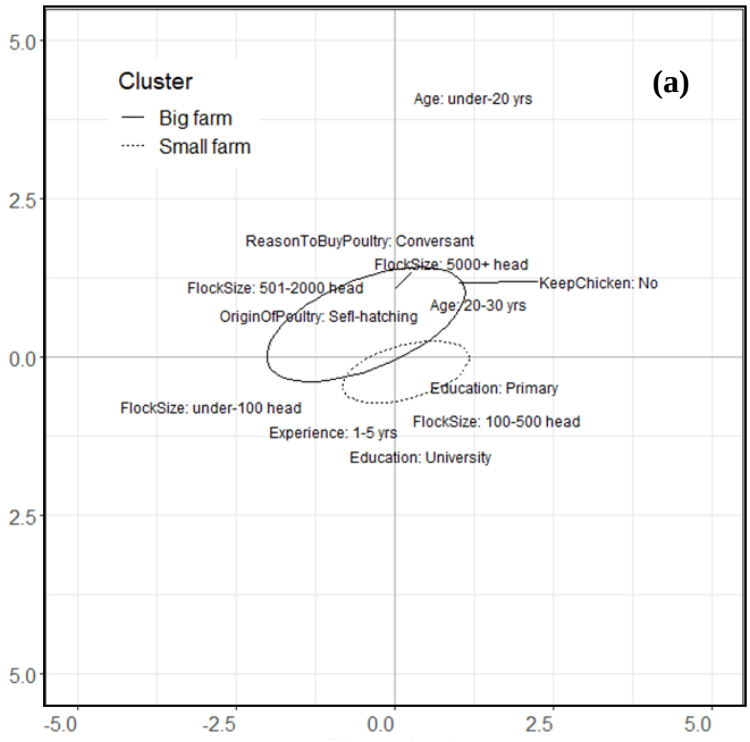
Explanatory variable	Samples	LPAIV positive	Coefficient (SE)	*z*	*P*-value	OR (95% CI)
Intercept	939	11	16.04 (1643.00)			
Sampling species						
Chicken	520	10	Ref	Ref	Ref	1.0
Duck	328	1	1.0580 (1.6550)	0.64	0.52	2.9 (0.1–98.9)a
Muscovy duck	80	0	-1.077 (4.071E+06)	0.00	1.00	NA
Age						
Under 20 year-old	59	1	Ref	Ref	Ref	1.0
20 - 30 year-old	280	10	-20.48 (1643.0000)	-0.00	0.99	0.0 (0.0–3.6)
31 - 40 year-old	280	0	-51.77 (3.123E+06)	0.00	1.00	NA
41 - 50 year-old	279	0	-49.50 (1.872E+06)	0.00	1.00	NA
Upper 50 year-old	30	0	-44.60 (1.310E+07)	0.00	1.00	NA
Gender						
Female	220	10	Ref	Ref	Ref	1.0
Male	708	1	-23.72 (1.643E+04)	-0.00	0.99	0.0 (0.0–3.0)
Experience						
Under 1 year	49	1	Ref	Ref	Ref	1.0
1 - 5 years	431	9	55.9200 (2.3400)	0.24	0.81	1.8 (0.0–2.3)
6 - 10 years	288	1	-0.0320 (2.5900)	-0.01	0.99	1.0 (0.0–7.6)
More 10 years	160	0	-1.3690 (4.62E+06)	0.00	1.00	NA
Keep Muscovy duck						
No	757	2	Ref	Ref	Ref	1.0
Yes	171	9	5.3380 (1.6600)	3.21	<0.01	208.2 (13.4–1.11E+04)
Random effects	Variance	SE				
Individual farm	1.46E-15	3.82E-08				

[a] Interpretation: After adjusting for the effect of sampling in chicken the odds of a bird being LPAIV positive if it was from a 'Chicken' was 2.9 (95% CI: 0.1–98.9) times the odds of a bird from a 'Duck' being LPAIV positive. Ref reference.

MCA

In the demographic section, two clusters were identified (Figure 20a; Table 18). The first (n=17) comprised predominantly large-scale farms with a poultry population of more than 500 heads (‘Big farm’; median=2,000; mean=2,072) that paid more attention to the quality of hatchlings by buying hatchlings from hatcheries they knew previously. The second (n=44) was mostly small-scale farms with under 500 heads of poultry (‘Small farm’; median=215; mean=347) that preferred to buy hatchlings from traders at a lower price.

The questionnaire survey demonstrated that none of the respondents knew about LPAIV, so that they could not differentiate between HPAI and LPAI cases. Two clusters were identified in the knowledge section (Figure 20b; Table 19) by confirmation of their general knowledge about AI. In the first cluster (n=51; ‘Correct knowledge’), most respondents obtained information about AI from local radios and believed that the separation of newly imported poultry could reduce AI risk. All respondents in this cluster agreed that eating sick birds might cause AIV infection in humans. All respondents in this cluster understood AI clinical signs, and the majority of them (54.9%) believed that AI symptoms might not be observed in sick ducks. In the second cluster (n=10; ‘Mixed knowledge’), the respondents rarely believed that the separation of newly imported poultry could reduce AI risk. Eighty percent of this cluster thought that AIV might not infect humans by eating sick birds. Most responders in this cluster were unsure about AI clinical signs but believed clinical signs in sick ducks.



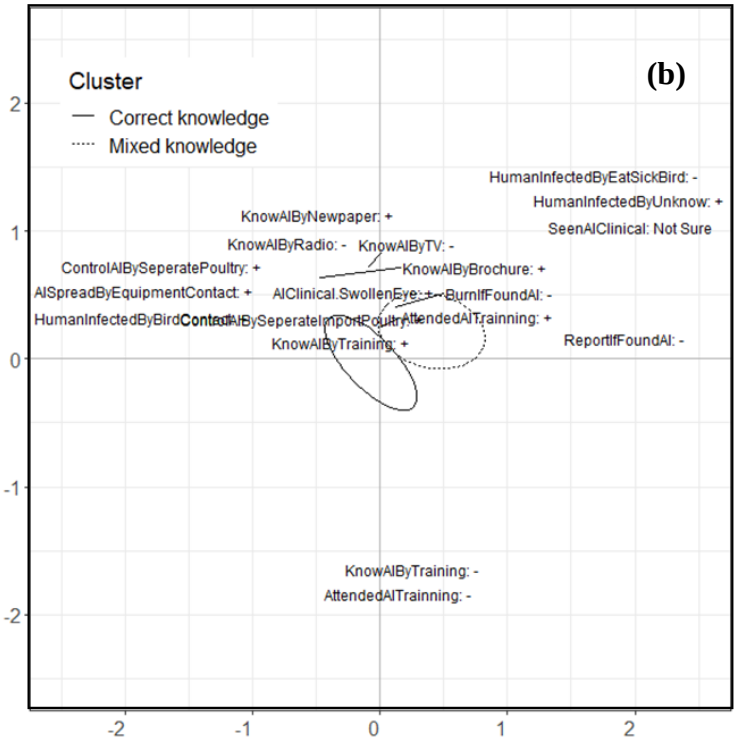


Figure 20. MCA biplot in each of four sections. MCA scatterplot shows questionnaire responses related to respondent demographics (a), knowledge (b), attitude (c), and practice (d). The clusters identified by the HCPC method were indicated by ellipses superimposed on each MCA scatterplot.

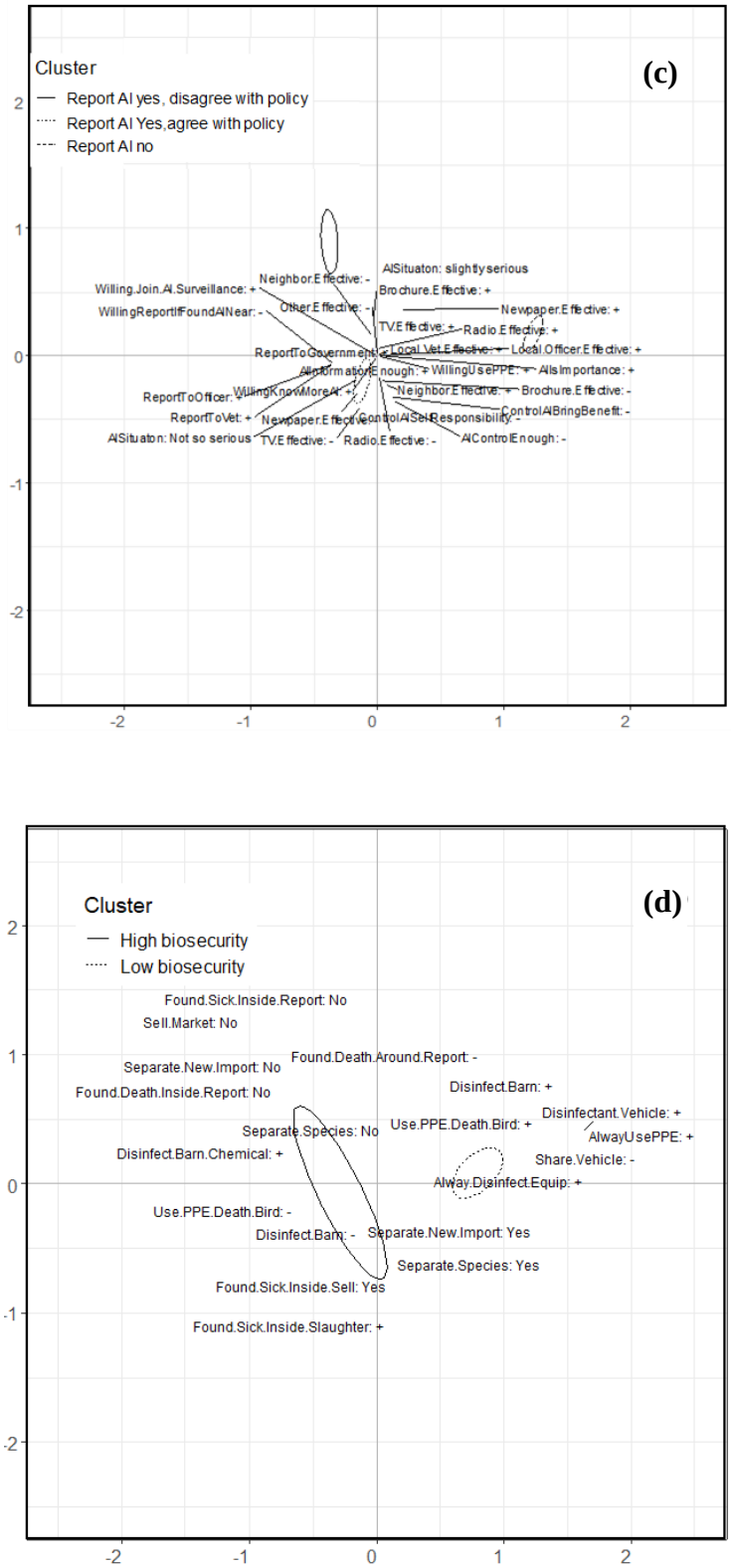


Figure 20 *(cont)*. **MCA biplot in each of four sections.**

Table 18. Numbers of respondents in each identified of the two respondent demographic cluster groups (n=61) and percentages of responses for each question type

Variable	Big farm (n=17)	Small farm (n=44)
Age		
Under 20 year-old	11.8	2.3
20 - 30 year-old	29.4	31.8
31 - 40 year-old	23.5	36.4
41 - 50 year-old	29.4	29.5
Upper 50 year-old	5.9	0.0
Gender		
Female	11.8	38.6
Male	88.2	61.4
Education		
No	11.8	15.9
Primary	29.4	34.1
High school	58.8	34.1
College	0.0	13.6
University	0.0	2.3
Experience		
Under 1 year	0.0	11.4
1 - 5 years	58.8	34.1
6 - 10 years	23.5	34.1
More 10 years	17.6	20.5
Keep chicken		
No	29.4	13.6
Yes	70.6	86.4
Keep duck		
No	64.7	52.3
Yes	35.3	47.7
Keep Muscovy duck		
No	82.4	79.5
Yes	17.6	20.5
Flock size		
Under 100 heads	0.0	15.9
100 - 500 heads	5.9	79.5
501 - 2000 heads	47.1	2.3
2001 - 5000 heads	47.1	0.0
More than 5000 heads	0.0	2.3

Table 18 *(cont)***.** Numbers of respondents in each identified of the two respondent demographic cluster groups (n=61) and percentages of responses for each question type

Variable	**Big farm (n=17)**	**Small farm (n=44)**
The most time to sell poultry		
Jan - Mar	100.0	100.0
Buy the hatchlings from the same commune		
No	58.8	27.3
Yes	41.2	72.7
Buy the hatchlings from the different commune		
No	41.2	72.7
Yes	58.8	27.3
The origin of the hatchlings		
Sefl-hatching	0.0	27.3
Hatchery	82.4	15.9
Trader	17.6	56.8
Reason to by the hatchlings		
Cheap	5.9	61.4
Convenience	35.3	38.6
Conversant	58.8	0.0

Table 19. Numbers of respondents in each identified of the two respondent knowledge cluster groups (n=61) and percentages of responses for each question type

Variable	Correct knowledge (n=51)	Mixed knowledge (n=10)
Know AI		
Yes	100.0	100.0
Know LPAI		
No	84.3	100.0
Yes	15.7	0.0
Know the different between HPAI and LPAI		
No	100.0	100.0
AI cause by virus		
Yes	100.0	100.0
AI cause by bacteria		
No	100.0	100.0
AI cause by parasite		
No	100.0	100.0
Do you know which species can be infected by AIV?		
No	100.0	100.0
Know AI by television		
No	15.7	20.0
Yes	84.3	80.0
Know AI by radio		
No	19.6	40.0
Yes	80.4	60.0
Know AI by newspaper		
No	86.3	80.0
Yes	13.7	20.0
Know AI by brochure		
No	86.3	80.0
Yes	13.7	20.0
Know AI by local officer		
Yes	100.0	100.0
Know AI by local vet		
Yes	100.0	100.0

Table 19 *(cont)***.** Numbers of respondents in each identified of the two respondent knowledge cluster groups (n=61) and percentages of responses for each question type

Variable	Correct knowledge (n=51)	Mixed knowledge (n=10)
Know AI by the training course		
No	13.7	10.0
Yes	86.3	90.0
Thought that AI is controllable		
Yes	100.0	100.0
AI can be controlled by vaccine		
Yes	100.0	100.0
AI can be controlled by keeping the good environment		
Yes	100.0	100.0
AI can be controlled by keep separate poultry		
No	72.5	90.0
Yes	27.5	10.0
AI can be controlled by separating the poultry newly import		
No	56.9	90.0
Yes	43.1	10.0
AI can be controlled by soap wash		
Yes	100.0	100.0
AI can spread by contact with the infected bird		
Yes	100.0	100.0
AI can spread by contact with the contaminated equipment		
No	76.5	90.0
Yes	23.5	10.0
AI can spread by contact with the contaminated cloth/boot		
No	100.0	100.0
Human can be infected by AI from the infected bird		
No	88.2	100.0
Yes	11.8	0.0
Human can be infected by AI from the contaminated equipment		
No	100.0	100.0
Human can be infected with AI by eating the sick bird		
No	0.0	80.0
Yes	100.0	20.0

Table 19 *(cont)***.** Numbers of respondents in each identified of the two respondent knowledge cluster groups (n=61) and percentages of responses for each question type

Variable	Correct knowledge	Mixed knowledge
	n=51	n=10
Human can be infected with AI by unknown source		
No	100.0	20.0
Yes	0.0	80.0
Know the AI clinical signs		
Yes	100.0	30.0
Not sure	0.0	70.0
AI clinical sign can be observe in chicken		
Yes	100.0	100.0
AI clinical sign can be observe in duck		
No	54.9	30.0
Yes	45.1	70.0
AI clinical sign can be observe in Muscovy duck		
No	100.0	100.0
AI clinical sign is depression		
Yes	100.0	100.0
AI clinical sign is edema in the comb		
Yes	100.0	100.0
AI clinical sign is eye swelling		
No	76.5	90.0
Yes	23.5	10.0
AI clinical sign is sudden death		
Yes	100.0	100.0
AI clinical sign is ruffed		
No	100.0	100.0
AI clinical sign is diarrhea		
No	100.0	100.0
Attended the AI training		
No	13.7	0.0
Yes	86.3	100.0
Know about vet law		
Yes	100.0	100.0
Know the purpose of the surveillance		
Early detection	90.2	90.0
Diagnosis	9.8	10.0

Three clusters were identified in the attitude section (Figure 20c; Table 20). For the first cluster (n=8; ‘Report AI but disagree with policy’), although all respondents were willing to report a disease notification to local veterinarians or officials when detected, they were not satisfied yet with the control measures applied. Furthermore, it was thought that AI control was not under their responsibility, which did not benefit their farming in this cluster. The members of the second cluster (n=46; ‘Report AI and agree with policy’) were relatively good attitude respondents because of their willingness to report a disease notification to both local veterinarians and officials when detected. They also agreed to the control measures applied in their area and understood that AI control was under their responsibility, which might be beneficial for their farming. In contrast, all members of the third cluster (n=7; ‘Don't report AI’) declared unwillingness to report a disease notification even when detected. Although they agreed to the control measures applied and understood that AI control was under their responsibility, they were unsure about the AI situation in their husbandry area.

For the practice section, two clusters were identified (Figure 20d; Table 21). Respondents comprising the first cluster (n=17; ‘High biosecurity’) mostly used PPE when slaughtering or handling dead birds. This cluster also disinfected equipment, vehicle, and the barn at high frequency. Furthermore, none of the respondents shared their vehicles for other purposes, except farming. Respondents comprising the second cluster (n=44; ‘Low biosecurity’) seemed to be more careless with a lower frequency of PPE use for slaughtering or handling dead birds. They commonly shared vehicles for other purposes without disinfection and disinfected barn with less frequency.

Table 20. Numbers of respondents in each identified of the three respondent attitude cluster groups (n=61) and percentages of responses for each question type

Variable	Report AI but disagree with policy (n=8)	Report AI and agree with policy (n=46)	Don't Report AI (n=7)
Willing to report if recognize an AI event around			
Yes	100.0	100.0	0.0
Not sure	0.0	0.0	100.0
Report AI event to the local officer			
No	0.0	0.0	100.0
Yes	100.0	100.0	0.0
Report AI event to the local vet			
No	0.0	0.0	100.0
Yes	100.0	100.0	0.0
Report AI event to the local government			
No	0.0	0.0	100.0
Yes	100.0	100.0	0.0
Do you think using PPE is safer for poultry contact?			
Yes	100.0	100.0	100.0
AI situation is important for your business			
Yes	100.0	100.0	100.0
Do you think AI situation in your area become more severe?			
No	37.5	15.2	57.1
Yes	62.5	84.8	42.9
Do you think the AI information provided to you was enough?			
Yes	100.0	100.0	100.0
Do you agree with the local Control measures?			
No	100.0	0.0	0.0
Yes	0.0	100.0	100.0
Do you think AI control is your benefit?			
No	87.5	0.0	0.0
Yes	12.5	100.0	100.0
Do you think AI control is your responsibility?			
No	100.0	4.3	0.0
Yes	0.0	95.7	100.0

Table 20 *(cont).* Numbers of respondents in each identified of the three respondent attitude cluster groups (n=61) and percentages of responses for each question type

Variable	Report AI but disagree with policy (n=8)	Report AI and agree with policy (n=46)	Don't Report AI (n=7)
Do you want to receive more AI information?			
Yes	100.0	100.0	100.0
Television is an effective way to collect the AI information			
No	12.5	30.4	14.3
Yes	87.5	69.6	85.7
Radio is an effective way to collect the AI information			
No	25.0	26.1	28.6
Yes	75.0	73.9	71.4
Brochure is an effective way to collect the AI information			
No	37.5	60.9	57.1
Yes	62.5	39.1	42.9
Newspaper is an effective way to collect the AI information			
No	37.5	63.0	28.6
Yes	62.5	37.0	71.4
Local officer is an effective way to collect the AI information			
Yes	100.0	100.0	100.0
Local vet is an effective way to collect the AI information			
Yes	100.0	100.0	100.0
Neighbor is an effective way to collect the AI information			
No	75.0	56.5	57.1
Yes	25.0	43.5	42.9
Willing to join the AI surveillance			
Yes	100.0	100.0	100.0

Table 21. Numbers of respondents in each identified of the two respondent practice cluster groups (n=61) and percentages of responses for each question type

Variable	High biosecurity (n=17)	Low biosecurity (n=44)
Use PPE when slaughtering		
Yes	100.0	100.0
The frequency of PPE using when slaughtering		
Some time	11.8	100.0
Always	88.2	0.0
Use PPE when handling the death bird		
No	0.0	93.2
Yes	100.0	6.8
Disinfectant equipment		
Yes	100.0	100.0
The frequency of disinfectant equipment		
Some time	0.0	88.6
Always	100.0	11.4
Disinfect vehicle		
Yes	88.2	0.0
No	11.8	100.0
Share vehicle		
Always	0.0	31.8
Some time	0.0	68.2
Never	100.0	0.0
Bury the dead bird if found it around		
Yes	100.0	100.0
Slaughter the dead bird if found it around		
No	100.0	100.0
Feed the dead bird to the other animal if found it around		
No	100.0	100.0
Throw the dead bird if found it around		
No	100.0	100.0
Burn the dead bird then disinfect the area if found it around		
Yes	100.0	100.0
Report the dead bird to the local authority if found it around		
No	5.9	13.6
Yes	94.1	86.4
Disinfect the barn		
Yes	100.0	100.0

Table 21 *(cont)***.** Numbers of respondents in each identified of the two respondent practice cluster groups (n=61) and percentages of responses for each question type

Variable	High biosecurity (n=17)	Low biosecurity (n=44)
The frequency of disinfect barn		
Some time	17.6	90.9
Always	82.4	9.1
Barn disinfectant method		
Water	100.0	0.0
Chemical	0.0	100.0
Separate the sick bird if found inside farm		
Yes	100.0	100.0
Slaughter the sick bird if found inside farm		
No	100.0	90.9
Yes	0.0	9.1
Sell the sick bird if found inside farm		
No	100.0	95.5
Yes	0.0	4.5
Treat the sick bird if found inside farm		
Yes	100.0	100.0
Report the sick bird to the local authority if found inside farm		
No	0.0	11.4
Yes	100.0	88.6
Feed the dead bird to the other animal if found inside farm		
No	100.0	100.0
Throw dead bird if found inside farm		
No	100.0	100.0
Burn or bury the dead bird if found inside farm		
Yes	100.0	100.0
Report the dead bird to the local authority if found inside farm		
No	0.0	29.5
Yes	100.0	70.5
Separate the newly imported poultry		
No	0.0	50.0
Yes	100.0	50.0
Keep separate species		
No	11.8	63.6
Yes	88.2	36.4
The method to sell the poultry		
Bring to market	0.0	34.1
Sell to trader	100.0	50.0
Self-consume	0.0	15.9

Multivariable logistic regression analysis for variables identified by MCA

The mixed-effects logistic regression model using clustering data was developed to assess all variables and reduce the bias from the elimination of the variables in the screening process. The fixed- and mixed-effects logistic regression model with estimated regression coefficients of the knowledge and attitude clusters is shown in Table 22 and Table 23, respectively. One knowledge cluster (‘Correct knowledge’) and two attitude clusters (‘Report AI but disagree with policy’ and ‘Report AI and agree with policy’) showed the potential to reduce the risk of LPAIV infection, although a significant difference was not observed. The proportions of unexplained variance at the farm and bird levels after adjustment for including the fixed effects in the model were 0.42 and 0.58, respectively. The AUC for the fixed-effects model was 0.91, indicating that the model possessed sufficient power to discriminate between AIV-positive and AIV-negative birds. In the mixed-effects model, the AUC was 0.99.

Table 22. A fixed-effects logistic regression model quantifying the association between clusters and LPAIV positivity

Explanatory variable	Samples	LPAIV positive	Coefficient (SE)	*z*	*P*-value	OR (95% CI)
Intercept	939	11	-3.0415 (0.7325)			
Knowledge						
Mixed knowledge	100	1	Ref	Ref	Ref	1.0
Correct knowledge	839	10	-2.1462 (1.0690)	-2.008	0.05	0.1 (0.0–0.6)[a]
Attitude						
Report AI but disagree with policy	79	2	Ref	Ref	Ref	1.0
Report AI and agree with policy	770	1	-3.5804 (1.2398)	-2.888	<0.01	0.0 (0.0–0.3)
Report AI no	90	8	1.2631 (0.8158)	1.548	0.12	3.5 (0.8–24.2)

[a] Interpretation: In the knowledge category, the odds of a birds being LPAIV positive if it was from a farm in 'Correct knowledge' cluster was 0.1 (95% CI: 0.0–0.6) times the odds of a birds from a farm in 'Mixed knowledge' cluster being LPAIV positive.
Ref reference.

Table 23. A mixed-effects logistic regression model quantifying the association between clusters and LPAIV positivity

Explanatory variable	**Samples**	**LPAIV positive**	**Coefficient (SE)**	***z***	***P*-value**	**OR (95% CI)**
Intercept	939	11	-3.48 (1.6333)			
Knowledge						
Mixed knowledge	100	1	Ref	Ref	Ref	1.0
Correct knowledge	839	10	-1.6654 (2.1070)	-0.79	0.42	0.2 (0.0–11.8)[a]
Attitude						
Report AI but disagree with policy	79	2	Ref	Ref	Ref	1.0
Report AI and agree with policy	770	1	-3.6116 (2.0797)	-1.737	0.08	0.0 (0.0–1.6)
Report AI no	90	8	0.2825 (2.0665)	0.137	0.89	1.3 (0.0–76.1)
Random effects	Variance	SE				
Individual farm	7.342	2.71				

[a] Interpretation: After adjusting for the effect of respondent knowledge category and attitude category the odds of a bird being LPAIV positive if it was from a 'Correct knowledge' cluster was 0.2 (95% CI: 0.0–11.8) times the odds of a bird from a 'Mixed knowledge' cluster being LPAIV positive.
Ref reference.

Discussion

Application of multi-approach countermeasures such as strengthening the active and passive surveillance, mass vaccination, education campaigns reduced the HPAI cases in Vietnam, leading to the minimization of substantial losses in the domestic poultry sector. However, field report of AI typical symptoms but not due to H5 HPAIV infection should raise the concern about the damage by LPAIV. The prevalence of AIV positivity in poultry at the backyard farms in this study was 3.9% (95% CI: 2.3%–6.1%) in which the HPAIVs were isolated in a single farm, returning LPAIV prevalence to 2.4% (95% CI: 1.2%–4.2%). This result was comparable to the previous studies in the southern Vietnam which were determinate the LPAIV prevalence in backyard farms range from 0.6% to 5.0% with the 95% CI as follow: 0.6% (95% CI: 0.1%–1.7%) in 2011, 1.7% (95% CI: 0.8%–3.0%) in 2012 [20-23,29], or recently, was 1.4% (95% CI: 0.7%–2.3%) in 2016, 5.0% (95% CI: 3.4%–7.2%) in 2017 [117], 0.8% (95% CI: 0.2%–2.0%) in 2018 [100]. These results indicated that the LPAIV prevalence was varied among the sample collection time. Overall, the LPAIV prevalence in backyard farms (range from 0.6% to 5.0%) was higher than one in high biosecurity farms (range from 0.0% to 2.3%) [100,117]. Although the detection of HPAIV in farms during the active surveillance was a sporadic event, the low biosecurity condition in the backyard/small-scale farms might promote the occurrence of outbreaks [29]. In addition, the circulation of multi-subtypes was confirmed in Vietnam. This situation promotes the reassortment event that leads to the antigenic shift. The report about the reassortment of LPAIV with H5N6 HPAIV was released in Vietnam [28,118]. Furthermore, mixing many species and a free-grazing farming model might enhance the frequency of reassortment events [119]. Based on these results, the countermeasures focusing on backyard farms more should be established to reduce the risk of AIV infection in region.

Considering the effect of individual birds, the unmeasured effect has existed in the individual bird-level because not all of the poultry are positive for LPAIV infection under the same condition in a farm. It means that the LPAI positivity at the individual-bird level comprised the measured effect in herd level and unmeasured effect in individual-bird level. In the mixed-effects model, the effects at herd level (or farm level) and individual-bird level were included to explain the unmeasured effects operating at both levels which

influenced the proportions of variance in LPAIV positivity. Multilevel analysis model provided the opportunity to separate the influence of the herd and individual bird on the risk of being LPAIV positive.

The strong correlation between aquatic birds and the circulation of AIVs including LPAIVs was confirmed in this study (Table 17). In the mixed model of the individual factors, keeping Muscovy duck during the farming practice was significantly at higher risk of the LPAIV positivity. This finding was consistent with the previous study which identified the role of Muscovy ducks as the promotor for AIV spreading [29]. However, only 84 out of 150 variables were assessed in this model due to the zero count of positive birds. Based on the results of the mixed model of the individual factors, the range of odds and the standard error implied that it might contain unrevealed factors, and keeping Muscovy duck might not be a sole variable contributing to LPAIV positivity in the total dataset. To incorporate the variables as much as possible, MCA analysis was performed to identify the clusters of responses for each section and then be used as explanatory variables in the multivariable analysis. The profiles of each cluster showed the effect on LPAIV positivity among clusters in each section. It was revealed that large-scale farms were more likely to consider the safety of the farming process through the quality of imported poultry, whereas small-scale farms prefer to buy the cheap hatchlings (Table 18). While most large-scale farms run poultry farming as the main business, the main business in most of the small-scale farms was rice padding, cattle farming, worker, etc. Therefore, small-scale farms pay less attention to poultry farming than large-scale farms. This would support that most small-scale farms lack resources and were unlikely to pay money for infrastructure for raising poultry, which is a side business [120,121]. Good practice by applying biosecurity measures can minimize the risk of LPAIV infection. It was confirmed in previous studies in the Mekong River Delta that good biosecurity practices, vaccination, and separation of poultry species significantly reduced AI risk in farms [122,123]. In the previous study, the countermeasures applied in LBMs might not appropriately prevent AIV introduction because the infection in birds might occur before entering LBMs [23] meaning the backyard farms could be one of the contributors.

In the fixed-effects logistic regression model, knowledge explanatory variable (‘Correct knowledge’ and ‘Mixed knowledge’) and attitude explanatory variable (‘Report AI but disagree with policy’, ‘Report AI and agree with policy’, and ‘Report AI no’)

showed a significant correlation with LPAIV infection at the bird level (Table 22). In detail, the farmers classified in the ‘Correct knowledge’ cluster had a lower fold in the odds of their birds being LPAIV positive compared to farmers classified as having a ‘Mixed knowledge’. The opposite trends were confirmed in the attitude variable when the cluster of farmers likely to report an outbreak of AI but disagree with the countermeasures against AIV by local authorities (‘Report AI but disagree with policy’) was classified as the reference category. Farmers who provided consistent responses in terms of their willingness to report an AI outbreak and support for the countermeasures applied by the local authorities (‘Report AI and agree with policy’) had a lower fold in the odds of their birds being LPAIV positive. In contrast, farmers who were unwilling to report an outbreak of AI to authorities (‘Report AI no’) had a higher fold in the odds. Although none of them showed a significant association with LPAIV positivity status, the outcome of the mixed-effects regression model at the farm level including a random effect was similar to one of the fixed-effects regression models in the tendency and magnitude of the point estimates of the regression coefficients. A good attitude by reporting the outbreak might mitigate the risk of AI infection in the future was confirmed on farms in the Mekong River Delta in the previous study [123]. It means that LPAI control should be focused on improving the specific knowledge of LPAI to enhance the awareness of LPAI, leading to change the attitude at the farm level.

Because the questionnaire used in this study was developed based on the past AIV surveillance, especially targeting HPAIV, specific and critical factors for LPAIV infection might not be fully covered. Further investigation, such as an unstructured survey covering specific knowledge of LPAI, would contribute to overcoming this issue by combining data from the community, which might not be included in this questionnaire study. Furthermore, this study did not measure the actual damages due to LPAIV infection, such as low egg or growth rates. By focusing on the potential risk factors of LPAIV, future studies should evaluate these outcomes to perform clearer and more direct epidemiological investigations to elucidate the damage of LPAIV infection.

The difference in farming systems and value chains might affect the ecology characteristics of AI. Therefore, effective AI control measures should consider the characteristics of the farmers in the specific region. Good knowledge is the key to controlling LPAI and the local authority is the best candidate to transfer the correct

knowledge about LPAI (100% of farmers got the AI information from the local veterinarian or local officer). This phenomenon was suited not only for LPAI but also for AI [117]. The long-term and intensive monitoring is the key to a deeper understanding of LPAI. The appropriate policy for LPAI control should be established based on the above scientific evidence.

Brief summary

Although many losses have been reported in HPAI, the impact of LPAI has also been confirmed mainly in farms but has been hardly evaluated due to the underestimation of its spread and damage. In 2019, a questionnaire study was conducted in southern Vietnam to identify the specific risk factors of LPAIV circulation and find associations between husbandry activities related to LPAI prevalence. The multilevel regression analysis indicated that keeping Muscovy duck during the farming practice contributed to LPAIV positivity. Moreover, through the analysis for the cluster of factors indicated no significant difference in the correlation of farmer characteristics and LPAI, farmers who were willing to report AI events and agreed with the local AI control policy had a slightly lower risk for LPAIV infection. These findings indicated that keeping the Muscovy duck without appropriate countermeasures might increase the risk of LPAIV infection. Furthermore, locally specific control measures are effective for LPAIV circulation, and improvement of the knowledge about biosecurity and attitude contributes to reducing LPAI damage.

Conclusion

To develop an effective AI control strategy, circulation dynamics and AIV characteristics should be considered carefully. In detail, numerous well-known studies of AI figured out the damage and relative risk of HPAI in poultry. Originally, stamping out is the high priority for combating HPAIV, and vaccination is an optional measure because mass vaccination in the complex situation in the field may facilitate antigenic drift caused by immunological selection pressure. Unlike HPAIV, LPAIV antigenicity was more conserved due to the local infection, which induces a relatively low selection pressure [124]. However, LPAIVs play a critical role in generating the potential pandemic strains by contributing genetic diversity via reassortment. Thus, the control strategy for LPAIV might consider reducing its prevalence using the vaccine as an optional countermeasure. Unfortunately, research on the genetic diversity and potential risk of LPAIV only played a minor role in the overall research on AIV. Therefore, the scientific evidence related to the evolution rate and factors affecting the evolution dynamic of LPAIV remains unclear. This study applies a multi-aspect approach for improving AI control strategy in Vietnam. By combining the virological and epidemiological studies, the findings of this thesis provide a new perspective for improving AI control and prevention.

The genetic diversity of LPAIV was assessed in Chapter I. A total of 1,361 AIVs of various subtypes were isolated in the surveillance from 2014 to 2018, in which H6 and H9 viruses were the dominant subtypes and H7N7 viruses were initially detected. The phylogenetic analysis of the HA genes revealed that Vietnamese H6 and H9 LPAIVs were classified into Group II and Y280/BJ94 sub-lineages, respectively, and clustered together with previous isolates in Vietnam and neighboring countries. H7 LPAIVs were clustered together with Cambodian isolates, but not with H7 LPAIVs previously isolated in Vietnam or Chinese H7N9 HPAIVs. The antigenicity of Vietnamese H6 and H7 viruses showed a slight diverse and formed into different antigenic groups from preexisting viruses, whereas H9 viruses isolated during the study period were almost identical. Conserved antigenicity of H9 isolates from poultry suggested that the viruses were maintained in the immunologically naive poultry population in Vietnam despite the high prevalence of H9 viruses. However, concerns regarding the damage caused by H9 viruses were raised due to the field reports from DAH that AI-typical clinical signs were

observed in the outbreak with a diagnosis of influenza A only. Although H9 viruses were classified as LPAIV, they could cause severe damage due to co-infection with other pathogens in the field. Therefore, to understand the pathogenesis of H6 and H9 LPAIVs in the field, experimental infection with or without other pathogens to poultry will be performed.

Unfortunately, a previous study in Vietnam indicated that interventions applied in LBMs were not effective enough to minimize the risk of AIVs. Therefore, the identification of stakeholders' contributions that increase the likelihood of AIV isolation in individual birds was the target of Chapter II. In the study area, birds sampled from PDSs had the highest prevalence (21.0%), followed by LBMs (14.0%), backyard farms (3.0%), and commercial poultry farms (0.6%). Adequate knowledge of AI was identified as a protective factor by demonstrating that respondents with a mixed (uncertain or inconsistent) level and a low level of knowledge about AI increased odds of birds being AIV positive compared to a good knowledge of AI respondents. These findings confirm the hypothesis that insufficient knowledge of AI might increase the risk of AIV positivity. To assist in this regard, the AI control strategy should focus more on PDSs by providing appropriate education programs specifically designed for those in each enterprise.

The risk factors of LPAI have not been precisely evaluated due to the underestimation of its spread and damage in farms. Therefore, the risk factors of LPAI in farms were investigated in Chapter III. A total of 2,019 AIVs were isolated from 2009 to 2019, with an overall prevalence of 7.7%. The distribution of subtypes differed between northern and southern Vietnam, with subtype H9 being the remarkably dominant subtype in the north, while H6 and H9 subtypes were equally circulating in the south. The epidemiological survey emphasized that raising aquatic birds, particularly Muscovy ducks, might increase the risk of LPAIV infection, whereas good behavior of reporting AI events and supporting AI control policy had a protective effect against LPAIV infection in farms. The differences in the distribution of host species in specific regions and the beliefs of the farmers in countermeasures implementation by the local authority indicated that locally specific control measures are effective for LPAIV circulation.

Finally, the necessity of AI control is undisputed but enhancing the effectiveness of countermeasures is a challenging task. Therefore, collaboration with multiple stakeholders employing different approaches should be the mainstream spirit in AI

control strategy development. Thus, the findings in this thesis provide more information regarding the evolution and impact of AIVs in the fields, which might contribute to improving the AI control strategy in Vietnam.

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Appendix 1

BASELINE QUESTIONNAIRE FOR AI INVESTIGATION
ON THE KNOWLEDGE, ATTITUDE, AND PRACTICES AT FARM

Date of investigation	_ _ / _ _ / _ _ _ _
Name of investigator	
Phone number	

Address

District		
Address		
Name of farmer		
Phone number of farmer		
Type of farm	☐ Chicken ☐ Duck ☐ Mix	☐ Biosecurity ☐ Backyard ☐ Other
Order collected sample	From: ……. To: ……	

X co-ordinate: …… Y co-ordinate: ……

A. GENERAL INFORMATION

1. How long have you involved in poultry farming?
   - ☐ Under 5 year
   - ☐ 1 - 5
   - ☐ 6 - 10
   - ☐ Over 10 year
2. What is your purpose?
   - ☐ Meat ☐ Egg ☐ Other:……
3. Does your farming model belong to the campaign/ program of local government?
   - ☐ Yes ☐ No

4. If the answer of Q3 is yes:

☐ Name of the campaign/ program: ……

☐ How much budget did you receive from this program?: ……....

☐ Is there any breed support? ……

☐ Is there any vaccination support? ……

☐ Is there any disinfectant support? ……

☐ Is there any farming technique support? ……..

☐ Other:……

5. How can you sell your poultry or product?

☐ Sell at market by yourself:

Name of market No1: ……..; Name of market No2: ……..

☐ Sell to trader:

Name of trader No1: ……..…..; Name of trader No2: ……

Name of trader No3: ……..…..

☐ Self-consumption

6. How often did you sell your poultry or product?

☐ Sell at market by yourself (how many times per day/week/month/year) :

……..……...

☐ Sell to trader (how many times per day/week/month/year) :

……..……

What time the trader usually come?

☐ 3-5; ☐ 6-8; ☐ 9-14; ☐ 15-17; ☐ After 17;

☐ unidentified

☐ Self-consumption

7. How can you contact to sell your poultry?

☐ Proactively contact the trader

☐ Proactively contact the seller at market

☐ The trader and seller order the number of poultry before they come.

☐ Is there any vaccination support? ……

☐ Is there any disinfectant support? ……

☐ Is there any farming technique support? ……

☐ Other:……

8. What is the main reason you decide to sell your poultry to one trader?

☐ Price

☐ Relation

☐ Follow the contract

☐ Follow the recommendation of local government

☐ Other:……

9. Does raising poultry is the main income of you?

☐ Yes; ☐ no; ☐ Other: ……

B. POULTRY INFORMATION

1. What is the approximate number of birds that you keep?

Chickens: ……..

Ducks: …….

Other: ……...……..

2. Do you separate the birds?

☐ Yes: How can you separate the birds?: ……

☐ No

3. What is the source of your poultry?

☐ Hatchery; Name, address: …….

☐ Hatch by yourself

☐ Other:……

4. Do you separate the new imported birds?

☐ Yes

☐ No

5. Do you vaccinate for your birds?

☐ Yes

What kind of vaccine:

……..

☐ No

6. Do you vaccinate the H5N1 vaccine for your birds?

☐ Yes

What is the name of vaccine? …….

How many time you vaccinated:……..

☐ No

7. Have you ever seen the AI outbreak in poultry?

☐ Yes

☐ In your poultry (dd/mm/yyyy):……

☐ In other farm: Village:…… Commune:…….

District:…….. Time:……..

☐ No

8. Do you disinfectant on your farming area frequently?

☐ Yes

How many time per day/ week/ month:…….

☐ Never

C. KNOWLEDGE

(Do not read the answers in this part)

1. Have you ever heard about AI (bird flu) H5N1?

☐ Yes ☐ No

If **No**, terminate the interview

2. What is the causative agent of AI?

☐ Genetic

☐ Weather

☐ Bio-factor: ☐ Bacteria ☐ Virus ☐ Parasite

☐ Other factor: ☐ Physical ☐ Chemical ☐ Toxic

☐ Don't know

3. In your opinion, how is AIV infected your poultry?

☐ Pathogen already in poultry ☐ Contact with infected or sick bird

☐ Contact with wild bird ☐ Contact with other animal

☐ Contact with farmer ☐ Contact with trader

☐ Other: ……..

4. In your opinion, where is the hot spot of AI?

☐ Backyard farm ☐ Commercial farm ☐ LBM

☐ Poultry delivery station ☐ Slaughtering house ☐ Other:……

5. From where did you learn about AI?

☐ TV ☐ Market manager

☐ Radio ☐ Animal health worker

☐ Newspaper ☐ Training course

☐ Brochure ☐ Other……

6. Have you ever seen the clinical signs of AI in poultry?

☐ Yes ☐ Not sure

☐ No

7. 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?.....

8. Do you afraid to be infected by AIVs?

☐ Very afraid ☐ Afraid ☐ Don't care

9. What kind of information do you prefer to know before you decide to buy the poultry?

☐ The health of poultry ☐ Confirm by local vet

☐ Vaccinated ☐ Don't care

☐ Other: ……

D. ATTITUDES

1. Have you ever seen the poultry infected by AIV?

☐ Yes ☐ Not sure

☐ No

2. If you thought you had a bird flu case in your farm or near your farm (neighbor farmer) would you report it? (If the answer is No/Not sure, skip Q3)

☐ Yes ☐ Not sure

☐ No

3. To whom would you be more likely to report suspected cases of bird flu in poultry?

☐ Market manager ☐ Local authority

☐ Veterinarian

4. Do you think your poultry can be infected by AIV?

☐ Yes ☐ Not sure

☐ No

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

☐ TV ☐ Market manager

☐ Radio ☐ Animal health workers

☐ Poster, brochures ☐ Family, friends, neighbors and colleagues

☐ Newpapers ☐ Other…….

E. PRACTICES

1. Do you use the PPE (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 ☐ I don't believe it help to protect from AI

   ☐ It is not convenience

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

   ☐ Every time ☐ Never

   ☐ Sometime

   ❖ 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

3. Do you spray disinfectant before and after you contact to poultry?

☐ Every time ☐ Never

☐ Sometime

4. What will you do when you find the dead birds in your farm? (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

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

☐ Keep them in separate from other poultry ☐ Slaughter for food

☐ Sell them as soon as possible ☐ Report to animal health workers

☐ Give them antibiotics ☐ Do nothing

6. Do you separate the new imported birds?

☐ Yes

☐ No

Thank you very much for participating in our survey.

Appendix 2

BASELINE QUESTIONNAIRE FOR AI INVESTIGATION
ON THE KNOWLEDGE, ATTITUDE, AND PRACTICES AT LBM

Date of investigation	_ _ / _ _ / _ _ _ _
Name of investigator	
Phone number	

Address

District	
Address	
Name of seller	
Phone number of seller	
Order collected sample	From: ……. To: ……

X co-ordinate: …… Y co-ordinate: ……

A. GENERAL INFORMATION

1. Where are you living?

   Address:……

2. What is your gender?

   ☐ Male

   ☐ Female

   How old are you? ……

3. How long have you involved in poultry selling?

   ☐ Under 5 year ☐ 6 - 10

   ☐ 1 - 5 ☐ Over 10 year

4. What are the poultry species and how much average quantity, do you buy and sell every day?

☐ Chicken (…….) ☐ Duck (……..) ☐ Muscovy duck (……) ☐ Goose (…….) ☐ Mix species (…….)

5. Where is your common selling area?

☐ Inside the market (name:…….) ☐ Outside the market

6. When is your busiest time for your business?

☐ Most: From: …… to ……; Reason: ……

☐ Least: From: …… to ……; Reason: ……

7. How many times do you bring your poultry to the market?

…… Times/ day ……times/week

8. Where is your poultry's source?

☐ From the farm: ☐ Farmer bring to you
☐ You come to the farm by yourself

☐ From the trader: ☐ Trader bring to you
☐ You come to the poultry delivery station by yourself

☐ From the other sellers

9. Can you describe some locations where you collect the poultry?

☐ Farm

- Commune 1: ……..; farm 1: ……; farm 2:……..
- Commune 2: ……..; farm 3: ……; farm 4:……..
- Other information:

……..

☐ Trader

- Trader 1: ……..; Address: ……
- Trader 2: ……..; Address: ……

☐ Other sellers

- Seller 1: ……..; Market 1: …….
- Seller 2: ……..; Market 2: …….

10. What is the main factor that affected your business?

☐ Price

☐ Disease

☐ Quarantine

☐ The quality of poultry

☐ The source of poultry

☐ Requirement of market

☐ Other: ……..

11.How can you manage your business?

☐ Based on the number of poultry from the trader

☐ Based on the number of poultry from the last business

☐ Fit the number of poultry for every day

☐ Based on the experience: ……

☐ Other: ……..

12.Does selling poultry is the main income of you?

☐ Yes; ☐ no; ☐ Other: ……

B. KNOWLEDGE

(Do not read the answers in this part)

1. Have you ever heard about AI (bird flu) H5N1?

☐ Yes ☐ No

If **No**, terminate the interview

2. What is the causative agent of AI?

☐ Genetic

☐ Weather

☐ Bio-factor: ☐ Bacteria ☐ Virus ☐ Parasite

☐ Other factor: ☐ Physical ☐ Chemical ☐ Toxic

☐ Don't know

3. In your opinion, how is AIV infected your poultry?

☐ Pathogen already in poultry ☐ Contact with infected or sick bird

☐ Contact with wild bird ☐ Contact with other animal

☐ Contact with farmer ☐ Contact with trader

☐ Other: ……..

4. In your opinion, where is the hot spot of AI?

☐ Backyard farm ☐ Commercial farm ☐ LBM

☐ Poultry delivery station ☐ Slaughtering house ☐ Other:……

5. From where did you learn about AI?

☐ TV ☐ Market manager

☐ Radio ☐ Animal health worker

☐ Newspaper ☐ Training course

☐ Brochure ☐ Other…….

6. Have you ever seen the clinical signs of AI in poultry?

☐ Yes ☐ Not sure

☐ No

7. 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?.....

8. Do you afraid to be infected by AIVs?

☐ Very afraid ☐ Afraid ☐ Don't care

9. What kind of information do you prefer to know before you decide to buy the poultry?

☐ The health of poultry ☐ Confirm by local vet

☐ Vaccinated ☐ Don't care

☐ Other:

……

C. ATTITUDES

1. Have you ever seen the poultry infected by AIV?

☐ Yes ☐ Not sure

☐ No

2. If you thought you had a bird flu case in your farm or near your farm (neighbor farmer) would you report it? (If the answer is No/Not sure, skip Q3)

☐ Yes ☐ Not sure

☐ No

3. To whom would you be more likely to report suspected cases of bird flu in poultry?

☐ Market manager ☐ Local authority

☐ Veterinarian

4. Do you think your poultry can be infected by AIV?

☐ Yes ☐ Not sure

☐ No

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

☐ TV ☐ Market manager

☐ Radio ☐ Animal health workers

☐ Poster, brochures ☐ Family, friends, neighbors and colleagues

☐ Newpapers ☐ Other…….

D. PRACTICES

1. Do you use the PPE (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 ☐ I don't believe it help to protect from AI

☐ It is not convenience

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

☐ Every time ☐ Never

☐ Sometime

❖ 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

3. Do you spray disinfectant before and after you contact to poultry?

☐ Every time ☐ Never

☐ Sometime

4. What will you do when you find the dead birds in your farm? (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

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

☐ Keep them in separate from other poultry ☐ Slaughter for food

☐ Sell them as soon as possible ☐ Report to animal health workers

☐ Give them antibiotics ☐ Do nothing

6. Do you separate the new imported birds?

☐ Yes

☐ No

7. How can you keep the remaining birds?

☐ At home

☐ At poultry delivery station

☐ At market

☐ Return to the farm

Thank you very much for participating in our survey.

Appendix 3

BASELINE QUESTIONNAIRE FOR AI INVESTIGATION ON THE KNOWLEDGE, ATTITUDE, AND PRACTICES AT PDS

Date of investigation	_ _ / _ _ / _ _ _ _
Name of investigator	
Phone number	

Address

District	
Address	
Name of trader	
Phone number of trader	
Order collected sample	From: ……. To: ……

X co-ordinate: …… Y co-ordinate: ……

A. GENERAL INFORMATION

1. Where are you living?

   Address:……

2. What is your gender?

   ☐ Male

   ☐ Female

   How old are you? ……

3. How long have you involved in poultry trading?

   ☐ Under 5 year ☐ 6 - 10

   ☐ 1 - 5 ☐ Over 10 year

4. What is the poultry product you run in your business?

☐ Live poultry

☐ Meat

☐ Egg

☐ Other:……

5. What are the poultry species and how much average quantity, do you buy and sell every day?

☐ Chicken (…….) ☐ Duck (……..) ☐ Muscovy duck (……) ☐ Goose (…….) ☐ Mix species (…….)

6. Where is your common business area?

☐ Within commune (name:…….) ☐ Within district (name:…….…..)

☐ Within province (name:……..…….) ☐ Interprovincial (name:…….)

☐ Other (……..…)

7. What is the range of your movement during a business day?

☐ < 10km ☐ 10 – 50 km ☐ 50 – 100 km

☐ 100 – 200 km ☐ 200 – 500 km ☐ > 500 km

8. When is your busiest time for your business?

☐ Most: From: …… to ……; Reason: ……

☐ Least: From: …… to ……; Reason: ……

9. How many times do you collect the poultry for your business?

…… times/day ……times/week

10. Where did you keep the poultry collected from the farm?

- ☐ To the market directly
- ☐ To the slaughterhouse
- ☐ To the consumption place (restaurants, food shop)
- ☐ To the intermediate place
- ☐ Keep at home before distributing to the market

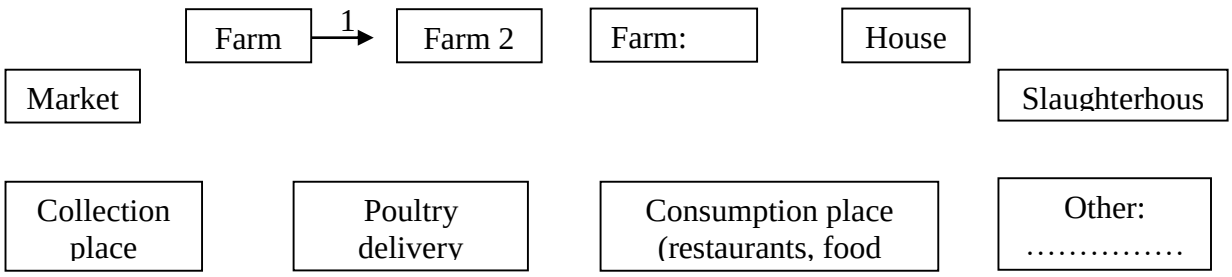
11. How many poultry was collected in a collection round?

Chicken: ……..; Duck: ……..….; Egg: …….; Other:……

12. What kind of vehicle was uses for your business?

- ☐ Motorcycle
- ☐ Truck
- ☐ Boat
- ☐ Other: ……

13. Can you draw the route you collect the poultry?



14. Can you describe some locations where you collect the poultry?

- ☐ Farm

  - Commune 1: ……..; farm 1: …….; farm 2:……
  - Commune 2: ……..; farm 3: …….; farm 4:……
  - Commune 3: ……..; farm 5: …….; farm 6:……
  - Other information:

  ……..

☐ Commercial Farm

- Commune 1: ……..; Commercial farm 1: …….; Commercial farm 2:……
- Commune 2: ……..; Commercial farm 3: …….; Commercial farm 4:……
- Other information:

……..

☐ Other traders

- Trader 1: ……..; Address: ……
- Trader 2: ……..; Address: ……

☐ Market

- Market 1: ……..; Address: ……
- Market 2: ……..; Address: ……

15. Where did you sell your poultry?

☐ Market

- Market 1: ……..…..…..; Market 2: …….;
- Market 3: ……...…..; Market 4: …….;

☐ Poultry delivery station

- PDS 1: ……...…..; PDS 2: …….;
- PDS 3: ……..…..…..; PDS 4: …….;

☐ Other traders

- Trader 1: ……..; Address: ……
- Trader 2: ……..; Address: ……

☐ Slaughterhouse

- Slaughterhouse 1: ……..; Address: ……
- Slaughterhouse 2: ……..; Address: ……

☐ Other consumption place (restaurants, food shop)

- consumption place 1: ……..; Address: ……
- consumption place 2: ……..; Address: ……

☐ Other : ……

16. What is the main factor that affected your business?

- ☐ Price
- ☐ Disease
- ☐ Quarantine
- ☐ The quality of poultry
- ☐ The source of poultry
- ☐ Requirement of market
- ☐ Other: ……..

17. How can you manage your business?

- ☐ Based on the number of poultry from the trader
- ☐ Based on the number of poultry from the last business
- ☐ Fit the number of poultry for every day
- ☐ Based on the experience: ……
- ☐ Other: …….

18. How many times you bring poultry to market/PDS every day?

To the market	☐	1 time/day	☐	2 - 3 times/day	☐	> 3 times/day
To the PDS	☐	1 time/day	☐	2 - 3 times/day	☐	> 3 times/day

19. Does selling poultry is the main income of you?

☐ Yes; ☐ no; ☐ Other: ……

B. KNOWLEDGE

(Do not read the answers in this part)

1. Have you ever heard about AI (bird flu) H5N1?

☐ Yes ☐ No

If **No**, terminate the interview

2. What is the causative agent of AI?

☐ Genetic

☐ Weather

☐ Bio-factor: ☐ Bacteria ☐ Virus ☐ Parasite

☐ Other factor: ☐ Physical ☐ Chemical ☐ Toxic

☐ Don't know

3. In your opinion, how is AIV infected your poultry?

☐ Pathogen already in poultry ☐ Contact with infected or sick bird

☐ Contact with wild bird ☐ Contact with other animal

☐ Contact with farmer ☐ Contact with trader

☐ Other: ……..

4. In your opinion, where is the hot spot of AI?

☐ Backyard farm ☐ Commercial farm ☐ LBM

☐ Poultry delivery station ☐ Slaughtering house ☐ Other:……

5. From where did you learn about AI?

☐ TV ☐ Market manager

☐ Radio ☐ Animal health worker

☐ Newspaper ☐ Training course

☐ Brochure ☐ Other….

6. Have you ever seen the clinical signs of AI in poultry?

☐ Yes ☐ Not sure

☐ No

7. 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?.....

8. Do you afraid to be infected by AIVs?

☐ Very afraid ☐ Afraid ☐ Don't care

9. What kind of information do you prefer to know before you decide to buy the poultry?

☐ The health of poultry ☐ Confirm by local vet

☐ Vaccinated ☐ Don't care

☐ Other:

……

C. ATTITUDES

1. Have you ever seen the poultry infected by AIV?

☐ Yes ☐ Not sure

☐ No

2. If you thought you had a bird flu case in your farm or near your farm (neighbor farmer) would you report it? (If the answer is No/Not sure, skip Q3)

☐ Yes ☐ Not sure

☐ No

3. To whom would you be more likely to report suspected cases of bird flu in poultry?

☐ Market manager ☐ Local authority

☐ Veterinarian

4. Do you think your poultry can be infected by AIV?

☐ Yes ☐ Not sure

☐ No

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

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

D. PRACTICES

1. Do you use the PPE (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
☐ I don't believe it help to protect from AI
☐ It is not convenience

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

☐ Every time
☐ Never
☐ Sometime

❖ 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

3. Do you spray disinfectant before and after you contact to poultry?

☐ Every time ☐ Never

☐ Sometime

4. What will you do when you find the dead birds in your farm? (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

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

☐ Keep them in separate from other poultry ☐ Slaughter for food

☐ Sell them as soon as possible ☐ Report to animal health workers

☐ Give them antibiotics ☐ Do nothing

6. Do you separate the new imported birds?

☐ Yes

☐ No

7. How can you keep the remaining birds?

- ☐ At home
- ☐ At poultry delivery station
- ☐ At market
- ☐ Return to the farm

Thank you very much for participating in our survey.

Appendix 4

INFORMATION TO READ TO RESPONDENT:

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

We are planning a study from August 2019 and September 2019 to identify potential risk factors of low pathogenicity avian influenza. The information will help finding appropriate control and prevention strategies for of low pathogenicity avian influenza 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. Le Thanh Tung, Director of Vinh Long Sub-Department of Animal Health

- Dr. Le Trung Kien, Vietnam Department of Animal Health

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

QUESTIONNAIRE FOR LPAI INVESTIGATION

ON THE KNOWLEDGE, ATTITUDE, PRACTICES, AND IMPACT AT FARMS

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

Address

Province	
District	
Commune	
Model	
Name of farmer	
Phone number of seller	
Order collected sample	

X co-ordinate: …… Y co-ordinate: ……

A. GENERAL INFORMATION

1. How old are you?

☐ Under 20 ☐ 41-50

☐ 21-30 ☐ Over 50

☐ 31-40

2. What is your gender?

☐ Male ☐ Female

3. What is your highest education?

☐ None ☐ High school

☐ Elementary ☐ College

☐ Middle school Other ……..

4. How long have you involved in poultry trading?

☐ Under 1 year ☐ 6-10 years

☐ 1-5 years ☐ Over 10 years

5. What type of birds do you usually sell?

☐ Chickens ☐ Pigeons

☐ Ducks ☐ Quails

☐ Muscovy duck ☐ Other……

6. What is the approximate number of birds that you have?

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 Address1: Prov……..., Dist……,Comm……

Address2: Prov……..., Dist……,Comm……

Address3: Prov……..., Dist……,Comm……

9. What source do you usually buy poultry from?

☐ Self-hatching ☐ Trader

☐ Hatchery ☐ Other:……..

10. 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

2. Have you ever heard about LPAI (low pathogenicity avian influenza)? (If no, skip Q3)

☐ Yes ☐ no

3. What is the difference between HPAI and LPAI?

☐ Mortality ☐ Clinical signs ☐ Infectivity ☐ Don't know

4. What is the causative agent of AI?

☐ Virus ☐ Bacteria ☐ Parasite ☐ Don't know

5. Which animals can be infected with AI?

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

6. From where did you learn about AI?

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

7. 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

8. 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.....

9. 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

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

- ☐ Contact with infected or sick bird
- ☐ Contact with contaminated equipment
- ☐ Other….
- ☐ Don't know

☐ Eat duck blood pudding

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

☐ Yes ☐ Not sure

☐ No

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

☐ Chicken ☐ Muscovy duck

☐ Duck ☐ Not at all

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

☐ Sleepiness ☐ Ruffled feathers

☐ Dark/red/blue comb and wattles ☐ Diarrhea

☐ Swollen and puffy looking eyes ☐ Other….

☐ Sudden death in large number ☐ Don't know

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

☐ Keep them in a closed building/separate from other poultry and animal ☐ Burn them

☐ Sell them ☐ Report to local authority

☐ Slaughter for food ☐ Give antibiotics

☐ Throw them away in river or pond ☐ Do nothing

☐ Kill them and bury them ☐ Other…….

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

☐ Sell off all your poultry ☐ Do nothing

☐ Follow animal health authority instruction ☐ Other…..

16. 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?.....

17. 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 ☐ Not sure

☐ No

18. Do you know the purpose of the surveillance?

☐ Yes ☐ Not sure

☐ No

If your answer is yes, can you explain:

……

……

……

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 ☐ Not sure

☐ No

2. To whom would you be more likely to report suspected cases of bird flu in poultry?

☐ Market manager ☐ Local authority

☐ Veterinarian

3. Do you think you will be safe from bird flu without using PPE in handling the poultry?

☐ Yes ☐ Not sure

☐ No

4. Bird flu issues are important for your business?

☐ Yes ☐ Not sure

☐ No

5. How serious a problem do you think bird flu is in Vietnam or your region?

☐ Very ☐ Not very

☐ Somewhat

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 ☐ Not sure

☐ No

8. Do you think the programs of AI control will give you more benefits?

☐ Very ☐ Not sure

☐ Somewhat

9. Do you think that for the control of AI is a part of your responsibility?

☐ Yes ☐ Not sure

☐ No

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 ☐ Market manager

☐ Radio ☐ Animal health workers

☐ Poster, brochures ☐ Family, friends, neighbors and colleagues

☐ Newspapers ☐ Other…….

12. Do you willing to participate in an AI surveillance?

☐ Yes

☐ No

D. PRACTICES

1. Do you use the PPE (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 ☐ I don't believe it help to protect from AI

☐ It is not convenience

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

☐ Every time ☐ Never

☐ Sometime

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 sanitize the lairage?

☐ Yes

☐ No

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

☐ Every day ☐ After selling batch

☐ Every week ☐ Never

☐ Every month

❖ If the answer is **Yes**: How do you sanitize 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 ☐ Slaughter for food

☐ Sell them as soon as possible ☐ Report to animal health workers

☐ Give them antibiotics ☐ 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 ☐ Bury or burn them

☐ Sell them ☐ Report to animal health workers

☐ Slaughter for food ☐ Other…….

☐ Throw them away

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. How do you usually sell your poultry products?

☐ Sell directly in the market ; Name of market:……

☐ Sell to the trader

☐ Seft-consumption

E. IMPACT

1. Contribution of poultry to your total income:

☐ Under 10%

☐ 10 – 30%

☐ 31% - 50%

☐ Over 50%

☐ Not related to income

2. How your income from poultry changed within the last six months?

☐ Increase

☐ Decrease

☐ No change

3. How does the requirement of the trader to the quality of poultry change??

☐ Increase

☐ Decrease

☐ No change

4. Do you want to invest more in your poultry business?

☐ Yes

☐ No

5. How much did you pay for the treatment of your poultry last year?

……

……

How did the treatment cost change compare to the previous year?

☐ Increase ☐ No change

☐ Decrease

Thank you very much for participating in our survey.