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

Duc-Huy Chu\textsuperscript{a,b}, Masatoshi Okamatsu\textsuperscript{a}, Keita Matsuno\textsuperscript{a,c}, Takahiro Hiono\textsuperscript{a}, Kohei Ogasawara\textsuperscript{a}, Lam Thanh Nguyen\textsuperscript{a}, Long Van Nguyen\textsuperscript{b}, Tien Ngoc Nguyen\textsuperscript{b}, Thuy Thu Nguyen\textsuperscript{b}, Dong Van Pham\textsuperscript{b}, Dang Hoang Nguyen\textsuperscript{b}, Tho Dang Nguyen\textsuperscript{b}, Thanh Long To\textsuperscript{b}, Hung Van Nguyen\textsuperscript{c}, Hiroshi Kida\textsuperscript{c,d}, Yoshihiro Sakoda\textsuperscript{a,c,*}

\textsuperscript{a}Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
\textsuperscript{b}Department of Animal Health, Ministry of Agriculture and Rural Development, 15/78 Giai Phong, Phuong Mai, Dong Da, Hanoi, Vietnam
\textsuperscript{c}Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Sapporo, Hokkaido 001-0020, Japan
\textsuperscript{d}Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido 001-0020, Japan
\textsuperscript{e}Sub-Department of Animal Health, 62 Nguyen Chi Dieu, Hue city, Vietnam

*Corresponding author: Yoshihiro Sakoda

Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
Tel: +81-11-706-5207; Fax: +81-11-706-5273

E-mail: sakoda@vetmed.hokudai.ac.jp
Abstract

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

Transmission of avian influenza (AI) viruses among wild and domestic birds is an important target of control measures aiming to minimize the risk to both human and animal health worldwide (Kilpatrick et al., 2006; Nomura et al., 2012; Okamatsu et al., 2013). Vietnam has a large population of poultry (approximately 308 million), and a majority of these animals (approximately 80%) are raised under backyard conditions at households in rural areas without biosecurity application (General Statistics Office of Vietnam. Results of the 2012 Rural, Agricultural and Fishery Census. https://www.gso.gov.vn/default_en.aspx?tabid=778; Hanh et al., 2007). In Vietnam, H5N1 highly pathogenic avian influenza (HPAI) viruses have caused a large number of outbreaks in poultry since 2003 (Minh et al., 2009). In our previous study, we conducted surveillance of AI viruses in live bird markets (LBMs) and households that raise poultry in a large number of Vietnamese provinces in 2009. We found that the prevalence of AI viruses was substantially higher in LBMs than in backyard farms, and H5N1 viruses were isolated from apparently healthy domestic ducks and chickens in LBMs (Okamatsu et al., 2013; Nomura et al., 2012). The findings suggest that in Vietnam, LBMs are more responsible for the amplification, maintenance, circulation, and transmission of AI viruses than backyard farms. Therefore, continuous surveillance of AI viruses in LBMs in Vietnam is essential to understand the distribution of AI viruses and to minimize the risk to public and animal health.

LBMs are ubiquitous and integral parts of the poultry industry in Vietnam and other developing countries in Asia (Wan et al., 2011; Indriani et al., 2010). In China, LBMs have become
a major source of human infection with H5 HPAI viruses and H7 low pathogenic AI viruses (He et al., 2014; Yu et al., 2014; Wan et al., 2011). When keeping live birds overnight in LBMs was banned, the rate of virus isolation declined (Leung et al., 2012). In addition, it was reported that closing LBMs effectively reduced the risk of virus transmission to the public (Fournié et al., 2011; He et al., 2014; Yu et al., 2014). However, banning LBMs in developing countries remains challenging because changing the traditional market style should take a long time. Therefore, government intervention to improve the biosecurity measures employed at LBMs has been thought to represent a promising strategy to minimize the transmission of viruses in Asian countries including Vietnam. To support for the evaluation of local authority on the current intervention, we surveyed the prevalence of AI viruses at nine LBMs with or without the intervention of Vietnam Avian and Human Influenza Control and Preparedness Project (VAHIP) in Thua Thien Hue province, located in the central region of Vietnam. The VAHIP was a project funded by the World Bank aiming to reduce the risk of AI virus transmission to humans (The World Bank, Projects and Operations. Vietnam - Avian and Human Influenza Control and Preparedness Project. http://documents.worldbank.org/curated/en/2011/03/14026433/vietnam-additional-financing-vietnam-avian-human-influenza-control-preparedness-project-environmental-impact-assessment.). The project developed the new model of LBM in selected provinces in Vietnam by deploying infrastructure for the poultry markets and operating periodic disinfection in these intervention LBMs. In this study, various subtypes of AI viruses were isolated during surveillance of LBMs with or without intervention in August and December, 2014. The representative isolates were
phylogenetically and antigenically analyzed to characterize the genetic and antigenic variation of
the AI viruses currently circulating in LBMs in Vietnam.

2. Materials and methods

2.1. Sample collection

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

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

2.2. Isolation and identification of AI viruses
Samples were resuspended in virus transport medium and centrifuged at 2,000 rpm for 5 min.

The supernatant was inoculated into the allantoic cavity of a 10-day-old chicken embryo. After incubation at 35°C for 30–48 h, allantoic fluids exhibiting hemagglutination activity were collected for subtyping of influenza viruses by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests with antisera to the reference influenza virus strains (Kida et al., 1979).

2.3. Sequencing and phylogenetic analysis

Viral RNA was extracted from the 250 μl of allantoic fluids by TRIzol LS Reagent (Life Technologies, USA) following the manufacturer’s protocol and reverse transcribed with the Uni12 primer (Hoffmann et al., 2001) and M-MLV Reverse Transcriptase (Life Technologies, USA). Full-length cDNAs of the eight gene segments were amplified by polymerase chain reaction with Ex-Taq (TaKaRa, Shiga, Japan) and gene-specific primer sets (Hoffmann et al., 2001). Direct sequencing of each gene segment was performed using 3500 Genetic Analyzer (Life Technologies, USA).

For phylogenetic analysis, nucleotide sequences of the isolates, together with those from a public database, were aligned using Clustal W. Phylogenetic trees were constructed using the maximum-likelihood (ML) method with 1,000 bootstrap replicates using MEGA 5.0 software (Tamura et al., 2011). The genome sequences identified in this study have been registered in GenBank/EMBL/DDBJ (Table 2).
2.4. Antigenic analysis

Polyclonal antisera were prepared from chickens immunized with reference AI virus strains that had been inactivated with formalin (Kida et al., 1979). Antigenic analysis of H5, H6 and H9 viruses was performed using polyclonal antisera by HI test.

2.5. Pathogenicity of an H5N6 AI virus in chickens

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

2.6. Knowledge, attitude, and practices survey

The KAP survey was designed to investigate the knowledge, attitude, and practice of individual poultry sellers that associated with AI in LBMs using questionnaires written in Vietnamese. A self-designed questionnaire set comprised 47 questions about the general background of the sellers, the source of poultry, and application of any biosecurity measures for the identification of possible risk factors contributing to AI virus circulation in LBMs.

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

3. Results

3.1. Identification of AI viruses circulating in intervention and non-intervention LBMs

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

### 3.2. Genetic and antigenic analysis of H5 viruses

The full-length nucleotide sequences of the HA genes of H5 representative isolates were phylogenetically analyzed and divided into two lineages: Eurasian and North American. The H5 viruses in the Eurasian lineage were identified on the basis of the nomenclature defined by WHO/OIE/FAO H5N1 Evolution Working Group in 2014 (Smith et al., 2015). The HA genes of six representative H5N6 Vietnam isolates were classified into the clade 2.3.4.4, which was newly established in 2014 (Smith et al., 2015) and is closely related to the A/environment/Zhenjiang/C13/2013 (H5N6) virus isolated in China (Qi et al., 2014) (Fig. 1). All the H5N6 isolates contain multiple basic amino acids, R and K, at the proteolytic cleavage site of the hemagglutinin protein, PLREKRRKR/GLF, identical to the typical cleavage site motif of H5 HPAI viruses. The residues at position 190, 225, 226, and 228 (H3 numbering) are associated with influenza virus receptor specificity. In these viruses, those residues were E, G, Q, and G, respectively; all of them are of avian-type motif (Paulson et al., 2013).

Four representative strains of the H5 isolate were antigenically analyzed by cross HI test (Table 3). Chicken antiserum against the reference virus of the clade 2.3.4.4, A/chicken/Kumamoto/1-7/2014 (H5N8) (Kanehira et al., 2015), effectively inhibited hemagglutination of A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6), A/duck/Vietnam/HU1-1151/2014 (H5N6), and A/muscovy duck/Vietnam/HU1-1151/2014 (H5N6). All
duck/Vietnam/HU2-26/2014 (H5N6) and partly inhibited hemagglutination of A/environment/Vietnam/HU1-1434/2014 (H5N6) causing a 4-fold reduction in HI titer. In contrast, these viruses exhibited low HI titers in comparison with homologous titers in reactions with the antisera of different clades (HI titer from 20 to 80).

3.3. Genetic and antigenic analysis of H6 viruses

The H6 HA genes were phylogenetically divided into two lineages: Eurasian and North American. The H6 viruses in the Eurasian lineage were clustered into five different sublineages: Group II, W312, Group III, Early and Group I, as described in our previous study (Okamatsu et al., 2013). All H6 viruses isolated in Vietnam in 2014 were found to belong to the Group II sublineage (Fig. 2), as were the viruses previously isolated in Vietnam between 2010 and 2012 and those isolated in China between 2003 and 2011. However, the H6 viruses isolated in this study and in our previous study occupied different clusters.

Four strains representative of the H6 isolates were antigenically analyzed by cross HI test (Table 4) using a panel of chicken antisera against four viruses of different sublineages. These H6 viruses weakly reacted with A/duck/Vietnam/OIE-4429/2010 (H6N2) antiserum, a virus of the same genetic sublineage, Group II. In reaction with viruses of other sublineages, these H6 viruses exhibited HI titers 4-fold lower than the homologous groups. In addition, these H6 viruses share equivalent HI titers with homologous hyperimmune serum against A/duck/Vietnam/HU1-637/2014 (H6N6) virus, while A/duck/Vietnam/OIE-4429/2010 (H6N2) virus reacted weakly with the
antiserum with 16-fold lower HI titers. These results suggest that the antigenicity of the H6 viruses we isolated differed from viruses isolated in Vietnam between 2010 and 2012 (Okamatsu et al., 2013).

3.4. Genetic and antigenic analysis of H9 viruses

The HA genes of H9 isolates were phylogenetically divided into two lineages: Eurasian and North American lineages. All H9 viruses isolated at LBMs in Thua Thien Hue province in 2014 were classified as Y280 sublineage of the Eurasian lineage (Okamatsu et al., 2013; Nomura et al., 2012). These viruses were genetically related to the A/chicken/Vietnam/OIE-1611/2012 (H9N2) virus isolated in the North Vietnam in 2012 and other viruses isolated from poultry in China in 1997 and 2012, which were also classified into the Y280 sublineage (Fig. 3).

Representative strains of the H9 isolates were antigenically analyzed by cross HI test (Table 5). All viruses from Vietnam reacted with the antiserum of A/duck/Hong Kong/Y280/1997 (H9N2) virus, which belonged to the Y280 sublineage. These Vietnamese H9N2 viruses reacted weakly with antisera of other H9N2 viruses classified into different sublineages, such as the G1 or North American lineages, and reacted moderately with antisera to Y349 sublineage virus. This suggests that the antigenicities of the H9N2 viruses isolated in Vietnam have been stable in the poultry population.

3.5. The neuraminidase genes of AI viruses isolated in LBMs
For neuraminidase (NA) gene segments, the names of groups were defined based on previous studies (Kim et al., 2013; Okamatsu et al., 2013). The N2 NA genes of H6 and H9 AI viruses were phylogenetically categorized into two groups, Group II and Y280 (Supplemental Fig. 2a). All N6 NA genes of the H5 viruses belonged to Group II and of H6 viruses belonged to Group I (Supplemental Fig. 2b).

3.6. Genetic diversity of AI viruses isolated in LBMs

The six internal gene segments of the AI viruses were then phylogenetically analyzed to investigate the genetic diversity of AI viruses circulating in Vietnamese LBMs (Supplemental Fig. 3). For each internal gene segment, the names of groups were defined as previously described (Kim et al., 2013; Okamatsu et al., 2013). In this study, the six internal gene segments were also classified into H6 Group I, H6 Group II, H6 Group III, JX8264 like, and Gs/Gd like, indicating that these viruses were closely related to the viruses isolated in China. For instance, the PB2 and PA genes of the A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6) were classified as JX8264 like. The NP, M, and NS genes of this H5N6 virus were grouped into Gs/Gd like and H6 Group I where Chinese viruses were also grouped into. Phylogenetic analysis also indicated that the PB2, PB1, PA, and NS internal genes of H9N2 viruses, A/chicken/Vietnam/HU1-1050/2014 and A/chicken/Vietnam/HU1-976/2014, were classified into the same groups: Vietnam II, Vietnam, and JX8286 like. However, other gene segments, NP and M genes, were classified into different groups (Supplemental Fig. 3).
3.7. Knowledge, attitude, and practices

A total of 52 sellers working at LBMs were interviewed during the first sampling collection, 29 sellers working at intervention LBMs and 23 working at non-intervention LBMs. All respondents working at intervention LBMs declared that their shops were cleaned by chemical disinfection twice per day before the LBM was opened and after closing their business. In contrast, only 17.4% of respondents working at non-intervention LBMs made this claim. In intervention LBMs, 52.0% of sellers acquired their poultry from within Thua Thien Hue province, within a 5 km radius. The other 48.0% of sellers imported their poultry from neighboring provinces, from within a radius of 50 km. In contrast, 100% of the sellers working at non-intervention LBMs sell poultry that originated from the local areas surrounding the LBMs.

4. Discussion

LBMs provide an ideal environment for genetic reassortment events and interspecies transmission of AI viruses (He et al., 2014; Yu et al., 2014; Okamatsu et al., 2013; Wan et al., 2011). In this study, 178 AI viruses were isolated from poultry in both intervention and non-intervention LBMs. The H5N6 viruses were isolated from apparently healthy domestic ducks, muscovy ducks, and an environmental sample at an intervention LBM, indicating that subclinical HPAI virus infections are endemic in domestic birds at LBMs. These results remind us that LBMs play an important role as a hotspot for the transmission of AI viruses, including HPAI viruses, in Vietnam. Although closure of LBMs or banning the storage of poultry overnight at the markets represents a
highly effective method to reduce amplification and persistence of viruses in LBMs (He et al., 2014; Yu et al., 2014; Leung et al., 2012; Fournié et al., 2011), it is hard to change the LBMs system in Vietnam due to well-established traditional cultures and the behavior of customers. Therefore, biosecurity measures were developed with the intention of reducing AI transmission. In this study, we conducted surveillance of AI viruses in both intervention and non-intervention LBMs in a central province of Vietnam. We sampled viruses on two occasions and found the AI virus prevalence at intervention LBMs to be 6.1%. The prevalence of AI viruses at non-intervention LBMs did not differ significantly from that at non-intervention LBMs, which was 5.6%. All the viruses identified in this study were isolated from apparent healthy LBM poultry. In addition, various subtypes of AI viruses were identified at intervention LBMs. The H6 and H9 viruses isolated in intervention LBMs were genetically similar to H6 and H9 viruses isolated at non-intervention LBMs. Although disinfection was reported to be performed twice per day at intervention LBMs, AI viruses still contaminated the environment. These results indicate that the current intervention LBMs remain several limitations for the control of AI contamination such as problems of periodic disinfection procedure and concentration of chemical disinfection. The intervention LBMs are generally larger capability to hold poultry than these of non-intervention LBMs meaning that intervention LBMs can contain poultry originating from many different sources, including neighboring provinces. In addition, KAP surveys indicated that the sources of poultry in intervention LBMs in Thua Thien Hue province are widely distributed from local area to other provinces. Therefore, the source of poultry may play an important role in AI transmission at LBMs,
and this factor should be further studied. As a next step, a risk analysis will be conducted to identify
the risk factors contributing to the appearance of various subtypes of AI viruses at intervention
LBMs.

The H5N1 HPAI viruses were first detected in Vietnam in 2001, and outbreaks have been
regularly reported since the end of 2003 (Minh et al., 2009). The H5 viruses circulating in Vietnam
were classified into seven major genetic clades: 1.1, 2.3.2.1, 2.3.4.1, 2.3.4.2, 2.3.4.3, 7.1, and 7.2
(Nguyen et al., 2014; Okamatsu et al., 2013; Nguyen et al., 2012). In this study, we report for the
first time the characterization of H5N6 viruses isolated in an intervention LBM in a center province
of Vietnam. The HA genes of the H5N6 viruses isolated from poultry and an environmental sample
at an intervention LBM in Thua Thien Hue province were classified as clade 2.3.4.4. Genetic
analysis indicated that these H5N6 viruses were closely related to an H5N6 virus isolated in
Zenjiang, China and differed slightly from the H5N6 viruses isolated in Laos, indicating an existing
genetic diversity within this new clade 2.3.4.4 (Food and Agriculture Organization of the United
Nations. Avian influenza A (H5N6): the latest addition to emerging zoonotic avian influenza threats
in East and Southeast Asia, 2014; www.fao.org/ag/empres.html). The H5 viruses found in northern
Vietnam may have been introduced from China. In April, 2014, the first outbreak of H5N6 was
found in a flock of chickens in Lang Son province, located near the border with China. Then
another outbreak was reported in a flock of ducks in Ha Tinh province, located in central part of
Vietnam and China (World Animal Health Information Database Interface, Disease Information,
Vietnam and China, AI vaccines are used to control H5 HPAI virus infections in poultry (Hu et al., 2015; Le et al., 2014). However, immunological selection pressure has driven development of antigenic variants of H5 HPAI viruses (Grund et al., 2011; Lee et al., 2004). The H5N6 HPAI viruses we identified were also antigenically distinct from the clade 2.3.4 virus used in the Re-5 vaccine, which has been applied in China and Vietnam (Hu et al., 2015; Le et al., 2014). Recently, several human H5N6 AI virus infections were reported, and avian-originated H5N6 viruses were also isolated from healthy pigs in China, although the H5N6 AI virus has not adapted to the swine population yet (Pan et al., 2016; Li et al., 2015). In Thua Thien Hue province, Vietnam, piglets, and poultry are often housed together in non-intervention LBMs. This LBM system may facilitate influenza virus reassortment and transmission of influenza viruses between poultry and from poultry to pigs or humans. Therefore, it will be important to monitor swine influenza, and strict controls should be applied to limit interspecies transmission of influenza viruses.

H6 and H9 AI viruses are widely distributed among poultry and wild birds in Asia (Huang et al., 2010; Moon et al., 2010). H9N2 viruses have become the most prevalent subtype detected in poultry populations in China since 2004 (Choi et al., 2004) and in Vietnam since 2009 (Okamatsu et al., 2013; Nomura et al., 2012). In this study, H6 and H9 viruses isolated from domestic ducks, chickens, and environment samples were genetically identical in both types of LBMs in Thua Thien Hue province, Vietnam. These viruses were phylogenetically closely related to viruses previously isolated in poultry in China. The HA genes of H9 viruses did not differ from those previously isolated in 2011 and 2012, indicating that these viruses were maintained in poultry in Vietnam.
Antigenic analysis of H6 viruses indicated that all the Vietnamese H6 viruses we isolated in 2014 exhibited low cross reactivity with antiserum of A/duck/Vietnam/OIE-4429/2010 (H6N2), another Group II virus. In addition, genetic analysis also indicated that the H6 viruses isolated in this study differed from other Vietnamese H6 viruses isolated in our previous study (Okamatsu et al., 2013). These results suggest that the antigenicity of these Vietnamese H6 viruses isolated in 2014 differed from those isolated in Vietnam between 2010 and 2012. Perhaps the H6 viruses circulating in Vietnam in 2014 exhibited altered antigenicity as a result of repetitive infections of the poultry population. Further studies should be conducted to characterize the antigenic variation of H6 viruses circulating in Vietnam.

In Vietnam, domestic birds are mainly raised in households in a free-range manner and are transported to LBMIs by their owners or poultry sellers. AI viruses are transmitted and spread within the poultry population. Although domestic birds are vaccinated against H5N1 in Vietnam (Le et al., 2014; Soares et al., 2010; Minh et al., 2009), HPAI viruses have silently spread in the poultry population. Our study provides initial evidences for the improvement of intervention strategies in study LBMIs. The intervention with supporting a good infrastructure as “a good hardware” need to comprise well with “a good software” like increasing of education level of people involved in poultry trade and improvement of hygiene procedures by local authority at LBMIs. Active surveillance program of AI and hygiene practice performance monitoring in LBMIs will be essential to eradicate HPAI from Vietnam as well as Asian countries.
Conflict of interest

None.

Acknowledgments

We thank Dr. T. Saito, National Institute of Animal Health, Japan for kindly providing viruses A/chicken/Ibaraki/1/2005 (H5N2), A/chicken/Yamaguchi/7/2004 (H5N1), and A/chicken/Kumamoto/1-7/2014 (H5N8). We also thank Dr. Luk S.M. Geraldine of the Tai Lung Veterinary Laboratory, Hong Kong SAR, China for providing A/peregrine falcon/Hong Kong/810/2009 (H5N1) virus. This research was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI (grant number 16J06369 to D-H. Chu) by the Program for Leading Graduate Schools from JSPS (grant number F01) and was partially funded by Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from Japan Agency for Medical Research and Development (AMED). D-H. Chu is supported by JSPS Research Fellowships for young scientists.
References


World Animal Health Information Database (WAHID) Interface, Disease Information.


**Figure captions**

**Fig. 1.** Phylogenetic tree for the influenza virus H5 HA genes. Full-length HA genes of six H5 subtype viruses, and reference strains were analyzed using the maximum-likelihood method with MEGA 5.0 software and divided into two lineages: Eurasian and North American. The Eurasian H5 viruses were identified on the basis of the nomenclature defined by WHO/OIE/FAO H5N1 Evolution Working Group, 2014 (Smith et al., 2015). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray. The viruses isolated in our previous study are underlined. The black circle indicates the viruses isolated in an intervention LBM.

**Fig. 2.** Phylogenetic tree for the influenza virus H6 HA genes. Nucleotides 51–943 (893 bp) of the HA genes of four viruses of H6 subtype and reference strains were analyzed using the maximum-likelihood method with MEGA 5.0 software and divided into two lineages: Eurasian and North American. The Eurasian H6 viruses were clustered into five different sublineages: Group II, W312, Group III, Early, and Group I (Okamatsu et al., 2013). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray, and the representative of each sublineage
is indicated in bold. The virus isolated in a previous study is underlined. The viruses isolated at intervention LBMs are indicated by black circles, and the virus isolated at a non-intervention LBM is indicated by a white circle.

**Fig. 3.** Phylogenetic tree for the influenza virus H9 HA genes. Full-length HA genes of eight viruses of H9N2 subtype, and reference strains were analyzed using the maximum-likelihood method with MEGA 5.0 software and divided into two lineages: Eurasian and North American lineages. The Eurasian H9 viruses were clustered into three different sublineages: Y280, G1, and Y439 (Nomura et al., 2012; Okamatsu et al., 2013). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications.

The viruses isolated in this study are highlighted in gray, and the representative of each sublineage is indicated in bold. The virus isolated in a previous study is underlined. The viruses isolated in intervention LBMs are indicated by black circles, and the viruses isolated in non-intervention LBMs are indicated by white circles. The H9N2 subtype of each virus strain is omitted.
<table>
<thead>
<tr>
<th>Type of LBMs</th>
<th>Name of LBMs</th>
<th>Latitude/Longitude</th>
<th>Number of samples</th>
<th>Number of isolates</th>
<th>% Positive (95% CI)</th>
<th>Subtype of isolates (number of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intervention</td>
<td>An Lo</td>
<td>16.545962/107.452388</td>
<td>500</td>
<td>18</td>
<td>3.6 (2.3–5.6)</td>
<td>H3N2 (1) H4N6 (1) H6N6 (1) H9N2 (1) H9N6 (10)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>16.511793/107.600336</td>
<td>500</td>
<td>61</td>
<td>12.2 (9.6–15.4)</td>
<td>H3N2 (4) H3N6 (1) H5N6 (8) H6N2 (9) H6N6 (3) H9N2 (36)</td>
</tr>
<tr>
<td></td>
<td>Thuy Phuong</td>
<td>16.433257/107.635256</td>
<td>496</td>
<td>13</td>
<td>2.6 (1.5–4.4)</td>
<td>H3N2 (2) H4N6 (1) H6N2 (1) H6N6 (5) H9N2 (3) H11N6 (1)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>1,496</td>
<td>92</td>
<td>6.1 (5.0–7.5)</td>
<td></td>
</tr>
<tr>
<td>Non-intervention</td>
<td>Phu Bai</td>
<td>16.408326/107.677989</td>
<td>300</td>
<td>40</td>
<td>13.3 (9.9–17.6)</td>
<td>H6N2 (3) H6N6 (1) H9N2 (55) H11N7 (1)</td>
</tr>
<tr>
<td></td>
<td>Phu Da</td>
<td>16.434797/107.710014</td>
<td>200</td>
<td>1</td>
<td>0.5 (0.1–2.8)</td>
<td>H3N2 (1) H9N2 (1) H6N6 (5) H9N2 (3) H9N6 (3)</td>
</tr>
<tr>
<td></td>
<td>Thanh Phu</td>
<td>16.424635/107.656268</td>
<td>199</td>
<td>12</td>
<td>6.0 (3.5–10.2)</td>
<td>H3N2 (1) H9N2 (1) H6N6 (5)</td>
</tr>
<tr>
<td></td>
<td>Quang Phuoc</td>
<td>16.57431/107.51984</td>
<td>212</td>
<td>6</td>
<td>2.8 (1.3–6.0)</td>
<td>H9N2 (6) H9N2 (15)</td>
</tr>
<tr>
<td></td>
<td>Tay Ba</td>
<td>16.537018/107.560679</td>
<td>338</td>
<td>25</td>
<td>7.4 (5.1–10.7)</td>
<td>H3N2 (9) H9N2 (9) H6N6 (15)</td>
</tr>
<tr>
<td></td>
<td>Vinh Thanh</td>
<td>16.431796/107.783475</td>
<td>300</td>
<td>2</td>
<td>0.7 (0.2–2.4)</td>
<td>H6N6 (1) H9N2 (5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>1,549</td>
<td>86</td>
<td>5.6 (4.5–6.8)</td>
<td></td>
</tr>
</tbody>
</table>

CI, confidence interval
<table>
<thead>
<tr>
<th>HA subtypes</th>
<th>Type of LBMs</th>
<th>Name of LBMs</th>
<th>Isolates</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5</td>
<td>Intervention</td>
<td>A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6)</td>
<td>LC069835 LC069867 LC069898 LC041323 LC069953 LC041324 LC070009 LC070040</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-1151/2014 (H5N6)</td>
<td>LC041310 LC041311 LC041312 LC041313 LC086330 LC041314 LC041315 LC041316</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/environment/Vietnam/HU1-1434/2014 (H5N6)</td>
<td>LC069834 LC069866 LC069897 LC041321 LC069952 LC041322 LC070008 LC070039</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-1507/2014 (H5N6)</td>
<td>LC069832 LC069864 LC069895 LC041317 LC069950 LC041318 LC070006 LC070037</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-1511/2014 (H5N6)</td>
<td>LC069833 LC069865 LC069896 LC041319 LC069951 LC041320 LC070007 LC070038</td>
<td></td>
</tr>
<tr>
<td>H6</td>
<td>Intervention</td>
<td>An Lo</td>
<td>A/duck/Vietnam/HU1-1245/2014 (H6N2)</td>
<td>LC069813 LC069846 LC069877 LC069908 LC069932 LC069963 LC069988 LC070019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>A/environment/Vietnam/HU1-1423/2014 (H6N2)</td>
<td>LC069836 LC041334 LC041335 LC041336 LC041337 LC041338 LC041339 LC041340</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-intervention</td>
<td>Than Phu</td>
<td>A/duck/Vietnam/HU1-637/2014 (H6N6)</td>
</tr>
<tr>
<td>H9</td>
<td>Intervention</td>
<td>An Lo</td>
<td>A/chicken/Vietnam/HU1-3/2014 (H9N2)</td>
<td>LC069838 LC069869 LC069900 LC041350 LC069955 LC069980 LC070011 LC070042</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-225/2014 (H9N2)</td>
<td>LC069842 LC069873 LC069904 LC069928 LC069959 LC069984 LC070015 LC070046</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-intervention</td>
<td>Phu Bai</td>
<td>A/chicken/Vietnam/HU1-381/2014 (H9N2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Than Phu</td>
<td>A/duck/Vietnam/HU1-675/2014 (H9N2)</td>
<td>LC069820 LC069853 LC069884 LC069915 LC069939 LC069970 LC069995 LC070026</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quang Phuoc</td>
<td>A/chicken/Vietnam/HU1-786/2014 (H9N2)</td>
<td>LC069843 LC069874 LC069905 LC069929 LC069960 LC069985 LC070016 LC070047</td>
</tr>
<tr>
<td>Lineage</td>
<td>Viruses</td>
<td>Clade</td>
<td>Antiserum to</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
<td>-------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vac-3</td>
<td>Mon/05</td>
</tr>
<tr>
<td>Eurasia</td>
<td>A/duck/Hokkaido/Vac-3/2007 (H5N1)</td>
<td>–</td>
<td>10,240</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td>A/whooper swan/Mongolia/3/2005 (H5N1)</td>
<td>2.2</td>
<td>80</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>A/whooper swan/Hokkaido/1/2008 (H5N1)</td>
<td>2.3.2.1</td>
<td>80</td>
<td>1,280</td>
</tr>
<tr>
<td></td>
<td>A/peregrine falcon/Hong Kong/810/2009 (H5N1)</td>
<td>2.3.4</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>A/muscovy duck/Vietnam/HU1-1144/2014 (H5N6)</td>
<td>2.3.4.4</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>A/duck/Vietnam/HU1-1151/2014 (H5N6)</td>
<td>2.3.4.4</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>A/environment/Vietnam/HU1-1434/2014 (H5N6)</td>
<td>2.3.4.4</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>A/muscovy duck/Vietnam/HU2-26/2014 (H5N6)</td>
<td>2.3.4.4</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>A/chicken/Kumamoto/1-7/2014 (H5N8)</td>
<td>2.3.4.4</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>A/chicken/Yamaguchi/7/2004 (H5N1)</td>
<td>2.5</td>
<td>640</td>
<td>10,240</td>
</tr>
<tr>
<td>North America</td>
<td>A/chicken/Ibaraki/1/2005 (H5N2)</td>
<td>–</td>
<td>160</td>
<td>640</td>
</tr>
</tbody>
</table>

The H5 isolates identified in the present study are shown in bold. Vac-3, A/duck/Hokkaido/Vac-3/2007; Mon/05, A/whooper swan/Mongolia/3/2005; Hok/08, A/whooper swan/Hokkaido/1/2008; HK/09, A/peregrine falcon/Hong Kong/810/2009; Km/14, A/chicken/Kumamoto/1-7/2014; Yama/04, A/chicken/Yamaguchi/7/2004; Ibr/05, A/chicken/Ibaraki/1/2005

Homologous titers are underlined. 

"−" indicates that the virus does not belong to clade 0–9.
<table>
<thead>
<tr>
<th>Lineage</th>
<th>Sublineage</th>
<th>Viruses</th>
<th>Antiserum to HK/960/80</th>
<th>VN/OIE-4429/10</th>
<th>VN/HU1-637/14</th>
<th>Aus/1/72</th>
<th>Mas/3740/65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eurasia</td>
<td>Early</td>
<td>A/duck/Hong Kong/960/1980 (H6N2)</td>
<td>5,120</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>A/duck/Vietnam/OIE-4429/2010 (H6N2)</td>
<td>640</td>
<td>5,120</td>
<td>640</td>
<td>160</td>
<td>2,560</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-637/2014 (H6N6)</td>
<td>640</td>
<td>1,280</td>
<td>10,240</td>
<td>80</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-1245/2014 (H6N2)</td>
<td>160</td>
<td>160</td>
<td>2,560</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/environment/Vietnam/HU1-1423/2014(H6N2)</td>
<td>320</td>
<td>640</td>
<td>2,560</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/environment/Vietnam/HU1-1426/2014(H6N2)</td>
<td>320</td>
<td>640</td>
<td>2,560</td>
<td>80</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/shearwater/South Australia/1/72 (H6N5)</td>
<td>2,560</td>
<td>40</td>
<td>80</td>
<td>1,280</td>
<td>640</td>
</tr>
<tr>
<td>North American</td>
<td></td>
<td>A/turkey/Masachusetts/3740/65 (H6N2)</td>
<td>2,560</td>
<td>1,280</td>
<td>40</td>
<td>1,280</td>
<td>5,120</td>
</tr>
</tbody>
</table>

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

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

Homologous titers are underlined.

"--" indicates that the virus does not belong to any lineage.
Table 5 Cross-reactivity of H9N2 influenza viruses with antisera by HI test

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Sublinage</th>
<th>Viruses</th>
<th>Antiserum to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HK/Y280/97</td>
</tr>
<tr>
<td>Eurasia</td>
<td>Y280</td>
<td>A/duck/Hong Kong/Y280/1997</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/chicken/Vietnam/HU1-3/2014</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-225/2014</td>
<td>2,560</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/chicken/Vietnam/HU1-381/2014</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/chicken/Vietnam/HU1-786/2014</td>
<td>20,480</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/chicken/Vietnam/HU1-1286/2014</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/environment/Vietnam/HU1-1424/2014</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Vietnam/HU1-1512/2014</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>A/quail/Hong Kong/G1/1997</td>
<td>1,280</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/duck/Hokkaido/49/1998</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>Y439</td>
<td>A/duck/Hokkaido/49/1998</td>
<td>640</td>
</tr>
<tr>
<td>North America</td>
<td>−</td>
<td>A/turkey/Wisconsin/1/1966</td>
<td>80</td>
</tr>
</tbody>
</table>

The H9 isolates identified in the present study are shown in bold.
HK/Y280/97, A/duck/Hong Kong/Y280/1997; HK/G1/97, A/quail/Hong Kong/G1/1997; Hok/49, A/duck/Hokkaido/49/1998; Wis/66, A/turkey/Wisconsin/1/1966
Homologous titers are underlined.
"−" indicates that the virus does not belong to any sublinage.
Fig. 1. Chu et al.

- Duck/Vietnam/HU1-1152/2014 (H5N6)
- Duck/Nha Trang/75c131/2014 (H5N6)
- Duck/Vietnam/HU1-1151/2014 (H5N6)
- Duck/Vietnam/HU1-1507/2014 (H5N6)
- Environment/Vietnam/HU1-1434/2014 (H5N6)
- Muscovy duck/Vietnam/HU1-1144/2014 (H5N6)
- Duck/Vietnam/HU1-1511/2014 (H5N6)
- Environment/Zhenjiang/C13/2013 (H5N6)
- Mallard duck/Shanghai/SH-9/2013 (H5N8)
- Duck/Shandong/Q1/2013 (H5N8)
- Duck/Laos/LPQ002/2014 (H5N6)
- Chicken/Laos/LPQ001/2014 (H5N6)
- Common moorhen/Vietnam/WBT226/2014 (H5N6)
- Black crowned night heron/Vietnam/WBT198/2014 (H5N6)
- Duck/Guangdong/GD01/2014 (H5N6)
- Muscovy duck/Vietnam/LBM631/2014 (H5N1)
- Duck/Beijing/CT01/2014 (H5N8)
- Mallard/Korea/W452/2014 (H5N8)
- Chicken/Kumamoto/1-7/2014 (H5N8)
- Turkey/Germany MV/R2472/2014 (H5N8)
- Duck/England/36254/2014 (H5N8)
- Chicken/Netherlands/14015526/2014 (H5N8)
- Peregrine falcon/Hong Kong/810/2009 (H5N1)
- Hunan/1/2009 (H5N1)
- Vietnam/HN31432M/2008 (H5N1)
- Anhui/1/2005 (H5N1)
- Chicken/Vietnam/NCVD-279/2009 (H5N1)
- Chicken/Vietnam/NCVD-20/2007 (H5N1)
- Whooper swan/Hokkaido/4/2011 (H5N1)
- Duck/Vietnam/OIE-2202/2012 (H5N1)
- Muscovy duck/Vietnam/OIE-3314/2011 (H5N1)
- Duck/Bac Lieu/07-06/2007 (H5N1)
- Goose/Guangdong/1/1996 (H5N1)
- Hong Kong/156/1997 (H5N1)
- Mallard/Hokkaido/24/2009 (H5N1)
- Duck/Hokkaido/Vac-1/2004 (H5N1)
- Duck/Hokkaido/Vac-3/2007 (H5N1)
- Duck/Pennsylvania/10218/1984 (H5N2)
- Chicken/Penghu/1103/2012 (H5N2)
- Chicken/Chunghua/0101/2012 (H5N2)
Fig. 2. Chu et al.