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Virological and epidemiological studies for the control of highly pathogenic avian influenza

高病原性鳥インフルエンザの制御に関する ウイルス学的及び疫学的研究

Nguyen Thanh Lam

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Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AIV(s)	avian influenza virus(es)
AMED	Japan Agency for Medical Research and Development
BEAST	Bayesian evolution analysis sampling trees
BSL	biosafety level
CAR	conditional autoregressive
CrI	credible intervals
DAH	Department of Animal Health, Vietnam
dpc	days post challenge
EID_{50}	50% egg infectious dose
FAO	The Food and Agriculture Organization of the United Nations
GISAID	Global Initiative on Sharing All Influenza Data
GSO	General Statistics Office of Vietnam
Gs/GD	A/Goose/Guangdong/1/1996 (H5N1)
GTR	general time reversible
HA	hemagglutinin
HI	hemagglutination inhibition
HPAI	highly pathogenic avian influenza
HPAIV(s)	highly pathogenic avian influenza virus(es)
IAV(s)	influenza A virus(es)
IC	immunochromatographic diagnosis
IRD	Influenza Research Database
IVPI	intravenous pathogenicity index
J-GRID	Japan Initiative for Global Research Network on Infectious Diseases
KAP	knowledge, attitude and practices
LBM(s)	live bird market(s)
LPAIV(s)	low pathogenic avian influenza virus(es)
MAb(s)	monoclonal antibody(es)
MAFF	Ministry of Agriculture, Forestry and Fisheries of Japan
MCMC	Markov chain Monte Carlo
MDCK	Madin–Darby canine kidney
MEM	minimum essential medium
mL	milliliter

mg	milligram
µl	microliter
ML	maximum likelihood
NA	neuraminidase
NCVD	National Center for Veterinary Diagnostics, Vietnam
NI	neuraminidase inhibition
NP	nucleoprotein
NT	neutralization
OIE	The World Organization for Animal Health
PA	polymerase acidic
PB1	polymerase basic 1
PB2	polymerase basic 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	Protein Data Bank
PFU	plaque forming unit
RAxML	randomized axelerated maximum likelihood
RNP	ribonucleoprotein
rpm	rounds per minute
RR	relative risk
RT-PCR	reverse transcription polymerase chain reaction
SDAH	Sub-Department of Animal Health, Vietnam
SD	standard deviation
SMR(s)	standardized mortality ratio(s)
SNP(s)	single-nucleotide polymorphism(s)
$TCID_{50}$	50% tissue culture infectious dose
ZIP	zero inflated Poisson
WHO	The World Health Organization

Notes

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Preface

Influenza viruses belong of the *Orthomyxoviridae* family which are enveloped, segmented, negative single-stranded RNA genome viruses. At present, the *Orthomyxoviridae* family consists of 7 genera: *Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus* and *Deltainfluenzavirus* (commonly known as influenza types A, B, C and D), *Thogotovirus*, *Isavirus* and *Quaranjavirus* (King et al., 2018). Only *Influenza A virus* species of the *Alphainfluenzavirus* genus can infect human, mammalian and avian species and cause cross-transmission between these species (Webster, 1972; Kida, 2008; Long et al., 2018). Influenza A virus (IAV) particles have a size range of 80–120 nm in diameter and a simple structure which includes envelope, capsid and a core containing genetic material (viral genome). The lipid envelope derived from the host cell is comprised by three integral membrane proteins: hemagglutinin (HA), neuraminidase (NA) and matrix 2 protein (M2). The matrix 1 (M1) protein forms a layer beneath the envelope and under the M1 layer is the core of the virus particle which is made of the ribonucleoprotein (RNP) complex. The RNP complex consists of the viral RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase proteins of 'polymerase basic 1 and 2' and 'polymerase acidic' subunits (PB1, PB2 and PA). The IAV genome comprises eight single-stranded RNA gene segments that typically encode for 10 or 11 viral proteins (Palese, 1977; Long et al., 2018). The HA is the major membrane glycoprotein of IAVs that has at least two functions: it recognizes sialic acid-containing receptors on the cell surface and mediates fusion of the viral envelope with the endosomal membrane of host cells, leading to the release of the nucleocapsid into the cytoplasm (Webster and Rott, 1987; Long et al., 2018). The HA might also have a role for virus budding and particle formation. Further, HA is also the primary target for humoral immune response in infected hosts to IAV infection (Yewdell et al., 1979).

IAVs are further classified into different subtypes based on antigenic relationships of the surface glycoproteins of the HA and NA. To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) have been recognized (Yewdell et al., 1979; Webster et al., 1992; Long et al., 2018). All subtypes have been detected in wild aquatic birds except for the recently discovered H17N10 and H18N11 viruses of which genetic materials were only detected in bats (Tong et al., 2013). It is well known that simultaneous infection of a single cell by two different IAVs can lead to gene exchanges so called genetic reassortment which generates a novel influenza virus. It is believed that most human pandemic IAVs arose in this manner (Webster et al., 1982; Long et al., 2018).

To initiate infection and replication, IAVs bind to neuraminic acids (sialic acids) on the surface of host cells. On the basis of binding specificity of HA protein to the sialylated glycan receptors, IAVs exhibit host restriction in different species. Avian influenza viruses (AIVs) were found to have a binding preference for sialic acids that are linked to the galactose in an α2,3 linkage. In contrast, human influenza viruses (commonly in H1, H2 and H3 subtype viruses) preferentially bind to sialic acids that are linked to galactose in an α2,6 linkage. Swine influenza viruses are able to bind both α2,3- and α2,6- sialic acids. These differences in receptor binding specificity are important determinants of virus host range and tissue tropism of IAVs (Rogers et al., 1983; Ito and Kawaoka, 2000; de Graaf and Fouchier, 2014; Long et al., 2018).

The evolution of IAVs is defined by their constant antigenic variation to escape the host immune response via two distinct mechanisms of variability in the surface HA and NA glycoproteins – antigenic shift and antigenic drift (Yewdell et al., 1979; Webster et al., 1982). Antigenic shift is a sporadic event caused by a genetic reassortment which generates a new combination of HA and NA subtypes. The new subtype virus might have distinct antigenicity in which population has no preexisting immunity. Antigenic drift is a continuous process that occurs as results from the accumulation of point mutations in

the viral HA and NA, which is driven by antibody-mediated selective pressure and a high rate of viral mutations due to the absence of proofreading ability of the viral RNA-dependent RNA polymerase. Antigenic drift confers IAVs an ability to escape from host immunity induced by previous infection or vaccination and it is major cause of annual influenza epidemics. In the laboratory, antigenic drift can be mimicked by virus propagation in the presence of polyclonal immune antisera or monoclonal antibodies targeting a single site (Yewdell et al., 1979).

Natural reservoirs of IAVs are the orders waterfowl species such as *Anseriformes*: ducks, geese, swans and *Charadriiformes*: gulls, terns, shorebirds (Webster, 1972; Kida, 2008; Long et al., 2018). IAV infection in these species typically causes no or mild diseases; however, phenotypic infection of IAVs in terrestrial poultry is highly variable. Based on pathogenicity in chickens, IAVs are classified into two pathotypes: highly pathogenic avian influenza viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) (Alexander, 2000). LPAIVs cause mild respiratory disease, depression and/or a decrease in egg production but sometimes can result in morbidity or mortality. On the other hand, infection with HPAIVs, caused by some IAVs of H5 and H7 subtypes, results in at least 75% mortality in the infected poultry. The World Organization for Animal Health (OIE) defines an AIV to be HPAIV under one of the following criteria: *(i)* if it is lethal for six, seven, or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infectious allantoic fluid or *(ii)* if it has an intravenous pathogenicity index (IVPI, the mean clinical score of ten 6-week-old chickens intravenously infected) greater than 1.2 or *(iii)* all H5 and H7 LPAIVs possessing a multibasic sequence at the HA cleavage site are also considered highly pathogenic (OIE, 2015).

One of the most high risk HPAIVs are Asian-origin H5Nx viruses descended from A/goose/Guangdong/1/1996 (H5N1) (Gs/GD) which was first isolated from sick

domestic geese in 1996 in Guangdong province, China. In 1997, outbreaks of H5N1 HPAI occurred in poultry with high mortality rates (70–100%) in Hong Kong. These viruses were identified as H5N1 reassortant viruses that had acquired its HA gene from the Gs/GD-lineage virus with a multibasic sequence at the cleavage site, its NA gene from another AIV, and its remaining six internal genes from other AIVs (Xu et al., 1999). Until late 2003, outbreaks of H5 HPAIVs were reported concurrently in eight countries in South East Asia including China, Cambodia, Indonesia, Japan, Korea, Laos PDR, Thailand and Vietnam and then rapidly disseminated over large portions of Asia, Europe and Africa (Sims et al., 2005). In addition, H5N1 HPAIVs of the Gs/GD-lineage imposed pandemic concerns since several fatal human cases caused by H5N1 HPAIVs were also reported in association with poultry outbreak occurrence.

Although, almost all of worldwide circulating H5 HPAIVs evolved from only the Gs/GD strain, reassortment and antigenic drift have created a large genetic and antigenic diversification of the lineage. Reassortment between H5 HPAIVs with other AIVs has led to replacement of most internal genes of the original H5N1 virus but the H5 HA has retained in all isolates and showed large variability (WHO/OIE/FAO H5N1 Evolution Working Group, 2014). Owing to the large genetic diversity of H5 HA, the standard nomenclature of H5 phylogenetic clade of H5 HAs was developed, first adopted in 2008, based on the evolution and divergence of H5 HPAIVs that evolved from the original HA gene of the Gs/GD lineage. At first, from 1996 to 2008, 10 distinct clades (0–9) had been recognized (WHO/OIE/FAO H5N1 Evolution Working Group, 2008); however currently, more than 40 distinct clades and subclades of the H5 HA gene have been identified (Smith et al., 2015).

For the control of HPAI in poultry, traditional strategies have comprised four basic components: *(i)* education, *(ii)* biosecurity, *(iii)* diagnostics and surveillance, and *(iv)* elimination of infected poultry through depopulation (Swayne and Suarez, 2000). The

fifth component, mass poultry vaccination, was recently admitted to increase host resistance and reduce virus load shed into the environment, thereby to minimize further spread in poultry and likelihood of zoonotic potentials. Therefore, eradication of HPAI can be achieved through the synergized strategy of these components (Swayne et al., 2000; Sims et al., 2016).

Due to serious damage and zoonotic concerns of Gs/GD-lineage H5 HPAIVs, mass vaccination policy has been implemented in several countries including China, Hong Kong, Vietnam, Indonesia and Egypt (Swayne et al., 2000). At first, vaccination in some countries seemed successful to reduce H5 HPAI incidence as China, Hong Kong, and Vietnam but outbreaks are still occurring and viruses have persisted in poultry (Swayne, 2012; Sims et al., 2016), meaning that vaccines and vaccination have some limitations for control and prevention of HPAI. Vaccination failure might result from multiple factors. One of the notorious factors is the emergence of antigenic variants of H5 HPAIVs under immune pressure from vaccination or natural exposure (Swayne et al., 2000). It has been assumed that vaccination might contribute to the rapid evolution and antigenic change of HPAIVs, generating antigenically drifted viruses to escape from vaccine-mediated protection (Cattoli et al., 2011b; WHO, 2018a). Several studies have reported that antigenically drifted H5 HPAIV mutants were selected and able to escape from neutralizing activities of monoclonal and/or polyclonal antibodies *in vitro* condition (Kaverin et al., 2002; Salzberg et al., 2007; Sitaras et al., 2014; Nguyen et al., 2018). Thus, the present study aimed to demonstrate the adverse effects of vaccination using an *in vivo* setting. Results of selection of antigenic variants under immune pressure from chicken vaccination are described in Chapter I. On the basis of Chapter I, this thesis was extended for the control of H5 HPAIVs with antigenic variation in the field through development of rapid diagnostic kit in Chapter II and monitoring virus evolution in field setting in Chapter III.

Emergence of antigenic variants triggers not only a failure of vaccination, but also a failure of diagnostic efficacy. For instance, in response of widespread H5N1 HPAIVs, an immunochromatographic diagnosis (IC) kit, Linjudge Flu A/H5, was previously developed in our laboratory as a rapid method to detect H5 AIVs. The original kit was manufactured by a single monoclonal antibody (MAb) recognizing an H5 LPAIV (Tsuda et al., 2007). Initially, the Linjudge Flu A/H5 showed its efficacy to detect H5 AIVs. However, the kit was found to have lower sensitivity to H5 HPAIVs isolated in recent years due to their antigenic variation. In the present study, a new advanced H5 IC kit, New Linjudge Flu A/H5, was established by a combination of two anti-H5 HA MAbs, a novel MAb produced for a clade 2.3.4.4 H5 HPAIV and the original MAb. In Chapter II, diagnostic efficacy and applicability of the new H5 IC kit is demonstrated. Therefore, this kit shows suitability for the control of diverse H5 HPAIVs in the field as described in Chapter III.

For the field study, Vietnam, one of the most affected countries of GS/GD-lineage H5 HPAIVs since 2003, was selected as a site of the field study to monitor and control of H5 HPAIV divergence. Unless intensive control measures have been implemented including mass poultry vaccination since mid-2005, H5 HPAIVs have persisted and caused thousands of outbreaks in poultry in Vietnam (Pfeiffer et al., 2007; Sims et al., 2016). H5 HPAIVs in Vietnam also exhibited a large genetic and antigenic divergence with existence of multiple clade/subclade variants of H5 HPAIVs. Intensive active surveillance of AIVs in Vietnam has been routinely conducted by our OIE reference laboratory for AIVs in Japan following previous studies (Nomura et al., 2012; Okamatsu et al., 2013; Chu et al., 2016). In the present study, our longitudinal surveillance program was routinely performed in geographically representative provinces in Vietnam to investigate evolutionary and epidemiological dynamics of recent H5 HPAIVs. Results of virological and epidemiological characterizations of currently circulating H5 HPAIVs

isolated in Vietnam are shown in Chapter III. In addition to the surveillance program, several epidemiological studies have been carried out to investigate risk factors for persistence of AIVs in Vietnam. Recently, Chu D.H *et al.* (2017) applied a logistic regression model and identified several knowledge, attitude and practice of sellers might be associated with positivity of AIVs including H5 HPAIVs in live birds markets (LBMs) in Vietnam. Therefore, the present epidemiological study was performed to further identify other risk factors for H5 HPAI outbreak occurrence towards better surveillance and control efforts. Results of the epidemiological study are shown in Chapter IV. Findings of this study will additionally fill gap in epidemiological knowledge of H5 HPAIV persistence in the field and contribute to provisional control measures against HPAI.

Chapter I

Selection of antigenic variants of an H5N1 highly pathogenic avian influenza virus in vaccinated chickens

Introduction

In 1996, H5N1 HPAIVs were first detected from a goose population in Guangdong, China (Xu et al., 1999). After its reemergence in 2003, the virus has subsequently caused devastating mortality in domestic poultry and spread rapidly worldwide (OIE, 2018). The continuous circulation of H5 HPAIVs in a variety of avian species and wild birds has resulted in considerable genetic diversity represented by multiple phylogenetic lineages of H5 HA gene, classified as 10 distinct clades (0–9) and sub-clades (WHO/OIE/FAO H5N1 Evolution Working Group, 2014; Smith et al., 2015). In addition to the genetic diversity, H5 HPAIVs have exhibited large antigenic divergence through the variability of the HA protein to escape from host immunity so-called antigenic drift (Yewdell et al., 1979; Webster et al., 1982). Antigenic drift mainly occurs by amino acid mutation(s) on the surface glycoprotein HA or NA, but the NA is usually less taken into account (Yewdell et al., 1979; Webster et al., 1982; Wilson and Cox, 1990; Long et al., 2018). Large antigenic variation have been well documented in human IAVs and human vaccine strains must be frequently updated for antigenic match with circulating viruses to ensure sufficient protection (Koel et al., 2013; Li et al., 2016). Owing to the antigenic and genetic diversity of the H5 HPAIVs, several vaccine candidates have been developed and routinely changed to match the circulating strains for poultry vaccination and preparedness for a potential human pandemic (Chen, 2009; Swayne, 2012; Zeng et al., 2016; WHO, 2018a; Wu et al., 2019).

The spread of Gs/GD-lineage H5 HPAIVs with their severe consequences has necessitated the implementation of vaccination campaigns in domestic poultry to control H5 HPAI in several countries including China, Vietnam, Indonesia and Egypt (Swayne, 2012; Zeng et al., 2016). Despite intensive vaccination efforts, infections caused by H5 HPAIVs have still become endemic in poultry in these countries and have spread

worldwide via wild bird migrations (Lycett et al., 2016). Multiple empirical studies have documented that antigenically drifted H5 HPAIVs have continuously been selected through poultry herd immunity, either in defined case reports or large regions where mass vaccination is administered (Kilany et al., 2010; Cattoli et al., 2011a; Ma et al., 2014; Swayne et al., 2015; Cuong et al., 2016; Nguyen et al., 2016). Indeed, escape mutants that were antigenically distinct from the original H5 HPAIVs were positively selected to evade neutralizing antibodies *in vitro* experiments (Kaverin et al., 2002; Salzberg et al., 2007; Sitaras et al., 2014; Nguyen et al., 2018). Furthermore, it has been experimentally demonstrated that H5 HPAIVs antigenically distinct from vaccine strains had a selective advantage for replication in vaccinated animals; consequently, antigenic variants seemed to be selected (Connie Leung et al., 2013; Herve et al., 2015; Sitaras et al., 2016a; Peeters et al., 2017; Salaheldin et al., 2017). However, these studies did not provide direct evidence for impacts of vaccination on the selection of antigenic variants in poultry.

The present study aims to elucidate whether vaccination in chickens accelerates the selection of antigenically drifted variants and its impacts on driving the antigenic evolution of H5 HPAIVs in an *in vivo* setting.

Materials and Methods

Viruses and cells

An H5N1 HPAIV genetically belonging to the clade 2.3.2.1c, A/whooper swan/Hokkaido/4/2011 (H5N1) (Ws/Hok/11), isolated from a dead whooper swan in Hokkaido, Japan (Sakoda et al., 2012) was used. The virus was propagated in 10-day-old embryonated chicken eggs at 35°C for 30–48 h and infectious allantoic fluids were stored at −80°C until use. Madin–Darby canine kidney (MDCK) cells maintained in minimum essential medium (MEM), supplemented with 0.3 mg/mL L-glutamine, 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 8 mg/mL gentamicin and 10% calf serum, were used for virus titration and plaque cloning.

Vaccine preparation and vaccination of chickens

Infectious allantoic fluids of Ws/Hok/11 were inactivated with 0.1% formalin for vaccine preparation. White-leghorn chickens hatched and raised in our laboratory were used. Five hundred microliters of diluted inactivated vaccine containing 16 hemagglutination units (HAU) were intramuscularly injected without adjuvant into the 4–10-week-old naïve chickens. The vaccines were not formulated with adjuvant for the immunization in order to prime improper vaccination-induced immunity in chickens. Three weeks after the immunization, sera of the vaccinated and non-vaccinated chickens were collected to measure antibody titers by the hemagglutination inhibition (HI) test.

Ethics statements

All of the animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 15-0063) and all experiments were performed according to the

guidelines of the committee. All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The Graduate School of Veterinary Medicine, Hokkaido University has accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since 2007.

Consecutive passages of Ws/Hok/11 in chickens

Two serial consecutive passage studies, study-I and study-II, were carried out using vaccinated and non-vaccinated chickens. In study-I, three chickens in each of the vaccinated and non-vaccinated groups were intranasally challenged with 200 µl of untitrated infectious allantoic fluids containing the wild-type Ws/Hok/11 at the first passage. Challenge titers in each passage were indicated in Table 1 and Table 4. All chickens were monitored for clinical signs for 7 days post-challenge (dpc). Clinical signs of chickens were numbered as '0–no clinical sign', '1–sick', '2–severely sick' and '3–dead' according to the intravenous pathogenicity index test (OIE, 2015). To examine virus shedding, oropharyngeal and cloacal swab samples of the infected chickens were collected daily and suspended in virus transport medium as described previously (Chu et al., 2016). The suspension was inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs for virus isolation. After 30–48 h incubation at 35°C, allantoic fluids showing hemagglutination activity were collected and stored at −80°C. The last virus isolated from cloacal swabs in each vaccinated and non-vaccinated group during the virus shedding period was used for the next challenge, resulting in a consecutive passage. Individual chicken in each group was housed in a self-contained isolator unit at a biosafety level (BSL)-3 facility at the Faculty of Veterinary Medicine, Hokkaido University, Japan.

To further validate the selection of antigenic variants from vaccination after updating the vaccine strain, another consecutive passage study was subsequently performed, study-II. A vaccine strain containing 16 HAU was prepared from the virus isolated from the ninth passage in the vaccinated group in study-I, named Ws/Hok/11-VacP9 and used to immunize chickens. The vaccinated chickens were consecutively challenged with the homologous virus of Ws/Hok/11-VacP9 at the first passage with the same protocols as in study-I.

Antiserum preparation and antigenic analysis

Antisera from chickens immunized with a single dose of inactivated wild-type Ws/Hok/11 and its antigenically drifted viruses isolated from the last consecutive passages in study-I and study-II were used for the antigenic analysis. To produce the antisera, naïve chickens were intramuscularly immunized with 500 µl of non-adjuvanted vaccine containing 256 HAU of inactivated virus. Twenty-one days after the immunization, the chickens were sacrificed for whole blood collection. The antisera were collected from the supernatants after the centrifugation at 3,000 rpm for 10 min and stored at −30°C until use.

Antigenic analysis was performed by neutralization (NT) and HI tests according to previously described protocols (Ohkawara et al., 2017). For the NT test, 2-fold serial dilutions of serum samples in MEM were mixed with 100 50% tissue culture infectious dose ($TCID_{50}$) of virus and incubated at 35°C for 1 h. Next, the mixture was added into a 96-well microtiter plate containing confluent monolayers of MDCK cells. After 1-h incubation, the suspension was removed and the cells were maintained in MEM. After incubation at 35°C for three days, the number of wells with cytopathic effects was counted in quadruplicate cultures. The NT titer was calculated as the reciprocal value of the highest serum dilution that caused complete neutralization in more than 50% of the

wells. For the HI test, 25 µl of 8 HAU of virus was added to 25 µl of 2-fold dilutions of serum in phosphate buffered saline (PBS) and the mixtures were incubated at room temperature for 30 min. After the incubation, 50 µl of 0.5% chicken red blood cell suspension in PBS was added and incubated at room temperature for 30 min. HI titer was calculated as the reciprocal value of the highest serum dilution that caused complete HI. The NT and HI results of three independent and reproducible experiments are presented.

Sequencing of viral HA and NA genes and visualization of mutation sites in the HA

Viral RNA extraction and amplification of full-length cDNAs of the HA and NA segments were performed as described previously (Chu et al., 2016). Direct sequencing of HA and NA genes of each virus was performed using an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Santa Clara, CA, USA). Complete amino acid sequences of the HA and NA were deduced from the cDNA information using Bioedit Sequence Alignment Editor v7.0.5 (Hall, 2016). Residues in the HA and NA are numbered according to the wild-type Ws/Hok/11 throughout the text. Methionine encoded by the AUG start codon was defined as position 1. The HA and NA sequences of the wild-type Ws/Hok/11 were previously deposited in the DDBJ/EMBL/GenBank database under the accession numbers of AB610972 and AB610974, respectively (Sakoda et al., 2012). The HA protein of Ws/Hok/11 was plotted on the 3-dimensional structure of an H5 HA obtained from the Protein Data Bank (PDB), accession number: 4KTH (Shore et al., 2013), by PyMOL presentation (DeLano Scientific, San Carlos, CA, USA).

Deep sequencing

Next generation sequencing method was applied to analyze the whole genome sequences and potential antigenic variants with low frequencies of the passaged viruses in study-I and II. Briefly, total viral RNAs were extracted from infectious allantoic fluids

using the QIAamp Viral RNA Mini kit (Qiagen, USA). MiSeq libraries were prepared using a NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and sequenced by the MiSeq system (Illumina, San Diego, CA, USA). Sequence reads were assembled de novo using CLC Genomic Workbench v10.0.1 (CLC bio, Aarhus, Denmark). Single-nucleotide polymorphisms (SNPs) were examined to identify quasispecies variants in viral population at a particular deduced amino acid position in the HA. SNPs with the coverage of at least 100 sequence reads were used for the analysis and cut-off frequency was set at value of more than 1% (Dinis et al., 2016).

Plaque cloning

To identify variants selected from consecutive passages, cloacal swabs of individual chickens in each group were used for plaque cloning using MDCK cells as previously described (Shichinohe et al., 2013). Ten plaques were selected using a sterile capillary pipette and suspended in MEM. Each cloned virus was propagated in 10-day-old embryonated chicken eggs at 35°C for 30–48 h. Residues of the HA of each cloned virus were determined as previously described. Numbers of viruses which harbor identical amino acids of the HA per 10 clones isolated from each individual chickens were determined.

Evaluation of variability of the HA1 protein residues using the information entropy

Approximately 1,000 HA1 sequences of H5 HPAIVs genetically belonging to clade 2.3.2 of the A/goose/Guangdong/1/1996 lineage were downloaded from the Influenza Research Database (IRD) on March 12, 2017. Duplicate sequences and laboratory-generated strains were excluded using web-available options. All downloaded strains were further edited to remove sequences with lengths less than 300 residues and

sequences containing miscellaneous residues. After these manipulations, 924 sequences were included for alignment by Clustal Omega (Sievers and Higgins, 2014).

The information entropy method was used to measure mutation frequency of each amino acid position in the HA1 (Pan and Deem, 2011; Suptawiwat et al., 2016). The entropy value at each amino acid position was determined as $H(x) = -\sum_{i=1}^{n20} piLnpi$; where *pi* is frequency of amino acid *i* at a defined position in the HA1 alignment. Entropy scores were calculated with Bioedit Sequence Alignment Editor v7.0.5 (Hall, 2016). The cut-off value was determined from a Z-score of 0.5 *[Z-score = (X-)/SD]* as previously described (Suptawiwat et al., 2016).

Results

Consecutive passages of Ws/Hok/11 in chickens

Two consecutive passage studies, study-I and study-II, of an H5N1 HPAIV (Ws/Hok/11) in vaccinated chickens were performed to generate antigenic variants of the H5 HPAIV under immune pressure from vaccination. These two studies were designed using the homologous combination of vaccine and challenge strains.

In study-I, nine consecutive passages were concurrently conducted in both vaccinated and non-vaccinated chickens. Inactivated vaccine of Ws/Hok/11 induced antibody responses against the homologous virus in the vaccinated chickens with the range of 4–32 HI titers (Table 1). Antibody responses of vaccinated chickens against challenge viruses were also confirmed and showed significantly lower HI titers from the third to ninth passages. Non-vaccinated chickens were confirmed as immunologically naïve with Ws/Hok/11 by HI titers less than the detection limit (Table 2). Viruses were thoroughly isolated in each consecutive passage during 1–7 dpc in vaccinated chickens and 1–4 dpc in non-vaccinated chickens (Table 1 and Table 2). In addition, it was evident that increased morbidity and mortality were observed in the vaccinated chickens following serial passages (Table 3). Particularly, all non-vaccinated chickens survived until 4 dpc in the first challenge with the wild-type Ws/Hok/11 and then survival duration was only 2 dpc in the subsequent passages (data not shown).

Next, consecutive passage study-II was subsequently performed using an updated vaccine strain to confirm the selection of antigenic drift after renewing vaccine. In study-II, chickens were vaccinated with an inactivated virus isolated from the nine passages in vaccinated chickens of study-I, named Ws/Hok/11-VacP9 and were challenged with the homologous virus strain in the first passage. A total of four consecutive passages were carried out in vaccinated and non-vaccinated chickens. Antibody titers of the vaccinated

chickens against the vaccine strain were found at comparable levels with study-I in the range of 2–32 HI titers (Table 4). Slight differences in antibody titers of several vaccinated chickens against vaccine and challenge strains were observed in the third and fourth passages. Non-vaccinated chickens were also confirmed to be immunologically naïve before challenge (Table 5). Similar to study-I, virus shedding periods of vaccinated chickens (1–6 dpc) were longer than those of non-vaccinated chickens (1–2 dpc) (Table 4 and Table 5). Overall, these results highlighted that the partial vaccination in poultry might protect vaccinated chickens from lethal infection in both studies, but also resulted in silent spread of H5 HPAIVs and prolonged virus shedding from vaccinated chickens.

Table 1. Virus recovery from chickens vaccinated with Ws/Hok/11 after challenging with passaged viruses derived from Ws/Hok/11 in study-I.

Passage No.	Chicken No.	HI titers of chicken sera against		Challenge strains (challenge titer)[a]	Virus isolation from oropharyngeal/cloacal swabs on days post challenge[b]							
		Vaccine strain	Challenge strains		0	1	2	3	4	5	6	7
1	1	8	8	Ws/Hok/11	-/-	+/-	-/+*	-/-	-/-	-/-	-/-	-/-
	2	4	4	($10^{7.3}$)	-/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-
	3	16	16		-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
2	4	16	16	Ws/Hok/11-VacP1	-/-	-/-	-/-	+/-	-/-	-/-	-/-	-/-
	5	16	16	($10^{7.8}$)	-/-	+/-	+/-	+/-	+/+	-/+*	-/-	-/-
	6	16	16		-/-	-/-	-/-	+/-	-/-	-/-	-/-	-/-
3	7	16	2	Ws/Hok/11-VacP2	-/-	+/-	+/-	+/-	+/+	-/+	-/-	-/-
	8	16	8	($10^{9.0}$)	-/-	+/-	+/-	+/-	+/+	-/+	-/+*	-/-
	9	16	4		-/-	+/-	+/-	+/+	+/+	-/+	-/-	-/-
4	10	16	<2	Ws/Hok/11-VacP3	-/-	+/-	+/-	+/-	+/+	+/+	-/+	-/-
	11	8	4	($10^{8.3}$)	-/-	+/-	+/-	+/-	+/+	+/+	-/+*	-/-
	12	16	<2		-/-	+/-	+/+	+/+	+/+	-/+†	NA	NA
5	13	16	<2	Ws/Hok/11-VacP4	-/-	+/+	+/+	+/+†	NA	NA	NA	NA
	14	8	<2	($10^{7.8}$)	-/-	+/-	+/+	+/+	+/-	+/+	+/+†*	NA
	15	32	2		-/-	+/-	-/-	+/-	+/+	-/-	-/-	-/-
6	16	8	<2	Ws/Hok/11-VacP5	-/-	+/-	+/+	+/+	+/+†	NA	NA	NA
	17	32	2	($10^{8.0}$)	-/-	+/-	+/-	+/+	-/-	-/-	-/-	-/-
	18	32	4		-/-	+/-	+/+	+/+	+/+	-/+*	-/-	-/-
7	19	32	<2	Ws/Hok/11-VacP6	-/-	+/+	+/+	+/+	+/+†	NA	NA	NA
	20	16	<2	($10^{7.3}$)	-/-	+/+	+/-	+/+	+/+†	NA	NA	NA
	21	32	<2		-/-	+/-	+/+	+/+	+/+	-/+*	-/-	-/-
8	22	32	<2	Ws/Hok/11-VacP7	-/-	+/-	+/-	+/+	+/+	-/+	-/+†*	NA
	23	32	<2	($10^{8.0}$)	-/-	+/-	+/+	+/+	+/+	+/+	-/-	-/-
	24	16	<2		-/-	+/-	+/-	+/-	+/+†	NA	NA	NA
9	25	16	2	Ws/Hok/11-VacP8	-/-	+/+	+/+	+/+	+/+	+/+	-/+†	NA
	26	8	<2	($10^{7.3}$)	-/-	+/-	+/+	+/+	+/+	-/+†	NA	NA
	27	8	<2		-/-	+/-	+/+	+/+	+/+	+/+	+/+	-/+†*

[a] Challenge titer is expressed as $TCID_{50}$
[b] Positive (+) or negative (-) results of virus isolation from swab samples
* Recovered virus was used for the next challenge
† Chicken died
NA: not available

Table 2. Virus recovery from non-vaccinated chickens after challenging with passaged viruses derived from Ws/Hok/11 in study-I.

Passage No.	Chicken No.	HI titer[a]	Challenge strains (challenge titer)[b]	Virus isolation from oropharyngeal/cloacal swabs on days post challenge[c]							
				0	1	2	3	4	5	6	7
1	40	<2	Ws/Hok/11	-/-	+/-	+/-	+/+	+/+†	NA	NA	NA
	41	<2	($10^{7.3}$)	-/-	+/-	+/+	+/+	+/+†*	NA	NA	NA
	42	<2		-/-	+/-	+/-	+/+	+/+†	NA	NA	NA
2	43	<2	Ws/Hok/11-NonvacP1	-/-	-/-	-/-	+/+†	NA	NA	NA	NA
	44	<2	($10^{7.8}$)	-/-	+/-	+/-	+/+†*	NA	NA	NA	NA
	45	<2		-/-	-/-	-/-	+/+†	NA	NA	NA	NA
3	46	<2	Ws/Hok/11-NonvacP2	-/-	+/-	+/+†	NA	NA	NA	NA	NA
	47	<2	($10^{7.8}$)	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	48	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA
4	49	<2	Ws/Hok/11-NonvacP3	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	50	<2	($10^{7.3}$)	-/-	+/-	+/+†	NA	NA	NA	NA	NA
	51	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA
5	52	<2	Ws/Hok/11-NonvacP4	-/-	+/+	+/+†	NA	NA	NA	NA	NA
	53	<2	($10^{9.0}$)	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	54	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA
6	55	<2	Ws/Hok/11-NonvacP5	-/-	+/-	+/+†	NA	NA	NA	NA	NA
	56	<2	($10^{8.0}$)	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	57	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA
7	58	<2	Ws/Hok/11-NonvacP6	-/-	+/+	+/+†	NA	NA	NA	NA	NA
	59	<2	($10^{9.0}$)	-/-	+/+	+/+†*	NA	NA	NA	NA	NA
	60	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA
8	61	<2	Ws/Hok/11-NonvacP7	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	62	<2	($10^{7.3}$)	-/-	+/-	+/+†	NA	NA	NA	NA	NA
	63	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA
9	64	<2	Ws/Hok/11-NonvacP8	-/-	+/+	+/+†	NA	NA	NA	NA	NA
	65	<2	($10^{7.8}$)	-/-	+/-	+/+†**	NA	NA	NA	NA	NA
	66	<2		-/-	+/-	+/+†	NA	NA	NA	NA	NA

[a] HI titers of chicken sera against Ws/Hok/11
[b] Challenge titer is expressed as $TCID_{50}$.
[c] Positive (+) or negative (-) results of virus isolation from swab samples
* Recovered virus was used for the next challenge
** Recovered virus was used as the last passaged virus
†Chicken died
NA: not available

Table 3. Clinical signs of chickens vaccinated with Ws/Hok/11 after challenging with passaged viruses derived from Ws/Hok/11 in study-I.

Passage No.	Chicken No.	HI titers of chicken sera against		Challenge strains (challenge titer) [a]	Score of clinical signs (dpc)[b]							
		Vaccine strain	Challenge strains		0	1	2	3	4	5	6	7
1	1	8	8	Ws/Hok/11	0	0	0*	0	0	0	0	0
	2	4	4	($10^{7.3}$)	0	0	0	0	0	0	0	0
	3	16	16		0	0	0	0	0	0	0	0
2	4	16	16	Ws/Hok/11-VacP1	0	0	0	0	0	0	0	0
	5	16	16	($10^{7.8}$)	0	0	0	0	0	0*	0	0
	6	16	16		0	0	0	0	0	0	0	0
3	7	16	2	Ws/Hok/11-VacP2	0	0	0	0	0	0	0	0
	8	16	8	($10^{9.0}$)	0	0	0	0	0	0	0*	0
	9	16	4		0	0	0	0	0	0	0	0
4	10	16	<2	Ws/Hok/11-VacP3	0	0	0	0	1	1	0	0
	11	8	4	($10^{8.3}$)	0	0	0	0	0	0	0*	0
	12	16	<2		0	0	0	0	0	3	3	3
5	13	16	<2	Ws/Hok/11-VacP4	0	1	1	3	NA	NA	NA	NA
	14	8	<2	($10^{7.8}$)	0	0	0	2	2	2	3*	NA
	15	32	2		0	0	0	0	0	1	0	0
6	16	8	<2	Ws/Hok/11-VacP5	0	0	1	2	3	NA	NA	NA
	17	32	2	($10^{8.0}$)	0	0	1	1	1	1	1	1
	18	32	4		0	0	0	0	1	1*	3	NA
7	19	32	<2	Ws/Hok/11-VacP6	0	0	1	1	3	NA	NA	NA
	20	16	<2	($10^{7.3}$)	0	1	2	2	3	NA	NA	NA
	21	32	<2		0	0	0	1	1	1*	0	0
8	22	32	<2	Ws/Hok/11-VacP7	0	0	1	1	1	2	3*	NA
	23	32	<2	($10^{8.0}$)	0	0	1	1	2	2	1	0
	24	16	<2		0	0	0	2	3	NA	NA	NA
9	25	16	2	Ws/Hok/11-VacP8	0	0	0	1	1	2	3	NA
	26	8	<2	($10^{7.3}$)	0	0	0	1	1	3	NA	NA
	27	8	<2		0	0	0	1	2	2	2	3*

[a] Challenge titer is expressed as $TCID_{50}$

[b] Clinical signs of chickens were numbered as '0–no clinical sign', '1–sick', '2–severely sick' and '3–dead'

* Recovered virus was used for the next challenge

NA: not available

Table 4. Virus recovery from the chickens vaccinated with Ws/Hok/11-VacP9 after challenging with passaged viruses derived from Ws/Hok/11-VacP9 in study-II.

Passage No.	Chicken No.	HI titers of chicken sera against		Challenge strains (challenge titer)[a]	Virus isolation from oropharyngeal/cloacal swabs on days post challenge[b]							
		Vaccine strain	Challenge strains		0	1	2	3	4	5	6	7
1	28	8	8	Ws/Hok/11-VacP9	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	29	2	2	($10^{8.0}$)	-/-	+/-	+/+	+/+	+/+	-/+*	-/-	-/-
	30	8	8		-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
2	31	2	2	Ws/Hok/11-	-/-	+/+	+/+	+/+	+/+	+/+†	NA	NA
	32	8	8	VacP9/VacP1	-/-	+/-	+/-	+/+	+/+	-/+	-/-	-/-
	33	16	16	($10^{7.0}$)	-/-	+/-	+/-	+/+	+/+	-/+	-/+*	-/-
3	34	8	8	Ws/Hok/11-	-/-	+/-	+/-	+/+	-/-	-/-	-/-	-/-
	35	16	16	VacP9/VacP2	-/-	+/-	+/-	+/-	-/+	-/+	-/+*	-/-
	36	4	2	($10^{7.5}$)	-/-	+/-	+-	+/-	-/-	-/-	-/-	-/-
4	37	2	<2	Ws/Hok/11-	-/-	+/-	+/+	+/+	+/+	+/+	-/-	-/-
	38	32	16	VacP9/VacP3	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	39	8	2	($10^{7.3}$)	-/-	+/-	+/+	+/+	+/+	-/+**	-/-	-/-

[a] Challenge titer is expressed as $TCID_{50}$

[b] Positive (+) or negative (-) results of virus isolation from swab samples

* Recovered virus was used for the next challenge

** Recovered virus was used as the last passaged virus

† Chicken died

NA: not available

Table 5. Virus recovery from non-vaccinated chickens after challenging with passaged viruses derived from Ws/Hok/11-VacP9 in study-II.

Passage No.	Chicken No.	HI titer [a]	Challenge strains (challenge titer) [b]	Virus isolation from oropharyngeal/cloacal swabs on days post challenge [c]							
				0	1	2	3	4	5	6	7
1	67	<2	Ws/Hok/11-VacP9	-/-	+/-	+/+†	NA	NA	NA	NA	NA
	68	<2	($10^{8.0}$)	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
2	69	<2	Ws/Hok/11-	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	70	<2	VacP9/NonvacP1 ($10^{9.3}$)	-/-	+/-	+/+†	NA	NA	NA	NA	NA
3	71	<2	Ws/Hok/11-	-/-	+/-	+/+†*	NA	NA	NA	NA	NA
	72	<2	VacP9/NonvacP2 ($10^{8.5}$)	-/-	+/-	+/+†	NA	NA	NA	NA	NA
4	73	<2	Ws/Hok/11-	-/-	+/+	+/+†	NA	NA	NA	NA	NA
	74	<2	VacP9/NonvacP3 ($10^{7.5}$)	-/-	+/-	+/+†**	NA	NA	NA	NA	NA

[a] HI titers of chicken sera against Ws/Hok/11-VacP9
[b] Challenge titer are expressed as $TCID_{50}$
[c] Positive (+) or negative (-) results of virus isolation from swab samples
* Recovered viruses were used for the next challenge
** Recovered virus was used as the last passaged virus
† Chicken died
NA: not available

Table 6. Clinical signs of the chickens vaccinated with Ws/Hok/11-VacP9 after challenging with passaged viruses derived from Ws/Hok/11-VacP9 in study-II.

Passage No.	Chicken No.	HI titers of chicken sera against		Challenge strains (challenge titer)[a]	Score of clinical signs (dpc)[b]							
		Vaccine strain	Challenge strains		0	1	2	3	4	5	6	7
1	28	8	8	Ws/Hok/11-VacP9	0	0	0	0	0	0	0	0
	29	2	2	($10^{8.0}$)	0	0	0	0	0	0*	0	0
	30	8	8		0	0	0	0	0	0	0	0
2	31	2	2	Ws/Hok/11-	2	0	0	0	1	2	3	NA
	32	8	8	VacP9/VacP1	4	0	0	0	0	0	0	0
	33	16	16	($10^{7.0}$)	8	0	0	0	0	0	0*	0
3	34	8	8	Ws/Hok/11-	0	0	0	0	0	0	0	0
	35	16	16	VacP9/VacP2	0	0	0	0	0	1	1*	0
	36	4	2	($10^{7.5}$)	0	0	0	0	0	0	0	0
4	37	2	<2	Ws/Hok/11-	0	0	1	1	1	1	0	0
	38	32	16	VacP9/VacP3	0	0	1	0	0	0	0	0
	39	8	2	($10^{7.3}$)	0	0	0	1	1	1**	1	1

[a] Challenge titer is expressed as $TCID_{50}$

[b] Clinical signs of chickens were numbered as '0–no clinical sign', '1–sick', '2–severely sick' and '3–dead'

* Recovered virus was used for the next challenge

** Recovered virus was used as the last passaged virus

NA: not available

Antigenicity and deduced amino acid sequences in the HA and NA of the passaged viruses

The passaged viruses isolated from the vaccinated and non-vaccinated chickens were antigenically characterized by NT and HI tests and genetically examined by deducing the amino acid sequences of the HA and NA genes. Chicken immune sera against wild-type Ws/Hok/11 and Ws/Hok/11-VacP9 strains were used to monitor antigenicity of the passaged viruses in study-I and study-II, respectively.

In study-I, the passaged viruses isolated from the vaccinated groups in the first and the second passages, Ws/Hok/11-VacP1 and Ws/Hok/11-VacP2, respectively, showed similar antigenicity to the parental virus Ws/Hok/11 with the same NT and HI titers (Table 7). However, the subsequent passaged viruses, from Ws/Hok/11-VacP3 to Ws/Hok/11-VacP9, were significantly antigenically distinct, defined by the more than 16-fold difference in NT and HI titers, from their parental viruses. In contrast, the passaged viruses isolated from non-vaccinated chickens, Ws/Hok/11-NonvacP9, did not exhibit any antigenic change over the nine passages. For the HA gene, after the third passage in the vaccinated chickens, an amino acid substitution of G179D was identified in the HA protein of the passaged viruses. This change was followed by the appearance of viruses with double amino acid substitutions of Q131R/G179D in the fourth passage and these substitutions were conserved until the ninth passage as the consensus sequence of Ws/Hok/11-VacP9. In addition, no mutation was found in the NA of these viruses. Further, no amino acid change in the HA and NA was observed in the passaged viruses recovered from non-vaccinated groups (Table 7). The results showed that antigenicity of the passaged viruses was changed after three passages in the vaccinated chickens in association with the mutation at position 179 in the HA.

In study-II, antigenic drift of the passaged viruses was identified after three consecutive passages in the vaccinated chickens as determined by substantial reduction

of antibody binding. The passaged viruses recovered from the first and second passages in vaccinated chickens, Ws/Hok/11-VacP9/VacP1 and Ws/Hok/11-VacP9/VacP2, respectively, were antigenically similar to Ws/Hok/11-VacP9. On the other hand, an approximately 8-fold difference in NT and HI titers were identified for Ws/Hok/11-VacP9/VacP3 and Ws/Hok/11-VacP9/VacP4 in comparison with Ws/Hok/11-VacP9. Further, passaged viruses isolated from non-vaccinated chickens from the fourth passage, Ws/Hok/11-VacP9/NonvacP4, had common antigenicity with Ws/Hok/11-VacP9 (Table 8). For amino acid sequences of the HA, in the vaccinated groups, the single amino acid substitution of R339G was identified in the HA of Ws/Hok/11-VacP9/VacP1 and Ws/Hok/11-VacP9/VacP2. Additional single substitutions of H256R and S144P in the HA were observed for the passaged viruses isolated from the third and fourth passages, respectively; these mutations were likely related to the antigenic change of the passaged viruses Ws/Hok/11-VacP9/VacP3 and Ws/Hok/11-VacP9/VacP4. For the NA, a single mutation of D179E was found after the first passage in the vaccinated groups; this mutation was unlikely associated with antigenic change of the passaged viruses. Further, consensus sequences of the HA and NA were highly conserved in the viruses recovered from non-vaccinated chickens (Table 8). Thus, the results from study-II definitively confirmed the selection of antigenic variants under immune pressure from vaccination, and antigenic variants persistently emerged under vaccination-primed immunity despite updating to the matched vaccine strain.

Table 7. Antigenicity and deduced amino acids in the HA of the passaged viruses in study-I.

Viruses[a]	Chicken No.[a]	NT titer[b]	HI titer[b]	Amino acid positions in the HA[c]	
				131	179
Ws/Hok/11	–	32	32	Q	G
Ws/Hok/11-VacP1	1	32	32	•	•
Ws/Hok/11-VacP2	5	32	32	•	•
Ws/Hok/11-VacP3	8	<2	2	•	D
Ws/Hok/11-VacP4	11	<2	2	R	D
Ws/Hok/11-VacP5	14	<2	2	R	D
Ws/Hok/11-VacP6	18	<2	2	R	D
Ws/Hok/11-VacP7	21	<2	2	R	D
Ws/Hok/11-VacP8	22	<2	2	R	D
Ws/Hok/11-VacP9	27	<2	2	R	D
Ws/Hok/11-NonvacP9	65	32	32	•	•

[a] The last virus recovered from a cloacal swab of the chicken which had the longest virus shedding period in each passage was used

[b] Chicken serum against Ws/Hok/11 was used. The homologous titers are underlined

[c] '•' indicates the same amino acids as Ws/Hok/11

Table 8. Antigenicity and deduced amino acids in the HA and NA of the passaged viruses in study-II.

Viruses[a]	Chicken No.[a]	NT titer[b]	HI titer[b]	Amino acid positions in the HA and the NA[c]			
				HA			NA
				144	256	339	179
Ws/Hok/11-VacP9	27	16	16	S	H	R	D
Ws/Hok/11-VacP9/VacP1	29	16	16	•	•	G	E
Ws/Hok/11-VacP9/VacP2	33	16	16	•	•	G	E
Ws/Hok/11-VacP9/VacP3	35	<2	<2	•	R	G	E
Ws/Hok/11-VacP9/VacP4	39	<2	<2	P	R	G	E
Ws/Hok/11-VacP9/NonvacP4	74	16	16	•	•	•	•

[a] The last virus recovered from a cloacal swab of the chicken which had the longest virus shedding period in each passage was used

[b] Chicken serum against Ws/Hok/11-VacP9 was used. The homologous titers are underlined

[c] '●' indicates the same amino acids as Ws/Hok/11-VacP9

Amino acid changes in the whole genomes of the passaged viruses during serial passages

To characterize the genetic evolution of the H5 HPAIV acquired during serial passages in chickens, whole genomes of the passaged viruses in study-I and study-II were sequenced by deep sequencing method and compared with their parental viruses. In study-I, 13 mutations were identified in the PB1, PB2, PA, HA, M1, M2, NS1 and NS2. In particular, in non-vaccinated group, three amino acid substitutions of M317V in the PB1, P208L in the NS1 and H56Y in the NS2 early emerged and remained highly conserved during the serial passages (Table 9). In study-II, except for the amino acid changes in the HA and NA which were previously mentioned, only two amino acid substitutions (V212I and I385V) occurred in the PB2 of the passaged viruses isolated from vaccinated chickens (Table 10). On the contrary, no amino acid substitution was identified in the passaged viruses recovered from non-vaccinated chickens (Table 10).

Table 9. Amino acid substitution in the whole genome of the passaged viruses in study-I.

Viruses	Amino acid substitution[a]												
	PB1		PB2	PA		HA			M1	M2	NS1		NS2
	195	317	292	44	263	131	179	237	174	28	208	221	56
Ws/Hok/11	I	M	I	V	A	Q	G	G	R	F	P	I	H
Ws/Hok/11-VacP1	•	•	•	•	•	•	•	•	•	•	•	•	•
Ws/Hok/11-VacP2	•	•	•	•	•	•	•	•	•	•	•	•	•
Ws/Hok/11-VacP3	V	•	•	•	•	•	D	•	•	•	•	•	•
Ws/Hok/11-VacP4	•	•	•	•	•	R	D	•	•	•	•	•	•
Ws/Hok/11-VacP5	•	•	•	•	•	R	D	•	•	•	•	•	•
Ws/Hok/11-VacP6	•	•	•	•	•	R	D	•	•	•	•	•	•
Ws/Hok/11-VacP7	•	•	•	•	•	R	D	•	K	•	•	•	•
Ws/Hok/11-VacP8	•	•	•	•	E	R	D	•	•	•	•	•	•
Ws/Hok/11-VacP9	•	•	•	•	E	R	D	•	•	•	•	•	•
Ws/Hok/11-NonvacP1	•	V	•	•	•	•	•	E	•	•	•	•	•
Ws/Hok/11-NonvacP2	•	V	•	•	•	•	•	•	•	•	L	•	Y
Ws/Hok/11-NonvacP3	•	V	•	•	•	•	•	•	•	•	L	•	Y
Ws/Hok/11-NonvacP4	•	V	•	I	•	•	•	•	•	•	L	•	Y
Ws/Hok/11-NonvacP5	•	V	•	•	•	•	•	•	•	•	L	•	Y
Ws/Hok/11-NonvacP6	•	V	•	•	•	•	•	•	•	•	L	•	Y
Ws/Hok/11-NonvacP7	•	V	•	•	•	•	•	E	•	•	L	•	Y
Ws/Hok/11-NonvacP8	•	V	•	•	•	•	•	E	•	•	L	V	Y
Ws/Hok/11-NonvacP9	•	V	V	•	•	•	•	•	•	L	L	V	Y

[a] '•' indicates the same amino acids as Ws/Hok/11

Table 10. Amino acid substitution in the whole genome of the passaged viruses in study-II.

Viruses	Amino acid substitution[a]					
	PB2		HA			NA
	212	385	144	256	339	179
Ws/Hok/11-VacP9	V	I	S	H	R	D
Ws/Hok/11-VacP9/VacP1	•	•	•	•	G	E
Ws/Hok/11-VacP9/VacP2	I	•	•	•	G	E
Ws/Hok/11-VacP9/VacP3	I	V	•	R	G	E
Ws/Hok/11-VacP9/VacP4	I	V	P	R	G	E
Ws/Hok/11-VacP9/NonvacP1	•	•	•	•	•	•
Ws/Hok/11-VacP9/NonvacP2	•	•	•	•	•	•
Ws/Hok/11-VacP9/NonvacP3	•	•	•	•	•	•
Ws/Hok/11-VacP9/NonvacP4	•	•	•	•	•	•

[a] '●' indicates the same amino acids as Ws/Hok/11-VacP9

Population analysis of variants with mutation in vaccinated chickens

To assess the selection of antigenic variants in each individually vaccinated chickens, HA sequences of ten cloned viruses obtained from cloacal swabs of individually infected chickens were determined. The deduced amino acid sequences of the HA gene were compared with those of the wild-type Ws/Hok/11 (Table 11).

In study-I, two distinct variants with amino acid substitutions G179V and G179D were isolated independently from vaccinated chicken No. 7 and No. 8 in the third passage, respectively; these variants accounted for 10/10 and 7/10 of cloned viruses, respectively, selected from the corresponding individual chickens. The variant harboring G179D isolated from chicken No. 8, which showed the longest period of virus shedding, was selected for the next passage. Until the ninth passage, all cloned viruses recovered from the three vaccinated chickens harbored the double mutations of Q131R/G179D in the HA. In non-vaccinated chickens, two viruses, including the Ws/Hok/11 and a variant possessing an amino acid substitution of G237E in the HA which was previously identified in the wild-type Ws/Hok/11, coexisted in the ninth passage. The result from plaque cloning analysis was reinforced by deep sequencing methods. Indeed, SNPs indicated that a large quasispecies population could exist during the consecutive passages (Table 12).

In study-II, multiple variants were identified through the consecutive passages with different amino acid substitutions in the HA including G339R, A143D/R339G, H256R/R339G, D179N/R339G and S144P/H256R/R339G (Table 13). These variants were isolated independently from vaccinated chickens during the consecutive passages (Table 11). In particular, the mutation H256R, which was identified as the cause of the antigenic change of the passaged viruses, emerged from the third passage as the dominant virus. Consequently, 9/10 variants with the substitutions H256R/R339G were maintained until the fourth passage, followed by 1/10 variant possessing a newly appeared mutation

S144P/H256R/R339G isolated from chicken No. 39. Noteworthily, the minor variant S144P/H256R/R339G found in the swab sample was previously identified as the passaged virus Ws/Hok/11-VacP9/VacP4 (Table 8 and Table 9). On the other hand, all cloned viruses recovered from non-vaccinated chickens through the fourth passage shared the identical HA sequence with Ws/Hok/11-VacP9. Taken together, the findings from study-I and study-II indicated that vaccination-derived immunity accelerated the selection of HA variants of the H5 HPAIV and replication of the H5 HPAIV in naïve chickens did not provide selective pressure for the mutation in either HA or NA.

Table 11. Number of plaque-cloned viruses with amino acid substitutions in the HA in study-I and study-II.

Study	Passage No.	Challenge strains	Chicken No.	Sample (dpc)	# of viruses[a]	Amino acid positions in the HA[b]						
						131	143	144	179	237	256	339
I	–	Ws/Hok/11	–	–	9‡	Q	A	S	G	G	H	R
					1	●	●	●	●	E	●	●
	3	Ws/Hok/11-VacP2	7	Cloacal (5)	10	●	●	●	V	●	●	●
			8*	Cloacal (6)	7‡	●	●	●	D	●	●	●
					3	●	●	●	●	●	●	●
			9	Cloacal (5)	7	●	●	●	●	●	●	●
					3	●	●	●	D	●	●	●
	9	Ws/Hok/11-VacP8	25	Cloacal (6)	10	R	●	●	D	●	●	●
			26	Cloacal (5)	10	R	●	●	D	●	●	●
			27*	Cloacal (7)	10‡	R	●	●	D	●	●	●
	9	Ws/Hok/11-NonvacP8	64	Cloacal (2)	7	●	●	●	●	E	●	●
					3	●	●	●	●	●	●	●
			65	Cloacal (2)	6‡	●	●	●	●	●	●	●
					4	●	●	●	●	E	●	●
			66	Cloacal (2)	6	●	●	●	●	●	●	●
					4	●	●	●	●	E	●	●
II	3	Ws/Hok/11-VacP9/VacP2	34	Cloacal (3)	9	R	●	●	D	●	●	G
					1	R	D	●	D	●	●	G
			35*	Cloacal (6)	10‡	R	●	●	D	●	R	G
			36	Oropharyngeal (3)	5	R	●	●	D	●	●	G
					5	R	●	●	N	●	●	G
	4	Ws/Hok/11-VacP9/VacP3	37	Cloacal (5)	8	R	●	●	D	●	R	G
					2	R	●	●	D	●	●	G
			38	Oropharyngeal (3)	9	R	●	●	D	●	●	G
					1	R	●	●	D	●	R	G
			39	Cloacal (5)	9	R	●	●	D	●	R	G
					1‡	R	●	P	D	●	R	G
	4	Ws/Hok/11-VacP9/NonvacP3	73	Cloacal (2)	10	R	●	●	D	●	●	●
			74	Cloacal (2)	10‡	R	●	●	D	●	●	●

[a] # of plaque-cloned viruses which contained identical HA sequences per 10 clones recovered from each individual chicken are indicated
[b] '●' indicates the same amino acids as Ws/Hok/11
* Viruses recovered from chickens used for the next challenge
‡ Representative HA sequence of the viruses previously identified as the consensus sequence of the passaged viruses in Table 7 and Table 8

Table 12. Quasispecies population defined by the codon sites in the HA of passaged viruses in study-I.

Viruses	Potential amino acid change in the HA positions[a] (Frequency value of particular amino acid change)																	
	2	5	45	50	51	124	131	147	179	198	210	218	237	388	434	436	521	529
Ws/Hok/11	–	–	A→T (38.5)	–	–	–	–	–	–	N→Y (38.5)	P→L (1.9)	T→A (1.0)	G→E (47.3)	–	–	–	–	Y→C (37.9)
Ws/Hok/11-VacP1	–	–	–	–	–	–	–	–	–	–	–	–	–	K→R (6.2)	–	–	–	–
Ws/Hok/11-VacP2	–	–	–	–	–	–	–	–	–	–	–	–	–	K→R (1.1)	–	–	–	–
Ws/Hok/11-VacP3	–	–	–	–	–	–	–	–	D→G (1.2)	–	–	–	–	–	–	–	–	–
Ws/Hok/11-VacP4	–	–	–	–	–	–	R→Q (1.5)	–	–	–	–	–	–	–	L→I (2.9)	–	–	–
Ws/Hok/11-VacP5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ws/Hok/11-VacP6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ws/Hok/11-VacP7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ws/Hok/11-VacP8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ws/Hok/11-VacP9	–	–	–	–	–	I→V (22.5)	–	–	–	–	–	–	–	–	–	V→I (10.1)	–	–
Ws/Hok/11-NonvacP1	–	–	–	–	–	–	–	–	–	–	P→S (2.9)	–	E→G (31.2)	–	–	–	–	–
Ws/Hok/11-NonvacP2	–	–	–	–	–	–	–	–	–	–	–	–	G→E (45.8)	–	–	–	–	–
Ws/Hok/11-NonvacP3	–	–	–	–	–	–	–	–	–	–	–	–	G→E (16.6)	–	–	–	–	–
Ws/Hok/11-NonvacP4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ws/Hok/11-NonvacP5	–	V→M (1.2)	–	–	–	–	–	–	–	–	–	–	G→E (5.4)	–	–	–	–	–
Ws/Hok/11-NonvacP6	E→K (1.0)	–	–	E→G (1.5)	K→R (3.9)	–	–	–	–	–	–	–	G→E (33.2)	–	–	–	–	–
Ws/Hok/11-NonvacP7	E→K (3.3)	–	–	–	–	–	–	V→M (2.8)	–	–	–	–	E→G (37.7)	–	–	–	V→A (1.2)	–
Ws/Hok/11-NonvacP8	–	–	–	–	–	–	–	V→M (1.4)	–	–	–	–	E→G (10.1)	–	–	–	–	–
Ws/Hok/11-NonvacP9	–	–	–	–	–	–	–	–	–	–	–	–	G→E (1.5)	–	–	–	–	–

[a] Arrow indicated potential amino acid substitution from residue in the consensus HA of the major virus to low-frequency variants in viral population

'–'indicates that no potential amino acid change was detected at the position with frequency higher 1%

Table 13. Quasispecies population defined by the codon sites in the HA of passaged viruses in study-II.

Viruses	Potential amino acid change in the HA positions[a] (Frequency value of particular amino acid change)													
	27	32	124	144	157	169	198	218	239	256	339	372	436	528
Ws/Hok/11-VacP9	_	_	I→V (22.5)	_	_	_	_	_	_	_	_	_	V→I (10.1)	_
Ws/Hok/11-VacP9/VacP1	_	_	_	_	_	_	_	T→A (1.0)	_	_	G→R (7.4)	_	_	_
Ws/Hok/11-VacP9/VacP2	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Ws/Hok/11-VacP9/VacP3	N→S (1.9)	_	_	_	_	_	_	_	_	R→H (22.9)	_	_	_	_
Ws/Hok/11-VacP9/VacP4	_	_	_	P→S (38.8)	_	_	_	_	_	_	_	_	_	_
Ws/Hok/11-VacP9/NonvacP1	_	_	_	_	_	K→R (1.3)	_	_	_	_	_	_	V→I (1.1)	_
Ws/Hok/11-VacP9/NonvacP2	_	_	_	_	_	_	_	T→A (1.2)	_	_	_	_	_	I→T (1.1)
Ws/Hok/11-VacP9/NonvacP3	_	V→I (1.2)	_	_	S→P (1.0)	_	N→S (2.2)	_	R→G (1.1)	_	_	S→N (2.6)	_	I→T (2.1)
Ws/Hok/11-VacP9/NonvacP4	_	_	_	_	_	_	N→S (42.8)	_	R→G (3.2)	_	_	_	_	_

[a] Arrow indicated potential amino acid substitution from residue in the consensus HA of the major virus to low-frequency variants in viral population
'–' indicated that no potential amino acid change was detected at the position with frequency higher 1%

Table 14. Antigenic characterization of plaque-cloned viruses recovered from study-I and study-II by cross HI test.

Viruses[a]	Chicken No.	HI titer[b]			Amino acid positions in the HA[c]						
		Ws/Hok/11	Ws/Hok/11-VacP9	Ws/Hok/11-VacP9/VacP4	131	143	144	179	237	256	339
Ws/Hok/11 #1	–	32	<2	4	Q	A	S	G	G	H	R
Ws/Hok/11 #8	–	32	<2	4	•	•	•	•	E	•	•
Ws/Hok/11-VacP3 #1	7	4	<2	4	•	•	•	V	•	•	•
Ws/Hok/11-VacP3 #2	8	4	16	4	•	•	•	D	•	•	•
Ws/Hok/11-VacP9 #1	27	4	16	4	R	•	•	D	•	•	•
Ws/Hok/11-VacP9/VacP1 #1	29	4	16	4	R	•	•	D	•	•	G
Ws/Hok/11-VacP9/VacP3 #10	34	4	16	4	R	D	•	D	•	•	G
Ws/Hok/11-VacP9/VacP3 #1	35	4	<2	8	R	•	•	D	•	R	G
Ws/Hok/11-VacP9/VacP3 #2	36	4	<2	4	R	•	•	N	•	•	G
Ws/Hok/11-VacP9/VacP4 #10	39	4	<2	32	R	•	P	D	•	R	G
Ws/Hok/11-VacP9/NonvacP4 #1	74	4	16	4	R	•	•	D	•	•	•

[a] '#' indicates plaque-clone identification number

[b] Chicken sera against Ws/Hok/11, Ws/Hok/11-VacP9 and Ws/Hok/11-VacP9/VacP4 were used
The homologous titers are underlined

[c] '●' indicates the same amino acids as Ws/Hok/11

Antigenic characterization of plaque-cloned viruses

To confirm the antigenic drift of the selected viruses, antigenicity of plaque-cloned variants identified in study-I and study-II were characterized by cross-reactivity HI with antisera against Ws/Hok/11, Ws/Hok/11-VacP9 and Ws/Hok/11-VacP9/VacP4. As expected, the variants harboring amino acid substitutions at position 179 (G179D, G179V) in study-I and 256 (H256R) in study-II were antigenically distinct from the homologous viruses Ws/Hok/11 and Ws/Hok/11-VacP9, respectively (Table 7 and Table 8). In addition, the minor population, Ws/Hok/11-VacP9/VacP3 #2, isolated from chicken No. 36 in study-II, containing the substitution D179N also exhibited distinct antigenicity in comparison to Ws/Hok/11-VacP9. Thus, the results suggested that 179 and 256 were likely the key positions for antigenic variation of the virus.

The positions of amino acid substitutions found in this study were plotted on the three-dimensional structure of the HA protein (Figure 1). All of these substitutions are located on the globular head domain of the H5 HA molecule and positions 179 and 256, associated with viral antigenicity, are located proximally to predicted antigenic sites of the HA based on the H1 HA antigenic mapping (Caton et al., 1982). The position 339 is unable to display in the visualization since this residue is positioned at the cleavage site which is unavailable in the crystal structure.

To analyze genetic patterns of the two key amino acid positions 179 and 256 in the HA found in this study, information entropy was applied to characterize variability of the residues (Pan and Deem, 2011; Suptawiwat et al., 2016) and compared amino acid representation at the sites with that of the genetic clade 2.3.2 H5 HPAIVs that are publicly deposited. Interestingly, position 179 possessed the highest entropy score of 0.96 in addition to the high entropy value of position 256 of 0.59, among all positions of the 924 HA1 sequences examined. These scores were remarkably higher than the average entropy used as the cut-off value of 0.17 (Figure 2A). This finding signified that positions 179

and 256 are highly mutated sites in the HA1 of clade 2.3.2 H5 HPAIVs. Furthermore, most of the amino acid substitutions at 179 and 256 found in this study (G179D, G179V, D179N and H256R) were identical with the amino acids observed in nature (Figure 2B).

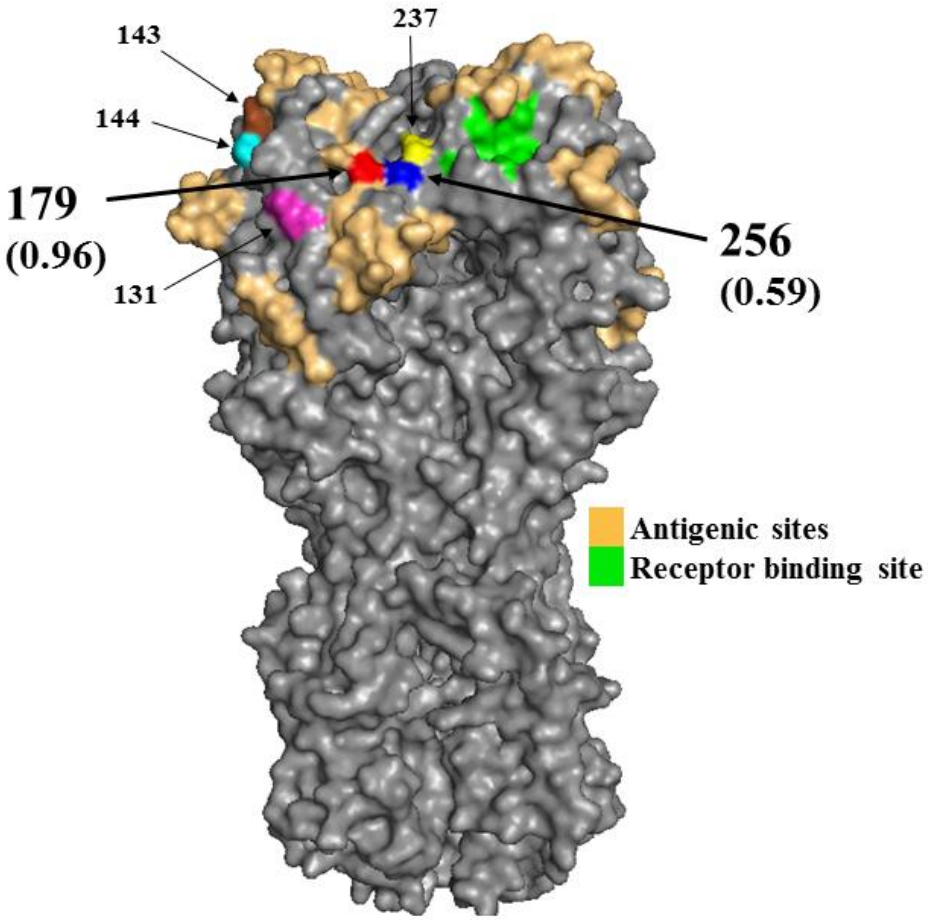

Figure 1. Amino acid substitutions in the HA associated with vaccination-induced immune escape. Crystallographic structure of the H5 trimer HA of A/Hubei/1/2010 (H5N1), PDB accession: #4KTH (Shore et al., 2013), is represented. The positions 179 and 256 in addition to 131, 143, 144 and 237 are shown in red, blue, pink, brown, cyan and yellow, respectively. The position 339 is unable to display in the visualization since this residue is positioned at the cleavage site which is unavailable in the crystal structure. The antigenic sites and receptor-binding domain of HA are shown in orange and green, respectively. Entropy values of the positions 179 and 256 are indicated in parentheses.

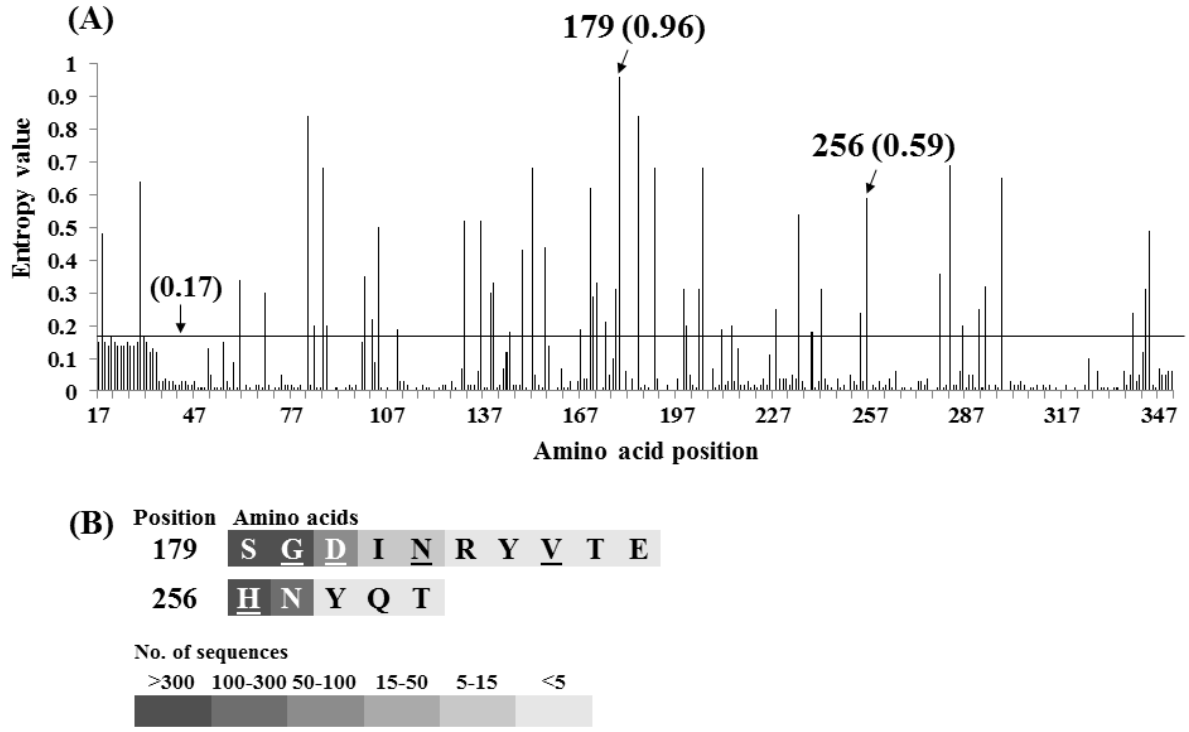

Figure 2. (A) Entropy value of 924 HA1 sequences of H5 HPAIVs belonging to the genetic clade and sub-clades 2.3.2. Entropy values of the positions 179 and 256 are indicated in parentheses. The line on the graph represents the cut-off value (Z-score = 0.5). **(B)** Amino acid residues at positions 179 and 256 of 924 HA1 sequences. Gradient background colors of each letter indicate the corresponding numbers of sequences. Underlined letters indicate the same amino acid residues as observed in this study.

Discussion

Mass vaccination campaigns in poultry have been implemented as a control strategy against the spread of H5 HPAIVs in some endemic countries in Asia. Initially, the programs were likely successful in reducing the burden of the disease (Ellis et al., 2005; Chen, 2009; Magalhaes et al., 2010). Nevertheless, H5 HPAIVs remained endemic in large poultry populations and eradication of H5 HPAIVs seemed to be unachievable in these countries. Empirically, vaccine failures mainly result from the appearance of antigenically drifted H5 HPAIVs (Kilany et al., 2010; Cattoli et al., 2011a; Ma et al., 2014; Swayne et al., 2015; Cuong et al., 2016; Nguyen et al., 2016). The present study clearly demonstrated the impacts of vaccination in driving the selection of antigenic variants by the consecutive passages of an H5N1 HPAIV in vaccinated chickens. Although the generation of antigenic variants under vaccination-mediated immunity has been well investigated in human influenza viruses using a mouse model (Hensley et al., 2009), our demonstration is the first report for H5 HPAIVs in chickens.

Two serial passage studies in vaccinated chickens were performed to clearly demonstrate the selection of antigenic variants under immune pressure conferred from the vaccination. The chickens vaccinated with the homologous vaccine strains were clinically protected from lethal infection in both studies; however, long-period virus shedding was maintained in these chickens (Table 1 and Table 4). This finding emphasized the disadvantage of vaccination in poultry that improper vaccination more likely increases the risk of further transmission by H5 HPAIVs from asymptomatically infected birds (Savill et al., 2006; Isoda et al., 2008; Cuong et al., 2016; Salaheldin et al., 2017). It is noteworthy that less proper vaccination was set in this study and the transmission of H5 HPAIVs might be prevented by optimal vaccination (Sitaras et al., 2016a; Sitaras et al., 2016b; Peeters et al., 2017).

Prior to initiating consecutive passages, pilot experiments using 2–256 HI titers of vaccinated chickens were pre-evaluated to obtain the most appropriate conditions for virus recovery in this study model (data not shown). As a result, single-shot vaccination was used to induce low immunity pressure in the vaccinated chickens. The vaccination mediated the average antibody titers of 2–32 HI thereby clinical signs of chickens were obviously alleviated after homologous lethal challenges in study-I and study-II. In fact, the vaccinated chickens were apparently healthy after the lethal challenges at the first and second passages (Table 3 and Table 6) in which the challenge viruses did not exhibit antigenic change. However, in the subsequent consecutive passages, the vaccinated chickens gradually displayed morbidity and mortality after challenge that were likely associated with the antigenic drifts of the passaged viruses. Surprisingly, antigenic variants were easily selected from vaccinated chickens during three consecutive passages, although an insufficient immunity level for chickens was adopted in our experimental design. On the other hand, severity of the infection in chickens could be affected by the adaptability of the H5 HPAIV acquired during serial passages, especially in non-vaccinated group in study-I (data not shown). Several amino acid substitutions in the internal genes, M317V in the PB1, P208L in the NS1 and H56Y in the NS2 (Table 9), likely contributed to the higher pathogenicity of the virus in chickens.

Although single-shot vaccination with the absence of adjuvant was applied to induce low immunity in the laboratory setting, our demonstration of the emergence of vaccination-derived antigenic variants is of practical value. The average immunity level from vaccination was previously evaluated as the serological potency to prevent morbidity in vaccinated chickens (Sitaras et al., 2016a; Sitaras et al., 2016b; Peeters et al., 2017) and was frequently documented in field reports as well (Henning et al., 2011; El-Zoghby et al., 2012; Poetri et al., 2014). Indeed, inconsistent immune responses with mass vaccinations under particular conditions against either vaccine or challenge strains

were observed, and multiple infection events more frequently occurred in large susceptible populations as in nature (Hafez et al., 2010; Kayali et al., 2013; Swayne et al., 2015). Therefore, it is probably impossible to ensure vaccination coverage at a robust titer for prevention of infection in a field context; this predicament consequently accelerates the emergence of antigenic variants. It is also necessary to imply that our demonstration might be only suitable at less protective immunity conferred from the non-adjuvanted vaccines. The fact that adjuvants are widely formulated to commercial vaccines to enhance and prolong robust immunity by vaccination (Lone et al., 2017). On the other hand, proper vaccination with sufficient efficacy has been highly recommended as a control measure against H5 HPAIVs and its antigenic variants in poultry (Sitaras et al., 2016a; Sitaras et al., 2016b; Peeters et al., 2017).

With the attempts to characterize antigenicity and fine antigenic structure of H5 HA, several neutralizing epitopes in the HA have been identified (Rudneva et al., 2010; Velkov et al., 2013). Moreover, previous studies revealed that antigenic transition of H5 HPAIVs was restricted to a few positions in the HA head domain during evolution (Cattoli et al., 2011b; Koel et al., 2014; Sitaras et al., 2014). This study newly identified multiple amino acid substitutions at positions 179 (G179D, G179V, D179N) and 256 (H256R) that were associated with antigenic drift of Ws/Hok/11, an H5 HPAIV belonging to the genetic clade 2.3.2.1c (Table 7, Table 8, Table 12 and Table 13).

Our antigenic analysis clearly indicated that the single amino acid substitutions at 179 and 256 are sufficient to lead to antigenic changes of Ws/Hok/11 (Table 14). In addition, these substitutions are positioned adjacent to the receptor-binding pocket and putative antigenic sites of the HA (corresponding H1 antigenic region) (Figure 1). Therefore, the results suggest that positions 179 and 256 are likely key residues for antigenic variation of the clade 2.3.2.1c H5 HPAIVs.

To clarify the impact of vaccination as the main driver of genetic and antigenic divergence of H5 HPAIVs, virus populations were determined by plaque cloning of samples collected from individually infected chickens. Obviously, multiple variants with amino acid substitutions in the HA were isolated from only the vaccinated chickens (Table 11). On the other hand, HA consensus sequences were highly conserved for viruses isolated from non-vaccinated chickens lacking protective immunity against H5 HPAIVs. Furthermore, in our study design, the viruses isolated from an individual bird, which had the longest virus shedding, were used for next passages; as a consequence, two distinct virus lineages were created with viruses with the amino acid substitution G179D in study-I and H256R in study-II being the dominant virus population. Further, the minority variants with different amino acids substitutions were isolated from other individually vaccinated chickens. Thus, it is assumed that antigenic variants with higher replication and transmission advantages in certain conditions could be selected to establish dominance, together with minor viruses as seen in nature. In particular, no mutation was found in the HA or NA during virus replication in non-vaccinated chickens (Table 11) despite quasispecies variants could persistently exist in the viral population. This indicates that virus propagation in the absence of vaccine-associated immune pressure negatively selected antigenic variants in this setting. Collectively, our results clarified that the genetic and antigenic divergence of H5 HPAIVs was mainly expedited by vaccination.

Several approaches, including information entropy, were applied to measure mutation frequency of a single position in the HA (Pan and Deem, 2011). Previous studies reported that numerous key sites with high entropy values were inferred as the antigenic epitopes in the HA during the evolution of H5 HPAIVs (Peng et al., 2014). This study further identified high variability of the positions 179 and 256 among clade and sub-clade 2.3.2 H5 HPAIVs (Figure 2A). Overall, it is reasonable to conclude that the highly

mutated sites 179 and 256 in the HA more likely contribute to the antigenic transition of the viruses in this clade. Indeed, antigenic drift of clade 2.3.2.1c H5 HPAIVs associated with the mutations at 179 and 256 was previously documented in the field reports (Marinova-Petkova et al., 2014; Nguyen et al., 2016). In addition, most amino acid substitutions identified in this study resembled the amino acids observed from field H5 HPAIVs (Figure 2B). These results also implied that the laboratory setting is valid for investigating and simulating the ongoing selection of antigenic variants in nature. The present study clearly demonstrated that antigenic drift rapidly and persistently occurred under vaccination-primed immunity despite updating the matched vaccine strain. Indeed, mass vaccination has been applied for more a decade in the endemic countries, but H5 HPAIVs have evolved more rapidly by continuous emergence of antigenically drifted variants (Swayne, 2012; WHO, 2018a).

In summary, the Chapter I study demonstrated the direct contribution of vaccination in chickens on the selection of antigenically drifted H5 HPAIVs and the updating of a matched vaccine strain could not prevent the emergence of antigenic variants. Therefore, vaccination should be used only as a tool in conjunction with primary strategies such as reinforcement of education, biosecurity, rapid diagnosis, surveillance and eliminating infected poultry for the sustainable control of H5 HPAI.

Brief summary

Vaccination-primed immunity in poultry has been suggested for selection of antigenically drifted HPAIVs. In this study, two consecutive passage studies of an H5N1 HPAIV in vaccinated chickens, namely, study-I and study-II, were carried out to select antigenic variants under immune pressure from the vaccination. In study-I, nine consecutive passages of a wild-type H5N1 HPAIV were carried out in chickens vaccinated with the homologous challenge strain. Antigenically drifted variants with mutations at position 179 in the HA were selected after three passages. Similarly, in study-II, a vaccination-mediated antigenic variant isolated in study-I was used as the vaccine and challenge strain to confirm further antigenic drift after updating the vaccine; after the third passage, additional antigenic variants with a mutation at position 256 in the HA were selected. Thus, this study demonstrated the contribution of vaccination in the selection of antigenic variants of H5 HPAIVs in chickens.

Chapter II

Rapid and broad detection of H5 hemagglutinin by an immunochromatographic kit using novel monoclonal antibody against highly pathogenic avian influenza virus belonging to the genetic clade 2.3.4.4

Introduction

IAVs have been classified into different subtypes according to their surface glycoproteins, HA and NA, in which 16 HA (H1–H16) and 9 NA (N1–N9) subtypes were recognized (Yewdell et al., 1979; Webster et al., 1992; Long et al., 2018). Among IAVs, an H5 subtype virus has become a major global concern for the poultry industry since its first emergence in Guangdong, China in 1996, causing HPAI with at least 75% fatality in infected birds (Xu et al., 1999). After its reemergence in 2003, Gs/GD-lineage H5 HPAIVs have consequently caused thousands of outbreaks in poultry and spread rapidly across the world via migratory wild birds (OIE, 2018). In addition, the zoonotic potential of the H5 HPAIV has been recognized since the first human case of an H5 HPAIV infection in Hong Kong in 1997 (Chan, 2002). To date, a total of 856 human infections with H5 HPAIVs were reported (WHO, 2018b). For controls of H5 HPAI in birds and H5 virus infection in humans, a simple, rapid and accurate diagnostic tool is in critical need (WHO, 2005).

The typical diagnoses of IAVs comprise serology, genetic identification and virus isolation; however, these methods are time-consuming and require appropriate facilities and biosafety (Okamatsu et al., 2016). In recent years, the simple and rapid IC technique, mainly based on antigen detection by MAbs, has been in focus because of its useful clinical diagnosis in humans and surveillance of infection in the field in birds (Okamatsu et al., 2016). Several IC kits for detecting IAV and their specific subtypes are widely used for these purposes (Tsuda et al., 2007; Manzoor et al., 2008; Mizuike et al., 2011; Wada et al., 2011; Baas et al., 2013; Sakurai et al., 2013; Sakai - Tagawa et al., 2014; Sakurai et al., 2015). Efficacy of IC diagnosis for IAV to detect internal NP, a highly conserved protein of IAV and large-quantity expression in cells (Yewdell et al., 1985), is stable. Meanwhile, detection of HA determining specific subtypes of influenza viruses remains

relatively less sensitive due to lower expression in cells and large variation of the surface protein (Okamatsu et al., 2016). In addition, persistent circulation of H5 HPAIVs in domestic poultry and wild birds has led to extensive antigenic diversification, so-called antigenic drift (Yewdell et al., 1979; Webster et al., 1982). This viral property has caused specific and sensitive diminution of MAb reactivity against varied antigens and consequently reduced efficacy of rapid diagnosis (Huzly et al., 2016; Overmeire et al., 2016). A previously developed H5 IC kit manufactured by a single MAb recognizing A/duck/Pennsylvania/10218/1984 (H5N2), Linjudge Flu A/H5 (Tsuda et al., 2007), reduced the sensitivity and specificity to detect recent H5 HPAIVs. Therefore the primary component of IC kit, MAbs specifically recognizing variable H5 HA antigens, should be formulated according to antigenic variation of circulating viruses for more effective detection (Okamatsu et al., 2016).

In the present study, an H5 IC rapid diagnostic kit (New Linjudge Flu A/H5) was developed using two MAbs comprising a newly generated MAb A32/2 against a clade 2.3.4.4 H5 HPAIV (Ohkawara et al., 2017) and A64/1 originally used in the Linjudge Flu A/H5. The New Linjudge Flu A/H5 showed higher specificity and sensitivity to a broad range of H5 HPAIVs isolated in recent years compared to the original Linjudge Flu A/H5 kit. In addition, its diagnostic efficacy was comparable with an influenza detection kit recognizing NP, ImunoAce Flu (NP). The diagnostic applicability of the New Linjudge Flu A/H5 was reinforced by detecting H5 HA antigens from the swabs and tissue homogenates of naturally infected birds and experimentally infected chickens with the most recent H5N6 HPAIVs in Japan. Moreover, oropharyngeal and cloalcal swabs collected from healthy chickens in commercial poultry farms were tested to demonstrate the new kit has no potential to give false-positive detection.

Materials and Methods

Development of the IC kit to detect H5 HA antigen

Two MAbs, A64/1 and A32/2, were used in the New Linjudge Flu A/H5. The MAb, A64/1, was previously produced using a hybridoma cell line against an H5 LPAIV, A/duck/Pennsylvania/10218/1984 (H5N2) (Soda et al., 2008) and a novel MAb, A32/2, was prepared in a similar method against a clade 2.3.4.4 H5 HPAIV, A/chicken/Kumamoto/1-7/2014 (H5N8) (Ohkawara et al., 2017). The new H5 IC kit was manufactured following similar protocol as previously described (Tsuda et al., 2007). Briefly, the mixture of the anti-H5 HA MAbs, A64/1 and A32/2, were conjugated with colloidal gold with a proper ratio. Anti-mouse immunoglobulin antibodies and the cocktail of A64/1 and A32/2 were then immobilized onto a nitrocellulose membrane to capture antibodies in the control and test judgment regions, respectively. Schematic diagram of the New Linjudge Flu A/H5 is shown in Figure 3.

Viruses

A total of 28 strains of influenza viruses including 26 strains of IAVs, 17 strains of non-H5 viruses, 9 strains of H5 viruses and 2 strains of influenza B viruses were used (Table 15 and Table 16). These viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs for 30–48 h at 35°C. The infectious allantoic fluid was collected and stored at –80°C until use.

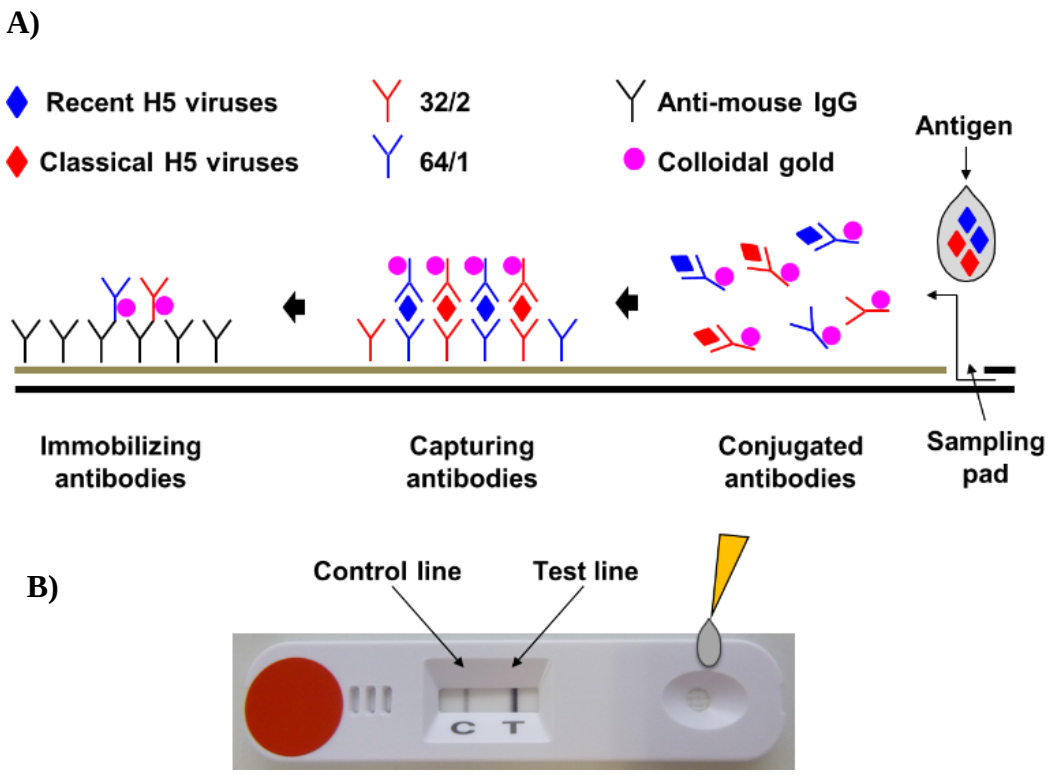

Figure 3. (A) Schematic diagram and **(B)** appearance of the New Linjudge Flu A/H5.

Virus titration

Virus titration was performed based on the $TCID_{50}$ value by using MDCK cells maintained in minimum essential medium supplemented with 0.3 mg/mL L-glutamine, 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 8 mg/mL gentamicin and 10% calf serum. Ten-fold dilutions of viruses in serum-free minimal essential medium were inoculated onto confluent monolayers of cells and incubated at 35°C for 1 h. After 72 h of incubation at 35°C, the cytopathic effects of the cells were observed. $TCID_{50}$ titers were calculated by the method of Read and Muench (Reed and Muench, 1938). In addition to $TCID_{50}$, the virus infectivity of H5 AIVs was measured as the 50% egg infectious dose (EID_{50}) by using 10-day chicken embryos. Dilution and titer calculation were performed as described in the $TCID_{50}$ method. The virus titration by EID_{50} and $TCID_{50}$ was performed using the same original working aliquot of each virus.

Evaluation of the specificity and sensitivity of the New Linjudge Flu A/H5, Linjudge Flu A/H5 and ImunoAce Flu (NP)

The detection efficacy of the present kit was compared with a human influenza commercial diagnosis kit, the ImunoAce Flu (NP antigen detection) (TAUNS Laboratories, Inc. Shizuoka, Japan) and the Linjudge Flu A/H5 kit (Tsuda et al., 2007). The test procedure was performed as previously described (Tsuda et al., 2007). In short, 10 μL sample solution was suspended in 90 μL of test solution (TAUNS Laboratories, Inc. Shizuoka, Japan) and the 100 μL suspension was applied to the sample port of each kit. Serial two-fold dilutions of each virus were tested. Results of the kit detection were recorded after 15 min of incubation at room temperature. A single colored line in the control judgment region (C) indicated the absence of H5 HA antigen. The concurrent presence of colored lines in both control and test judgment lines (T) indicated a positive test for H5 HA antigen in the samples. Results of antigen detection were indicated by +/-.

The intensity of the positive test line was further recorded on a scale from + to 6+. To standardize the visual judgment of the test line, the optical absorbance value was measured by the fluorescent immunochromato reader DiaScan 10-T (Otsuka Electronics Co., LTD., Osaka, Japan). The detection limit showing the lowest virus titer detectable by each kit was calculated by the equivalent proportion of the original virus titers to the last dilution that was able to yield positive detection. The detection limit was expressed as $\log_{10}$ EID_{50}/test and $\log_{10}$ $TCID_{50}$/test as previously described.

In addition, oropharyngeal and cloalcal swabs collected from 25 healthy chickens in commercial poultry farms were tested to examine cross reactivity of the New Linjudge Flu A/H5 with the field specimens. These samples were also confirmed to be negative with IAV by virus isolation using embryonated chicken eggs as previously described.

Swabs and tissue homogenates of naturally infected birds and experimentally infected chickens with H5N6 HPAIVs

A dead black swan and a dead whooper swan suspected of having natural infections with H5 HPAIVs were transferred to our laboratory for diagnosis. Two H5N6 HPAIVs were isolated from these birds and named A/black swan/Akita/1/2016 (H5N6) and A/whooper swan/Hokkaido/X12/2017 (H5N6), respectively (Okamatsu et al., 2017). Simultaneously, tissue homogenates of these birds were prepared for evaluation with the IC kits. Ten percent tissue homogenates were prepared in transport medium (minimal essential medium containing 10 000 U/mL Penicillin G, 10 mg/mL Streptomycin, 0.3 mg/mL Gentamicin, 250 U/mL Nystatin and 0.5% bovine serum albumin fraction V) as test samples and titration of infectivity. Swab samples from these naturally infected birds were not tested since multiple swabbings were formerly performed and used for emergency diagnosis and virus isolation, the subsequent swabs collected in the necropsy might not give appropriate results for kit evaluation. In addition to the natural cases,

experimental infection of chickens was performed. Briefly, 12-week-old white-leghorn chickens hatched and raised in our laboratory were used in this study. Three chickens were intranasally infected with $10^{8.4}$ EID_{50} of A/black swan/Akita/1/2016 (H5N6). Each chicken was housed in a self-contained isolator unit at the BSL3 facility in our laboratory. All chickens were monitored every 24 hours after the inoculation according to the standard protocol (OIE, 2015). After two days post inoculation, swabs and organs of dead chickens were collected for kit evaluation as described above. The test samples were diluted five-fold with the test solution and tested as previously described. The viral infectivity titers in the swabs and tissue homogenates were measured and expressed as $\log_{10}$ $TCID_{50}$/test.

Ethics statements

All the animal experiments were authorized by the Hokkaido University Animal Care and Use Committee (approval numbers: 13-0138) and all experiments were performed per the guidelines of the committee. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Results

Specificity of the New Linjudge Flu A/H5

The diagnostic specificity of the New Linjudge Flu A/H5 was compared with that of the original Linjudge Flu A/H5 kit and ImunoAce Flu (NP) by using a panel of 18 reference strains of IAVs including H1–H16 subtypes together with two strains of influenza B viruses (Table 15). The New Linjudge Flu A/H5 specifically detected each of the H5 AIVs tested and did not show cross-reactivity with any of the other HA subtypes (Table 16). The IAV and influenza B virus samples were positively tested with ImunoAce Flu (NP).

To demonstrate the New Linjudge Flu A/H5 had no potential to give false-positive detection, a total of 50 oropharyngeal and cloalcal swabs confirmed to be negative with IAVs were used for the kit evaluation. It was evident that the New Linjudge Flu A/H5 did not yield any false positivity with the field negative samples (Table 17).

Table 15. Specificity of the New Linjudge Flu A/H5, Linjudge Flu A/H5 and ImunoAce Flu (NP) with IAVs and influenza B viruses.

Viruses	Subtypes or lineages	Results [a]		
		New Linjudge Flu A/H5	Linjudge Flu A/H5	ImunoAce Flu (NP)
A/duck/Tottori/723/1980	H1N1	–	–	6+
A/Hyogo/YS/2011	H1N1(pdm09)	–	–	6+
A/duck/Hokkaido/17/2001	H2N3	–	–	6+
A/duck/Mongolia/4/2003	H3N8	–	–	6+
A/Hokkaido/M1/2014	H3N2	–	–	6+
A/duck/Czech/1956	H4N6	–	–	6+
A/duck/Pennsylvania/10218/1984	H5N2	5+	5+	6+
A/turkey/Massachusetts/3740/1965	H6N2	–	–	6+
A/seal/Massachusetts/1/1980	H7N7	–	–	6+
A/turkey/Ontario/6118/1968	H8N4	–	–	6+
A/turkey/Wisconsin/1966	H9N2	–	–	6+
A/chicken/Germany/N/1949	H10N7	–	–	6+
A/duck/England/1/1956	H11N6	–	–	6+
A/duck/Alberta/60/1976	H12N5	–	–	6+
A/gull/Maryland/704/1977	H13N6	–	–	6+
A/mallard/Astrakhan/263/1982	H14N5	–	–	6+
A/duck/Australia/341/1983	H15N8	–	–	6+
A/black-headed gull/Sweden/5/1999	H16N3	–	–	6+
B/Hokkaido/M2/2014	B/Victoria	–	–	3+
B/Hokkaido/30-4/2014	B/Yamagata	–	–	6+

[a] Positive/negative result of each test is indicated by +/–. Intensity of the positive test line was further recorded on a scale from + to 6+ based on visual judgments

Table 16. Detection limit of the New Linjudge Flu A/H5, Linjudge Flu A/H5 and ImunoAce Flu (NP) to detect H5 HA antigens.

Viruses	Subtypes	Clades	Original virus titers[a]	Detection limits[a]		
				New Linjudge Flu A/H5	Linjudge Flu A/H5	ImunoAce Flu (NP)
A/duck/Pennsylvania/10218/1984	H5N2	–	9.6/7.5	4.8/2.8	4.8/2.8	4.6/2.6
A/chicken/Taiwan/0502/2012	H5N2	–	8.2/6.0	4.2/2.0	4.2/2.0	4.2/2.0
A/Muscovy duck/Vietnam/OIE-559/2011	H5N1	1.1	9.3/8.5	6.3/5.5	6.3/5.5	5.3/4.5
A/whooper swan/Mongolia/3/2005	H5N1	2.2	9.2/7.5	5.2/3.5	5.2/3.5	4.9/3.2
A/whooper swan/Hokkaido/4/2011	H5N1	2.3.2.1c	8.6/7.8	6.5/5.7	–[b]	3.7/2.9
A/duck/Vietnam/HU3-16/2015	H5N1	2.3.2.1c	9.8/8.0	6.1/4.3	7.8/6.0	5.8/4.0
A/chicken/Kumamoto/1-7/2014	H5N8	2.3.4.4	8.8/5.2	4.8/1.2	5.8/2.2	3.8/0.2
A/duck/Vietnam/HU1-1151/2014	H5N6	2.3.4.4	9.5/7.5	5.5/3.5	–[b]	5.8/3.8
A/black swan/Akita/1/2016	H5N6	2.3.4.4	9.1/8.0	5.4/4.3	–[b]	5.4/4.3

[a] Original virus titers and detection limits were indicated by $\log_{10}$ EID_{50} / $\log_{10}$ $TCID_{50}$
[b] '–' indicates negative result of the test with undiluted virus titer

Table 17. Specificity of the New Linjudge Flu A/H5 with the swab samples from 3 experimentally infected chickens and 26 healthy chickens.

Result of kit detection	Virus isolation (oropharyngeal/cloalcal)	
	Positive[a]	Negative[b]
Positive	3/3	0/0
Negative	0/0	26/26

[a] Results of virus isolation and detection of the New Linjudge Flu A/H5 with the swab samples from experiementally infected chickens

[b] Result of virus isolation and detecion of the New Linjudge Flu A/H5 with the swab samples from 26 healthy chickens

Sensitivity of the New Linjudge Flu A/H5

The sensitivity of the new H5 kit was then evaluated with nine strains of H5 influenza viruses including LPAIVs and HPAIVs. Serial two-fold dilutions of each virus stock were concurrently examined by the New Linjudge Flu A/H5, the Linjudge Flu A/H5 and ImunoAce Flu (NP). The detection limit shows the lowest virus titer which was detectable by the individual tests (Table 16). The result indicates that the minimal detection limit of the New Linjudge Flu A/H5 was in the range of $10^{4.2}$–$10^{6.5}$ EID_{50}/test against all H5 influenza viruses examined in this study. On the other hand, the Linjudge Flu A/H5 detected HA antigens from classical clade 1.1 and 2.2 H5 HPAIVs with a comparable detection limit to the New Linjudge Flu A/H5 ($10^{5.2}$–$10^{6.3}$ EID_{50}/test). However, for recent H5 HPAIVs classified in clade 2.3.2.1c and 2.3.4.4, the detection limit of the original Linjudge Flu A/H5 is higher and the kit could not detect several strains in these sub-clades. Furthermore, the comparable range of detection limits of the New Linjudge Flu A/H5, the Linjudge Flu A/H5 and ImunoAce Flu (NP) were observed to detect LPAIVs and HPAIVs in a clade of 2.2 viruses (Table 16). Slight difference of limit detection was observed between virus titration in EID_{50} and $TCID_{50}$ amongst examined H5 AIVs. However, due to inefficient replication of A/chicken/Kumamoto/1-7/2014 (H5N8) strain in MDCK cells, infectivity of the virus titrated by $TCID_{50}$ was much lower compared with EID_{50}.

Detection of H5 HA antigen from swabs and tissue homogenates of naturally infected birds and experimentally infected chickens with H5N6 HPAIVs

The applicability of the New Linjudge Flu A/H5 for the diagnosis of H5 influenza infection was demonstrated by detecting H5 HA antigen in infected animals. Two naturally infected birds, a black swan and a whooper swan, confirmed infection with H5N6 HPAIVs and three chickens experimentally infected with an H5N6 HPAIV,

A/black swan/Akita/1/2016 (H5N6) were used for kit evaluation. A dead black swan from a local zoo was found on November 15, 2016 and stored at –20°C for shipment. Necropsy was performed on November 20, 2016 in the BSL3 facility in our laboratory. Similarly, the carcass of the whooper swan, a wild bird, was found on January 15, 2017 and transferred to our laboratory for necropsy on January 17, 2017.

In addition, three chickens were intranasally challenged and died two days post inoculation. Swab and tissue samples were then collected from black swan and dead chickens for subsequent kit testing. Viral titration in the swabs and tissue homogenates were performed to determine diagnostic sensitivity of the kits in clinical specimens. It was clear that the New Linjudge Flu A/H5 and ImunoAce Flu (NP) could detect the presence of H5 antigen and NP antigen, respectively in most specimens of infected birds; however, the presence of H5 antigen could not be detected with the original Linjudge Flu A/H5 kit (Table 18). Interestingly, the New Linjudge Flu A/H5 could detect H5 antigen in swabs samples from infected chickens that normally contain low virus load ($10^{2.2}$–$10^{3.4}$ $TCID_{50}$/test). On the other hand, all three kits evaluated in this study did not show non-specific reactivity with specimens with the absence of H5 antigen from the uninfected chicken as control (Table 17). All results emphasize the applicability of the New Linjudge Flu A/H5 for on-site diagnosis.

Table 18. Antigen detection from the swabs and tissue homogenates of naturally infected birds and experimentally infected chickens with H5N6 HPAIVs.

Birds[a]	Infection	Swabs[b]		Tissue homogenates[b]					
		Trachea	Cloacal	Brain	Trachea	Lung	Kidney	Spleen	Colon
Black swan	Natural	NT[c]	NT[c]	3+,–,5+ (5.1)	+,–,4+ (2.1)	+,–,6+ (3.1)	+,–,5+ (3.1)	+,–,5+ (2.8)	–,–,6+ (2.1)
Whooper swan	Natural	NT[c]	NT[c]	–,–,4+ (2.1)	–,–,4+ (0.8)	+,–,5+ (2.8)	+,–,6+ (3.8)	+,–,6+ (3.3)	–,–,3+ (1.3)
Chickens	Experimental	2+,–,6+ (3.4)	+,–,6+ (3.4)	+,–,6+ (3.3)	3+,–,6+ (4.1)	4+,–,6+ (3.3)	4+,–,6+ (3.8)	5+,–,6+ (4.1)	2+,–,6+ (3.8)
	Experimental	+,–,6+ (2.2)	+,–,6+ (2.9)	+,–,4+ (1.8)	+,–,6+ (2.1)	3+,–,6+ (5.1)	+,–,6+ (3.6)	4+,–,6+ (2.8)	+,–,6+ (2.8)
	Experimental	+,–,6+ (2.9)	+,–,6+ (2.2)	+,–,6+ (3.3)	+,–,6+ (2.8)	4+,–,6+ (3.8)	2+,–,6+ (4.1)	6+,–,6+ (4.1)	+,–,6+ (3.1)
	None	–,–,– (–)	–,–,– (–)	–,–,– (–)	–,–,– (–)	–,–,– (–)	–,–,– (–)	–,–,– (–)	–,–,– (–)

[a] The black swan and whooper swan were naturally infected with A/black swan/Akita/1/2016 (H5N6) and A/whooper swan/Hokkaido/X12/2017 (H5N6), respectively. The chickens were experimentally inoculated with A/black swan/Akita/1/2016 (H5N6).

[b] Results of antigen detection of the New Linjudge Flu A/H5, Linjudge Flu A/H5, ImunoAce Flu (NP). The number in parentheses is the virus infectivity as $\log_{10}$ $TCID_{50}$/test. (–) indicates virus infectivity under limit of detection

[c] NT indicates samples were not tested. Since the first swabs were used for emergency diagnosis and virus isolation, the subsequent swabs collected in the necropsy might not give appropriate results for kit evaluation

Discussion

For the controls of H5 HPAI in birds and H5 virus infection in humans, rapid and accurate diagnosis of the causative viruses plays a vital role to facilitate subsequent countermeasures (WHO, 2005; Swayne, 2012). Multiple studies have documented that antigenicity of the H5 HPAIVs is highly divergent; several strains of H5 HPAIVs belonging to distinct clades and sub-clades impede serological diagnosis by antisera against their earlier strains (WHO, 2018a). The changing antigenicity of the H5 HPAIVs has reduced the sensitivity of the IC technique against specific H5 subtypes viruses. Our laboratory testing has revealed the Linjudge Flu A/H5, previously established by our laboratory, failed to detect several H5 HPAIVs isolated in recent years (Table 16). In this study, a new H5 IC kit was developed using a combination of two MAbs. The MAb, A64/1, generated from an H5 LPAIV, A/duck/Pennsylvania/10218/1984 (H5N2), which was applied in the original Linjudge Flu A/H5 and a new MAb, A32/2, generated from A/chicken/Kumamoto/1-7/2014 (H5N8), an H5 HPAIV belonging clade 2.3.4.4 (Ohkawara et al., 2017). The novel MAb, A32/2, was selected amongst the four generated MAbs against A/chicken/Kumamoto/1-7/2014 (H5N8) since the A32/2 exhibited broad cross-clade reactivity with recently isolated H5 HPAIVs (Ohkawara et al., 2017). Furthermore, an escape mutant selected by the MAb possess an amino acid substitution at the relatively conserved position in the receptor domain and the amino acid substitution was found in several H5 strains. However, this MAb could not detect a few classical strains of H5 HPAIVs and LPAIVs (Ohkawara et al., 2017). The combination of A32/2 and A64/1 aimed to compensate the recognizing reactivity of each MAb for broad detection of H5 influenza viruses of the new kit. The New Linjudge Flu A/H5 shows apparent improvement over the original kit in terms of sensitivity and specificity to detect more recent H5 HPAIVs (Table 16). The sensitivity of the New Linjudge Flu A/H5 was

determined and compared with that of Linjudge Flu A/H5 and ImunoAce Flu (NP) by using distinct LPAIVs and HPAIVs (Table 16). The minimal detection limit of each kit was determined as both standard methods of EID_{50} and $TCID_{50}$ (Tsuda et al., 2007; Manzoor et al., 2008; Baas et al., 2013; Sakai - Tagawa et al., 2014). Since, this study examined various strains of H5 AIVs, each strain has different genetic backgrounds that might significantly affect virus replication in certain conditions either in chicken embryos or MDCK cells. Therefore, considerable differences in EID_{50} and $TCID_{50}$ were observed in this study (Table 16). These results suggested that virus titration assays should be appropriately selected to evaluate diagnosis efficacy of rapid detection techniques against influenza viruses. On the other hand, virus infectivity of the samples from infected birds was titrated by $TCID_{50}$ to minimize usage of live chicken embryos. Our evaluation indicates that the detection limit of ImunoAce Flu (NP) is in the range of $10^{3.7}$–$10^{5.8}$ EID_{50}/test which is almost comparable to other commercial kits to detect viral NP protein (Sakai - Tagawa et al., 2014). Significantly, the minimal detection limit of the New Linjudge Flu A/H5, in this study, is almost equal to that of the ImunoAce Flu (NP) to detect H5 HA antigen from LPAIVs and clade 2.2 and 2.3.4.4 HPAIVs; meanwhile, NP-targeting detection kits are generally more sensitive than that of anti-HA kits when using cultured viruses (Mizuike et al., 2011). The detection limit of the present kit is comparable to the commercially available kits for specific subtype influenza viruses for H5 subtype (Tsuda et al., 2007; Wada et al., 2011; Sakurai et al., 2013) or for H7 subtype (Manzoor et al., 2008; Baas et al., 2013). Although, combination of two MAbs, A64/1 and A32/2, allowed significantly improved sensitivity and specificity compared to the original Linjudge Flu A/H5 to detect recently expansive H5 HPAIVs, the sensitivity level of the New Linjudge Flu A/H5 as well as the ImunoAce Flu (NP) generally remained relatively low. This poses further challenges to improve the diagnostic efficacy of our methods in terms of sensitivity enhancement.

Point-of-care applicability of the New Linjudge Flu A/H5 was examined by detecting H5 antigen from clinical specimens of infected animals. A zoo bird and a wild bird, confirmed to be naturally infected with H5N6 HPAIVs, were transferred to our laboratory for further virological diagnosis. The New Linjudge Flu A/H5 could detect H5 HA antigen from most tissue homogenates tested. Owing to considerable roles of migratory birds in the transmission of the H5 HPAIVs, the surveillance of H5 HPAI and LPAI in wild birds has become a global emphasis (Rose et al., 2006; Lycett et al., 2016). Ordinary specimens, such as oropharyngeal and cloacal swabs collected within 24 h of dead or captured birds are preferred for viral detection; other necropsied organs are generally histopathologically examined to determine the cause of infection (Rose et al., 2006). Here, it is suggested the internal organs, which normally retain high viral loads, could be used for the scrutiny of viral detection in wild birds in addition to swab samples. Early and accurate diagnosis of the New Linjudge Flu A/H5 would be significant to minimize complexity of sample handlings and shipments in case of negative cases, or to conduct proper corresponding protocols with infection of H5 HPAI in wild birds, especially in remote areas. In addition, diagnostic efficacy was evaluated by detecting H5 HA antigen in chickens intranasally infected with the isolate, A/black swan/Akita/1/2016 (H5N6). The result of visual judgment of the kit was in accordance with virus titration. In particular, the New Linjudge Flu A/H5 detected H5 HA antigen from swab samples despite low virus titer, which was elusive in previous studies studies (Tsuda et al., 2007; Manzoor et al., 2008; Slomka et al., 2012) and highlighted the advanced improvement for the new kit. Non-specific reactivity was not observed in samples with the absence of H5 antigen, indicating the specificity of the New Linjudge Flu A/H5 in clinical diagnosis.

It was previously concluded that the point-of-care rapid antigen detection kits are appropriate for wildlife surveillance of AIVs despite low specificity and sensitivity of the methods (Cattoli and Capua, 2007). In this study, the New Linjudge Flu A/H5 achieves

highly sensitive and specific diagnosis against H5 HPAIVs regardless of the divergence of antigenicity of newly isolated viruses. This study emphasizes the ability of the New Linjudge Flu A/H5 to detect H5 HA antigens from broadly circulating viruses of H5 HPAIVs belonging to clade 2.3.2.1c and 2.3.4.4 (Dhingra et al., 2016; Lycett et al., 2016; Okamatsu et al., 2017; Si et al., 2017) and LPAIVs. It is conceivable to indicate that our New Linjudge Flu A/H5 could detect these occasionally emerged H5 viruses since the new kit efficiently detected their H5 LPAI prototype strains. Nevertheless, it is also necessary to further evaluate the diagnostic efficacy of the New Linjudge Flu A/H5 to detect a broader range of H5 AIVs.

Development of the New Linjudge Flu A/H5, in fact, was initiated as an adjunct tool for laboratory rapid diagnosis against H5 HPAI. The New Linjudge Flu A/H5 has been frequently used and its applicability was truly recognized by detecting recent multiple outbreaks of H5 HPAI in Japan (Okamatsu et al., 2017). Furthermore, the newly validated kit might carry practical significance on preparedness for a potential human pandemic caused by H5 HPAIVs. Although, diagnosis efficacy of the New Linjudge Flu A/H5 was clearly demonstrated, the result might only give preliminary conclusion on the infection and classical standard techniques such as virus isolation and antigenic and genetic characterization are essential for further diagnosis validity and virological investigation (Okamatsu et al., 2016).

In summary, the Chapter II study is the first report to evaluate diagnostic efficacy of the point-of-care rapid antigen detection method to detect H5 HPAIVs belonging to clade 2.3.4.4 which expansively spread and cause multiple outbreaks in domestic and wild birds in over the world (Lycett et al., 2016; Okamatsu et al., 2017; Si et al., 2017). This study revealed suitability of the New Linjudge Flu A/H5 for surveillance of H5 AIVs in domestic and wild birds and its significance for urgent control measures against H5 HPAIVs.

Brief summary

Gs/GD-lineage H5 HPAIVs have persistently caused outbreaks in domestic poultry and wild birds worldwide and sporadically infected humans. Rapid and accurate diagnosis is one of the key strategies for the control of H5 HPAIVs. However, the sensitivity of the diagnosis of H5 HPAIVs has gradually reduced due to extensive antigenic variation during their evolution. Particularly, the previously developed IC diagnosis kit for H5 AIVs, Linjudge Flu A/H5, exhibits reduced detection of H5 HPAIVs isolated in recent years. The present study established a new advanced H5 rapid IC detection kit (New Linjudge Flu A/H5) by a combination of two anti-H5 HA MAbs, A64/1 previously applied in the Linjudge Flu A/H5 and A32/2, a novel monoclonal antibody generated from a clade 2.3.4.4 H5 HPAIV. The new kit broadly detected all classical and recent H5 AIVs and showed a higher specificity and sensitivity than the original Linjudge Flu A/H5 with recently circulating H5 HPAIVs. Furthermore, the applicability of the New Linjudge Flu A/H5 was demonstrated by detecting antigens from the swabs and tissue homogenates of naturally infected birds and experimentally infected chickens with H5N6 HPAIVs belonging to the genetic clade 2.3.4.4. This study, therefore, can provide an effective point-of-care rapid antigen detection kit for the surveillance of H5 AIVs and as a prompt countermeasure against the current widespread of the clade 2.3.4.4 H5 HPAIVs in domestic and wild birds.

Chapter III

A systematic study towards evolutionary and epidemiological dynamics of currently predominant H5 highly pathogenic avian influenza viruses in Vietnam

Introduction

Materials and Methods

Results

Discussion

Brief summary

Chapter IV

Spatiotemporal and risk analysis of H5 highly pathogenic avian influenza occurrence in Vietnam during 2014–2017

Introduction

Materials and Methods

Results

Discussion

Brief summary

Conclusion

The aim of this thesis study is to investigate evolutionary and epidemiological dynamics of Gs/GD-lineage H5 HPAIVs which are considered as representative model for other HPAIVs. The whole dissertation can be defined as a systematic study covering basic research in laboratory to practical implementation in the field using interdisciplinary analyses. With obtained results, this thesis study comprehensively elucidated some of virological and epidemiological aspects of Gs/GD-lineage H5 HPAIVs which are causing huge damage to poultry industry and zoonotic concerns worldwide. Therefore, findings of this study carry novelty and significances to provide new insights of basic knowledge and leads to better control and prevention of HPAI.

Regarding H5 HPAI, despite intensive efforts on vaccination campaigns for disease control, viruses have persisted in some enzootic countries for more than decade and a haft. The present study provided conclusive laboratory evidence regarding direct contribution of vaccination in chickens to antigenic drift of H5 HPAIVs and update of a matched vaccine strain even could not prevent the emergence of antigenically drifted variants. Therefore, mass vaccination in poultry has limitation for the control and prevention of H5 HPAIVs. More recently, poultry vaccination campaign has been in place for the control of newly emerged H7 HPAIVs in China, thus evolution of H7 HPAIVs are presumed to be divergent as its counterpart H5 HPAIVs due to immune selection pressure from vaccination. Therefore, poultry vaccination should be taken into careful reconsideration and implemented only as an assistant tool for the control of HPAI.

In response of detecting antigenically drifted H5 HPAIVs, particularly the spreading 2.3.4.4 HPAIVs and human infection preparedness, an advanced rapid diagnostic kit, New Linjudge Flu A/H5 IC, was developed to replace of our original Linjudge Flu A/H5. Diagnosis efficacy of the New Linjudge Flu A/H5 exhibited greater than the original kit

regarding sensitivity. In addition, the applicability of the New Linjudge Flu A/H5 was demonstrated through detecting antigens from the swabs and organs of naturally infected birds for the first time and experimentally infected chickens with H5N6 HPAIVs belonging to the genetic clade 2.3.4.4. This study revealed suitability of the New Linjudge Flu A/H5 for surveillance of H5 AIVs in domestic and wild birds and its significance for control measures against H5 HPAIVs in the field.

In the field setting, the present study systematically elucidated distinct virological and epidemiological characterizations of currently circulating H5 HPAIVs in Vietnam through longitudinal active surveillance program. This study has provided multiple important results towards recent H5 HPAIVs in Vietnam: (*i*) this study highlights importance of routine surveillance and public sharing for the monitoring and control of H5 HPAIVs; (*ii*) H5 HPAIVs persisted most parts of Vietnam and domestic ducks and Muscovy ducks are likely important species for virus detection in active surveillance; (*iii*) clade 2.3.2.1c and 2.3.4.4 H5 HPAIVs are currently predominant in Vietnam and show distinct phylogeographic evolution; (*iv*) these predominant viruses exhibited large antigenic distance from its progenitor viruses and commercial poultry vaccines currently used in Vietnam, meaning that vaccine and vaccination might be less effective for the control of recently circulating H5 HPAIVs; (*v*) importantly, the present study discovered phylodynamics of H5 HPAIVs and highlights necessity of preventive control of transboundary spillovers to Vietnam.

The last part of the thesis study is to investigate epidemiology of recent outbreak occurrence of H5 HPAIVs in poultry in Vietnam. A total of 139 H5 HPAI outbreaks were reported during the period 2014 to 2017. Consistent with previous studies, several risk factors were identified to be associated with an increased risk of H5 HPAI outbreak reports. These indicators could be considered as stable predictors for early morning and target for monitoring H5 HPAVs in Vietnam.

To sum up, it is undeniable that eradication of causative agents from susceptible species is the utmost task for the control of HPAI but this work would be great controversial challenge under reality complex. Therefore, continuous control efforts should be taken in association with evolutionary dynamics of HPAIVs. Further systematic studies are highly important to take over effects of socioeconomics impacts and effective control measures in the field.

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