獸醫學博士 學位論文

Development of highly productive and mammalian non-pathogenic recombinant avian influenza vaccine strains

고생산성, 포유류 무병원성 재조합 조류인플루엔자 백신주 개발

2020년 2월

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

(수의미생물학)

안 세 희

Development of highly productive and mammalian non-pathogenic recombinant avian influenza vaccine strains

By
Se-Hee An

Advisor: Professor Jae-Hong Kim, D.V.M., Ph.D.

A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology

February. 2020

College of Veterinary Medicine
The Graduate School
Seoul National University

Development of highly productive and mammalian non-pathogenic recombinant avian influenza vaccine strains

지도교수 김 재 홍

이 논문을 수의학 박사 학위논문으로 제출함

2019년 10월

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

(수의미생물학)

안 세 희

안세희의 박사 학위 논문을 인준함

2019년 12월

위 원 장 권 혁준 (인)

부 위 원 장 김 재홍 (인)

위 원 송 창선 (인)

위 원 성 한우 (인)

위 원 조 성범 (인)

Abstract

Development of highly productive and mammalian non-pathogenic recombinant avian influenza vaccine strains

Se-Hee An

(Advisor: Jae-Hong Kim, D.V.M., Ph.D.)

College of Veterinary Medicine

The Graduate School

Seoul National University

Avian influenza viruses (AIVs) are highly infectious pathogen of waterfowls and can overcome species barriers to infect mammals by acquiring cumulative mutations. Low

pathogenic avian influenza viruses (LPAIVs) cause mild or moderate clinical symptoms, and highly pathogenic avian influenza viruses (HPAIVs) cause severe clinical symptoms in domestic poultry. Many subtype H5 HPAIVs have caused pandemics in poultry and wild birds. In Korea, H9N2 LPAIVs have caused continuous problems like decreased egg production since recurrent outbreaks in 1999 and an inactivated vaccine has been successfully applied in the field. Since 2003, seven epidemics caused by H5Nx HPAIVs have occurred and 'stamping out' policy has been conducted. However, stockpiling of HPAI antigen bank for emergency vaccine production has established in Korea since 2018.

Recently, vaccine strains for inactivated avian influenza vaccines have been generated by adaptation of field AIVs to embryonated chicken eggs (ECEs) or by reverse genetics technique. The Hoffmann's vector system adopts 2+6 reverse genetics scheme using hemagglutinin (HA) and neuraminidase (NA) genomes of field AIVs and 6 internal genomes coding PB2, PB1/PB1-F2, PA/PA-X, NP, M1/M2, and N2/NEP of A/Puerto Rico/8/1934 (PR8) strain, and has been broadly used for generation of productive vaccine strains. Although the PR8 internal genes are necessary for generation of productive recombinant virus, they possess important mammalian pathogenicity-related mutations in PB2, PA and NS1 genes. In addition, some of PR8-derived recombinant vaccine strains doesn't show enough replication efficiency in ECEs to be a inactivated vaccine strain. To generate more efficacious vaccine strains, it is recommended to match T and B cell epitopes of internal proteins to field strains for better protection, but in some cases rescue of recombinant virus possessing 8 genomes of field strain was unsuccessful.

Since 2007, a commercial LPAI vaccine strain A/chicken/310_E20//2001(H9N2)

(01310) was established by 20 times of passages through ECEs, acquiring high embryonic pathogenicity as well as high replication efficiency. The high pathogenicity resulted in early embryonic death and decrease of virus productivity because amount of harvested allantoic fluid were decreased. Because the PB2 gene of 01310 virus was prototypic and mammalian non-pathogenic, generation of complete recombinant virus containing 8 genomes of 01310 in 293T cells was impossible. The Hoffmann's vector system has fixed 3'end promoter constellation that polymerase (PB1, PB2 and PA) genes have weak (Cytidine at fourth nucleotide, C4) but others have strong (Uridine, U4) promoters. To solve the difficulty of 01310 virus rescue, I changed the promoters of polymerases genes from C4 to U4 for compensation of weak activity of PB2 in 293T cells, and finally succeeded in virus rescue. Moreover, 01310 NS1/NEP gene was exchanged with a mammalian non-pathogenic NS1/NEP gene originated from an embryo non-pathogenic H9N2 virus (0028) to understand the role of NS1/NEP genes in embryo pathogenicity, and recombinant 01310 virus bearing 0028 NS1/NEP (r310-NS28) was generated. The r310-NS28 showed decreased embryo pathogenicity and increased productivity in terms of mean death time of embryo and total hemagglutinin unit, respectively. The introduction of mammalian pathogenicity-related mutations G139D/N, S151T, and GSEV to EPEV into 0028 NS1/NEP gene also increased embryo pathogenicity.

Clade 2.3.4.4 H5Nx HPAIVs have spread world widely and clade 2.3.4.4a H5N8 virus was selected for emergency vaccine stockpiling program in Korea. However, the conventional PR8-derived recombinant H5N8 vaccine strain showed poor replication efficiency in ECEs and could replicate in the lungs of mice. To solve these problems,

recombinant H5N8 viruses possessing several mutations (H103Y, K161E and L317P in HA, and S369N in NA) identified in previous study were generated to increase virus titer in ECEs. Only the H103Y increased virus titer significantly, but also increased mammalian pathogenicity. The increased mammalian pathogenicity was removed by replacing PR8 PB2 with 01310 PB2 genes without change in virus titer. Interestingly, H103Y introduction also increased heat-stability of hemagglutinin. The optimized recombinant H5N8 virus completely protect from challenge with clade 2.3.4.4 H5N6 HPAIV in chickens, and induced age-dependent humoral immunity in ducks.

Furthermore, evolution pattern of H5 HPAIV derived by massive vaccination was analyzed to find strategy for future HPAIV vaccine strain development and vaccination program. It is well-known that continuous vaccination against HPAIVs facilitates appearance of antigenic variants. Clade 2.3.2.1c H5N1 HPAIVs have evolved from clade 2.3.2 H5N1 HPAIVs under vaccine-induced immune pressure, and amino acid sequences of HA proteins (n = 647) were compared to identify chronological variations. During the evolution clade 2.3.2.1c viruses acquired S144N and V223I mutations around the receptor binding site (RBS) of HA in order. The S144N mutation generated real N-linked glycosylation site (NGS) but it was less preferable NGS to 158N considering significantly higher frequency of 158NGS than 144NGS among HA proteins of H5 AIVs. The 144N and 158N are located in separate epitopes and their glycosylation may shield epitopes to result in humoral immunity evasion, and acquisition of 144NGS may be result of vaccination. The recombinant clade 2.3.2.1c H5N1 viruses having single reverse mutation (N144S or I223V) and combined reverse mutations (N144S and I223V) were generated and

characterized. The recombinant virus with single reverse mutation had lower viral titer in ECEs and avian receptor binding affinity. However, both reverse mutations increased replication efficiency in ECEs and mammalian cells, and pathogenicity in mice. Interestingly, the I223V mutation dramatically decreased thermo-stability possibly by destabilizing heterotrimer interaction of HA proteins. Therefore, clade 2.3.2.1c H5N1 HPAIVs may have evolved to escape avian immunity in the cost of decreased viral fitness and mammalian pathogenicity.

As results of the study, the innate defects and limits of Hoffmann's vector system affecting efficacy and pathogenicity of recombinant vaccine strains were improved by modification of 3'-end promoter constellation and exchanging PR8 genes with less pathogenic avian-origin PB2 and NS1/NEP genes. The common functions of NS1 and H103Y in chicken and mammalian cells may reinforce the important role of chickens for evolution of mammalian pathogenicity of AIVs. The developed highly productive and mammalian non-pathogenic H9N2 and clade 2.3.4.4 H5N8 vaccine strains may be useful and contribute to produce more efficacious vaccines, and the experimental and bioinformatics data from clade 2.3.2.1c virus investigation also helpful to develop more efficacious vaccine strains in the future.

Keywords: *Reverse genetics; Avian influenza A virus; Inactivated recombinant vaccine; High productivity; Mammalian non-pathogenicity*

Student number: 2015 - 21820

Contents

List of figures

List of tables

List of supplementary data

Abbreviations

AIV	Avian influenza virus
IAV	Influenza A virus
HPAIV	Highly pathogenic avian influenza virus
LPAIV	Low pathogenic avian influenza virus
PR8	A/Puerto Rico/8/34
RBS	Receptor binding site
HA	Hemagglutinin
NA	Neuraminidase
NS	Non-structural protein
NP	Nucleoprotein
M	Matrix protein
M2e	N-terminal ectodomain of M2 protein
NCR	Non-coding region
vRNA	Viral genomic RNA
vRNP	Viral ribonucleoprotein
α2,3 SA	sialic acid α2,3-linked to galactose
α2,6 SA	sialic acid α2,6-linked to galactose
ECE	Embryonated chicken egg
SPF	Specific pathogen free
EID_{50}	50 % chicken embryo infectious dose

MDCK	Madin-Darby canine kidney cell
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
$TCID_{50}$	50% tissue culture infectious dose
PBS	Phosphate buffered saline
dpi	Days post inoculation
hpi	Hours post inoculation
wpv	Week post vaccination
wpc	Week post challenge
dpc	Days post challenge
BEI	Binary ethylenimine
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
HRP	Horseradish peroxidase
HAU/HAT	Hemagglutination unit/Hemagglutination titer
HI	Hemagglutination inhibition
MDT	Mean death time
PRR	Pattern-recognition receptor
TLR	Toll like receptor
RIG-1	Retinoic acid inducible gene-1
PAMP	Pathogen-associated marker pattern
ISG	Interferon stimulated genes
DC	Dendritic cell

CTL	CD8+ cytotoxic T cell
Th cell	Helper T cell
TNF-α	Tumor necrosis factor alpha
NOS2	Nitric oxide synthase 2
NK	Natural killer cell
ADCC	Antibody dependent cell cytotoxicity
APC	Antigen presenting cell

General introduction

Influenza A viruses (IAVs) have eight segmented negative-sense RNA and are divided into subtypes according to two surface glycoprotein, hemagglutinin (HA) and neuraminidase (NA) (McCAULEY and Mahy, 1983; Schild et al., 1980). Aquatic birds are natural reservoirs of all subtypes and avian influenza viruses (AIVs) can be transsmitted from wild waterfowls to domestic poultry, and vice versa (Webster et al., 1992). AIVs have two forms of pathotype, low pathogenic avian influenza virus (LPAI) and highly pathogenic avian influenza virus (HPAI), based on severity of disease in domestic poultry. LPAI causes mild and moderate clinical symptoms like decreased egg production, but some H5 and H7 LPAI viruses can be converted into HPAI during circulation in poultry (Munster et al., 2005; Stephenson et al., 2004). HA protein was considered as major antigenic and pathogenic determinant of AIVs, but viral pathogenicity was also result of complex interaction between other genes like polymerase protein gene (PB2, PB1, and PA) and non-structural protein gene (NS) during viral infection and replication in host (Hale et al., 2008b; Hatta et al., 2001).

Since the HPAI H5N1 outbreak in Geese in Guangdong province in 1997 (Gs/Gd/1/96), GS/Gd-lineage HPAI H5N1 circulated in China and diversified into genetically distinct several lineages (Li et al., 2004; Smith et al., 2006a). During co-circulation of multiple clades and subclades in China and South East Asia, clade 2.3.2 became dominant and rapidly evolved into clade 2.3.2.1 and further 2.3.2.1a, b and c in 2009 (Group, 2014; Team, 2014). Since then, clade 2.3.2.1c has spread across continents

and detected in some countries until now (Bi et al., 2016; Laleye et al., 2018b; Nguyen et al., 2019; Parvin et al., 2019). Fujian-lineage H5N1 (clade 2.3.4) dominant in China and South East Asia in late 2005 was diversified into clade 2.3.4.4 in 2008 and clade 2.3.4.4 was globally reported since 2014 (Claes et al., 2016; Lycett et al., 2016; Smith et al., 2006a). Unlike other H5N1 HPAI viruses, clade 2.3.4.4 HA was reasserted with multiple NA subtypes, called clade 2.3.4.4 H5Nx, and evolved into four genetic sub-groups (Lee et al., 2016a; Lee et al., 2017c; Lycett et al., 2016).

In the HPAI endemic countries, inactivated whole virus vaccines have been used and they were focused on eliciting anti-HA and NA neutralizing antibodies because HA and NA are main targets of host humoral immunity and neutralizing antibodies against them are the most powerful means to prevent viral infection (Johansson et al., 1989). However, there is need for continual updating vaccine strain to newly emerging HPAI viruses because neutralizing antibodies are not effective to heterologous subtypes and AIVs have rapid antigenic variation strategy to escape humoral immunity (Carrat and Flahault, 2007).

Most of inactivated HPAI vaccine were generated in a 6+2 reassortant of which only HA and NA genes were exchanged according to circulating field HPAI strains by reverse genetics system (Li et al., 2014). The A/Puerto Rico/8/1934(H1N1) (PR8) was frequently used as a donor virus of six internal genes to recombinant HPAI vaccine strain because PR8-derived recombinant can be generated rapidly and high growth in embryonated chicken eggs (ECEs) (Li et al., 2014; Webby et al., 2004a). However, internal genes like nucleoprotein gene (NP), and matrix protein gene (M) have conserved epitopes

of T cell immunity and using homologous internal genes to AIVs improve heterosubtypic protection and PB2 gene of PR8 have many important mammalian pathogenic mutations like E627K (Lee et al., 2017a).

The Hoffmann's reverse genetics system and universal primers for cloning genes to the bi-directional plasmid (pHW2000) are also based on high growth PR8 virus (Hoffmann et al., 2000b; Hoffmann et al., 2001a). AIVs have non-coding regions (NCRs) in both 5'- and 3'-ends of all viral genomic RNA (vRNA) and the first 12 and 13 nucleotides in NCRs are very conserved between AIVs (Neumann et al., 2004). The NCRs have important role in viral replication and the fourth nucleotide in the 3'-end is known as promoter. Genome segments having uridine at the fourth nucleotide (U4) replicate and transcript higher than genome with cytidine (C4) (Jiang et al., 2010; Lee et al., 2017b). The universal primer sets of Hoffmann's reverse genetics system were constructed including the NCRs of each vRNA segments and the fourth nucleotides in NCRs were identical with high-growth PR8 (Hoffmann et al., 2001a).

In Korea, H9N2 LPAIV had been occurred since 1996 and evolved continuously to distinct Korean-like lineage virus (Lee et al., 2007c). Also, several times of HPAI outbreaks caused severe economic loss in poultry industry. After the first outbreak of clade 2.5 HPAI H5N1 in 2003, diverse sub-clades HPAI were introduced periodically by migratory birds (Clade 2.2 H5N1 in 2006, clade 2.3.2.1 in 2008, and clade 2.3.2.1c in 2010) (Kim et al., 2010; Lee et al., 2005; Lee et al., 2008). In early 2014, clade 2.3.4.4 H5N8 (group A and B) was reported in Korea and group A viruses was lasted until 2016, followed by continuous outbreaks of clade 2.3.4.4 H5N6 (group C) during 2016 -2017 (Kwon et al.,

2018; Kwon et al., 2017a; Lee et al., 2017c).

In order to develop a vaccine strain for control of LPAI, the A/chicken/Korea/01310/2001(H9N2) (01310) virus passaged 20 times in ECEs (01310 CE20) acquiring high replication efficiency in ECEs have been used as inactivated LPAI vaccine strain (Choi et al., 2008c). Though inactivated HPAI vaccine had not been permitted in Korea, emergency vaccine strain and rapid developing method of highly productive and safe vaccine should be prepared.

This study sought to address the limitations of the PR8-basd Hoffmann's reverse genetics systems, such as the failure to produce recombinant AIV containing eight genomes of 01310 for elimination of high virulence in ECEs and mammalian pathogenic factor possessed by internal genes of PR8. It also aimed to solve the low productivity problem in ECEs of PR8-based recombinant H5N8 vaccine strains and to reveal the antigenic variation of clade 2.3.2.1c virus induced by mass vaccination.

Literature review

1. Influenza A Virus : structure and life cycle

Influenza A viruses (IAVs) belong to family *orthomyxoviridae* and contain eight negative-sense single-stranded RNA genome segments (Webster et al., 1992). IAVs are divided into subtypes according to d:fferences in surface glycoproteins, hemagglutinin (HA) and neuratminidase (NA), and there are 18 HA subtypes and 11 NA subtypes (H17 and H18, N10 and N11 were isolated only in bats) (Gamblin and Skehel, 2010; Wu et al., 2014). Aquatic birds are natural reservoirs of IAVs but these viruses also infect in domestic poultry and mammals.

IAVs have spherical and pleomorphic virion and envelope is host-derived lipid bilayer that HA, NA, and M2 protein was embedded (Webster et al., 1992). Underneath the viral membrane, matrix is formed by M1 protein binding with viral ribonucleoprotein (vRNP). The vRNP consists of viral RNA (vRNA) encapsidated with nucleoprotein (NP) molecules and trimeric polymerase protein complex (PB1, PB2, and PA) at the ends (Eisfeld et al., 2015). Each RNA genomes are translated into 16 proteins with different functions (Shi et al., 2014).

Infection of IAVs start by binding of HA protein to host cell receptor and IAVs enter cells by endocytosis (Skehel and Wiley, 2000). The endosomal acidification trigger conformational change of HA protein and fusion of viral

membrane with endosomal membrane by exposing HA2 fusion peptide (Wiley and Skehel, 1987). M2 ion channel also promotes acidification of virion, and vRNP is released from M1 protein (Schnell and Chou, 2008). The released vRNP in cytoplasm are transported into cell nucleus through nuclear localization signals and nuclear pore complex (Boulo et al., 2007). In the nucleus, the viral RNA dependent RNA polymerase initiate transcription and replication of vRNA into mRNA and complementary RNA (cRNA). The 5'-cap of viral mRNA is originated from host cellular mRNA by binding of PB2 cap binding domain to host 5'-capped RNA and cleavage of 8-14 nucleotides downstream of the cap by PA (Dias et al., 2009; Plotch et al., 1981). Viral mRNA is exported from nucleus and translated into viral protein by cellular ribosome (Shapiro et al., 1987). Newly synthesized polymerase proteins and NP are transported to the nucleus and progeny vRNPs are generated. Progeny vRNPs are exported to cytoplasm by M1 and NEP proteins and incorporated into progeny virus particles with HA, NA, M2 and M1 (Boulo et al., 2007; Rossman and Lamb, 2011). Viral particles bud from apical plasma membrane where viral components (envelope, M1, and vRNP) are transported, and released from host cell membrane through M2-mediated membrane scission and cleavage of sialic acid off the cell by NA (Rossman and Lamb, 2011).

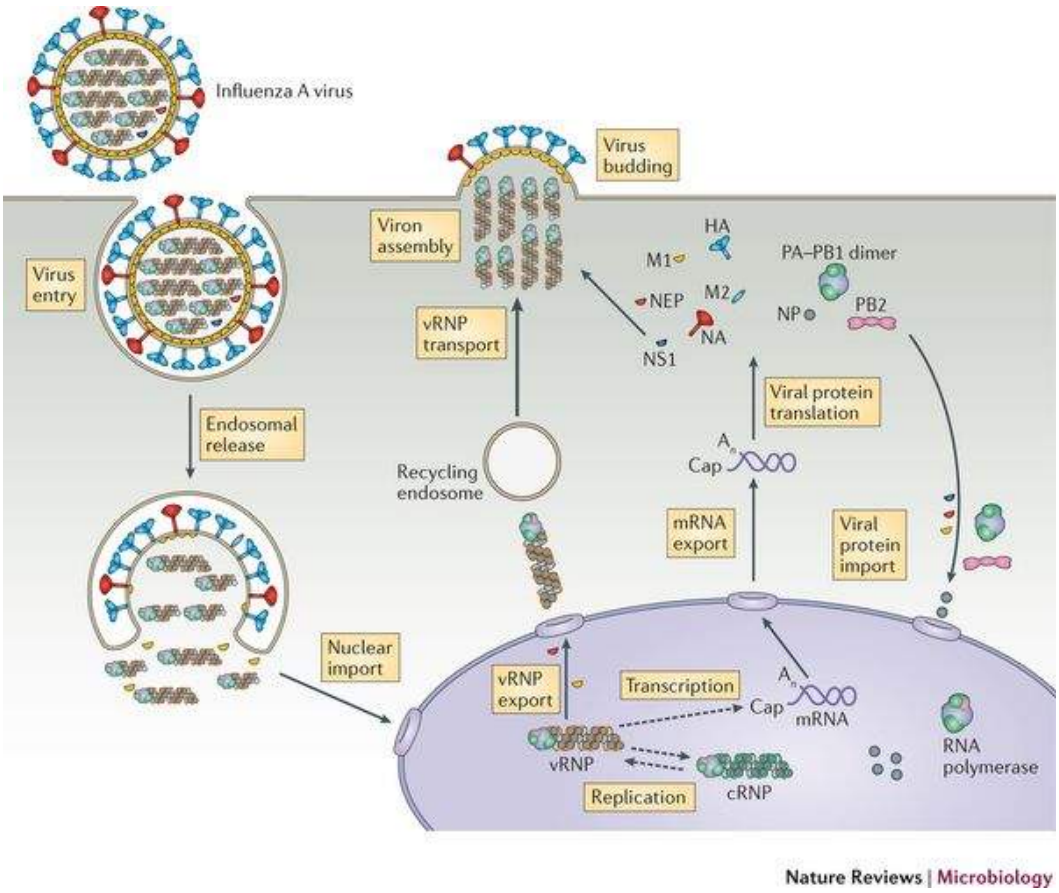

Figure L.1. Influenza A virus life cycle. (Te Velthuis and Fodor, 2016)

2. Immune response

2.1. Innate immunity

Infection of IAVs is sensed by innate immune cell through pattern-recognition receptors (PRRs) such as toll like receptors (TLRs) and retinoic acid inducible gene-1 (RIG-1) recognizing viral RNA as pathogen-associated marker pattern (PAMP) (Pang and Iwasaki, 2011). Signal from PRRs stimulate proinflammatory cytokines and type I interferons having antiviral activity of inhibiting protein synthesis and viral replication (Heil et al., 2004). Also, interferon stimulated genes (ISGs) like Mx gene having antiviral activity and dendritic cells (DCs) are stimulated by type I interferon (Holzinger et al., 2007). DCs detect virions and apoptotic bodies of infected cells by their dendrite extending between airway epithelial cell, and they migrate to draining lymph node to present antigen to T cell (GeurtsvanKessel and Lambrecht, 2008). Degraded viral protein by DCs (epitopes) presented by MHC class I transported to cell membrane and recognized by CD8+ cytotoxic T cells (CTL), and MHC clad II presenting epitopes are recognized by CD4+ T helper (Th) cells (Watts, 1997).

When alveolar macrophages are activated by infection of alveoli, macrophage phagocyte influenza virus infected cells and produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF-α) (Jayasekera et al.,

2006; Peper and Van Campen, 1995; Tumpey et al., 2005). The NOS2 and TNF-α causes pathology of influenza virus, and HPAI virus infect not only alveolar macrophages but also blood-derived macrophages that produce large amount of pro-inflammatory cytokines (Peiris et al., 2010).

Natural Killer (NK) cells recognize and lyse antibody bound influenza virus infected cells by cytotoxicity receptors, which is known as antibody dependent cell cytotoxicity (ADCC) (Mandelboim et al., 2001).

2.2. Adaptive immunity

2.2.1. Humoral immunity

Antibody responses to HA, NA, M and NP protein are induced by influenza virus infection, and anti-HA and NA antibodies are important for protective immunity (Johansson et al., 1989). The anti-HA antibodies neutralize influenza virus by binding to HA and inhibiting viral entry to cell, and mediate phagocytosis of virus by Fc receptor expressing cell and ADCC. However, influenza virus frequently mutates HA protein to evade humoral immunity and antibody formed against the relatively conserved stem region of HA have broad neutralizing ability (Ekiert et al., 2009). Unlike anti-HA antibodies, antibodies to NA protein does not have neutralizing ability but inhibit cleavage activity of NA and spread of progeny viruses (Webster et al., 1987; Webster et al., 1988).

Matrix protein 2 (M2) present in viral membrane protein at low concentration. The N-terminal ectodomain (M2e) of M2 protein exposed outside are highly conserved between other subtypes and antibodies to M2e allow broad cross-protection against influenza virus (Neirynck et al., 1999).

NP protein is internal protein having very conserved sequences among influenza viruses and attractive target for developing universal vaccine like M2e (Epstein et al., 2005; Shu et al., 1993). Although anti-NP antibodies do not neutralize influenza virus, these antibodies protect against influenza infection and induce complement mediated infected cell lysis (Fujimoto et al., 2016; Sambhara et al., 2001).

2.2.2. Cellular immunity

T cells are not able to protect against infection, but they reduced severity of disease by viral clearance. Naïve T cell expand and differentiate into variety of cells when they recognize antigen presented by MHC molecule of antigen presenting cell (APC) (Paul and Seder, 1994). Effector T cells migrate to lung for clearance of virus infected cells, and then memory T cells are established and respond to influenza virus re-infection.

CD4+ T cells are activated by recognizing antigen on MHC II molecule and polarized into T helper 1 (Th1) cells producing IFN-γ or T helper 2 (Th2) cells

secreting IL-4, IL-5 and IL-13 (Romagnani, 1994). The dominant function of CD4+ T cells is promoting B cell immunity and enhancing effector CD8+ T cell response through cell to cell interaction and secretion of cytokines (Swain et al., 2012). CD4 +T cells, called T_{FH} cells, help B cells in B cell follicles and efficient CD8+ T cell priming of APC through PRR-independent activation of APCs (Crotty, 2011). Additionally, CD4+ T cells develop into effector T cell and showed anti-viral functions. Effector CD4+ T cells secret cytokines regulating inflammation or activating macrophage to drive further inflammation, and directly lyse influenza infected cells through FAS-dependent and perforin-dependent manners (Brown et al., 2006; Román et al., 2002).

Viral clearance is mainly dependent of CD8+ T cell and the CD8+ cytotoxic T lymphocytes (CTL) recognize epitopes from conserved internal proteins like NP, M and polymerase proteins (Bender et al., 1992; Gotch et al., 1987). After antigen presented MHC I molecule of APC activates CD8+ T cell, primed CD8+ T cell divided into Tc1, Tc2 and Tc17 cells. Tc1 and Tc2 have Fas and perforin dependent cytolytic activity, and Tc17 recruit other immune cells by producing cytokines and chemokines (Hamada et al., 2013; Topham et al., 1997).

Following influenza virus clearance, the memory T cell immunity is established and rapidly react to subsequent influenza virus infection. The memory CD8+ T cells remain stable in the secondary lymphoid organs but they are also found in lung and other organs for a while (Masopust et al., 2001). In secondary

influenza infection, antigen-specific CD8+ T cell in lung mediate the first immune response that reducing viral amplification and the memory CD4+ T cell enhance protection from influenza infection by secreting IFN-γ (Hikono et al., 2006; Teijaro et al., 2010). Unlike neutralizing antibodies that are inefficient to protection of heterosubtypic strains, cellular immunity formed against relatively conserved internal proteins contributes to cross protection.

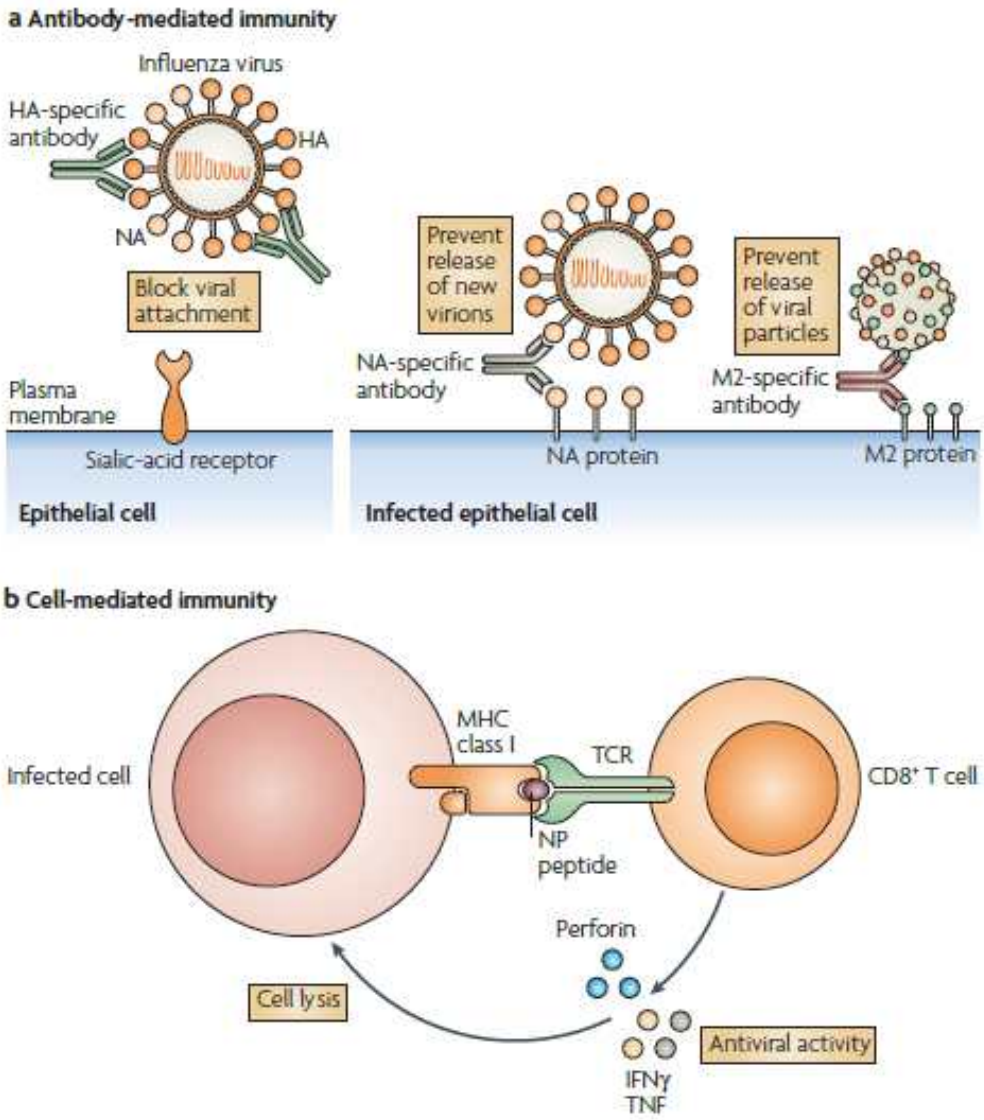

Figure L.2. The adaptive immune response during infection with influenza virus. (Subbarao and Joseph, 2007)

3. Avian influenza vaccine

3.1. Inactivated vaccine

The oil-emulsion inactivated whole AIV vaccine is the most widely used in poultry. The seed virus is selected among field strains and propagated in embryonated chicken eggs (ECEs). However, there are variation of replication efficiency in ECEs between AIVs and high antigen yield in ECEs is important for vaccine productivity and immunogenicity. Classically, a seed virus poorly growing in ECEs is passaged several times to increase quantity of antigen, but egg-adapted virus have potential of acquiring antigenic and pathogenic change (Choi et al., 2008a). Whole inactivated vaccines are generally cross-protective against diverse field strains and more immunogenic than other vaccine types because immune responses are stimulated not only in producing neutralizing HA and NA antibodies but also in cellular immunity about internal proteins (Lin et al., 2006; Swayne et al., 2000).

Commercial inactivated whole virus vaccines are prepared by chemical inactivation with formalin, β-propiolactone, or binary ethyleneimine, and produced as water-in-oil formulations. The oil emulsion vaccine is released slowly and continuously boost immune response. Also, local inflammation is induced at vaccine injecting site, which attract APCs and activate antigen specific humoral immunity (Jansen et al., 2005).

3.2. Recombinant vaccine generated by reverse genetics

Currently used inactivated whole virus HPAI vaccines in poultry are 6+2 recombinant vaccine generated by mixing HA and NA genes of circulating strain and six internal genes of good-growing strains (Li et al., 2014). Using reverse genetics technique allows rapid generation of vaccine seed virus and manipulating genomes to have desired traits such as mutating cleavage site of HA. Helper virus-independent plasmid-based reverse genetics system of AIVs needs co-transfection of plasmids for viral RNP with eight plasmids for viral RNA at first (Neumann and Kawaoka, 2002). However, recently developed reverse genetics system reduced numbers of transfecting plasmids by using bi-directional vector (Hoffmann et al., 2000a).

The H5 and H7 subtype live attenuated vaccines are not recommended in poultry because adaptation or reassortment with field strains have potential to make novel HPAI viruses. However, live LPAI vaccines can protect HPAI viruses and induced stronger immune response than inactivated vaccine (Beard, 2003). The problems like respiratory symptoms and egg-drop caused by live LPAI vaccination can be solved by the reverse genetics system (Lee and Suarez, 2005). The NS truncated AIVs showed decreased replication and attenuation of infectivity that are suitable traits for live LPAI vaccine because NS1 protein evade host immune system (Cauthen et al., 2007). Chimeric AIV vaccine expressing the HA-NA

ectodomain of NDV instead of NA of H5N1 AIVs generated by reverse genetics system provides immunity about both NDV and AIV (Park et al., 2006).

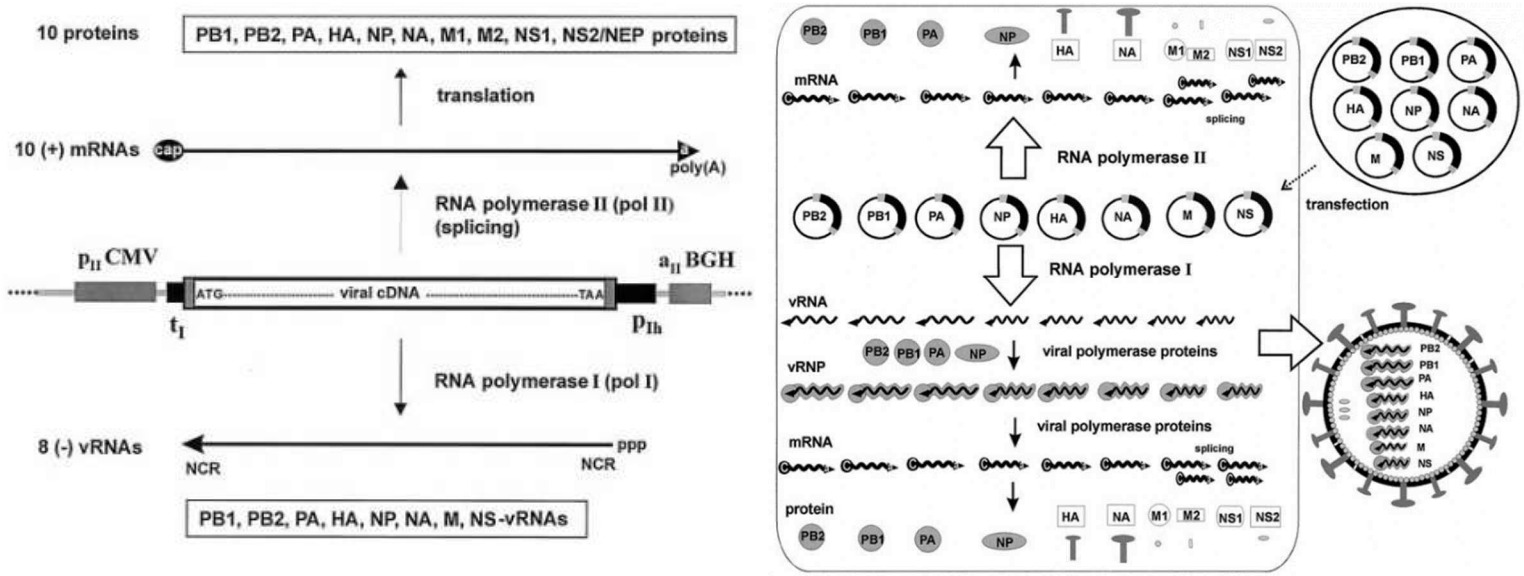

Figure L.3. Schematic representation of bi-directional plasmid and eight plasmid based reverse genetics system. (Hoffmann et al., 2000b)

3.3. AIV protein subunit vaccine

The HA protein is the most protective antigen to use subunit vaccines, and baculovirus expressed HA protein is protective in chickens (Chambers et al., 1988; Crawford et al., 1999). However, neutralizing antibodies to HA protein protect against closely matched HA and supplementation of NA protein induced balanced and broad immune response (Johansson et al., 2002). The M2 protein and NP protein have highly conserved sequence among AIVs and evaluated as universal vaccine, but they do not show enough protective activity in chickens (Epstein et al., 2005; Neirynck et al., 1999). However, baculovirus derived M1 protein produce virus-like-particles with HA and NA protein and it is immunogenic in muscovy ducks (Prel et al., 2007).

3.4. Vector vaccine expressing AI proteins

Vector vaccine induce rapid and broad immunity to bivalent antigens (vector virus and AIV), and have less risk in production process. Fowlpox virus (FPV) vector and Newcastle disease virus (NDV) vector are used for AI vector vaccine in poultry. FPV vector AI vaccines are designed to use in 1-day-old chick, and have several advantages; high level biosecurity of hatchery, automatic administration system (*in ovo*), and protection at younger age (Bublot et al., 2005). Also, FPV-based AI vaccine has short onset of protection and longer duration of

immunity (Swayne et al., 1997). NDV vector AI vaccine is administered by spray or drinking water, stimulating mucosal immunity (Ge et al., 2007). However, repeated administrations of NDV-based AI vaccine are need to provoke enough immunity in field conditions, and administration timing must be carefully considered to allow replication of NDV vector virus without inhibition of maternal antibodies against NDV.

3.5. DNA vaccine

Plasmid DNA vaccine expressing HA protein of AIVs provide protection in poultry and protective level is variable according to promoter, inserted gene, administration routes, and presence of adjuvants (Robinson et al., 1993; Suarez and Schultz-Cherry, 2000). The HA gene transcribed and translated in host cell, and HA protein is presented as antigen by MHC I molecule stimulating cytotoxic T cell. Thus, HA protein expressed DNA vaccine stimulate both humoral immunity and cellular immunity like live AI vaccine, but it is not used in poultry because of high manufacturing costs and multiple vaccinations requirement to achieve enough immunity.

Chapter I

Generation of highly productive recombinant H9N2 avian influenza viruses by 3′-end promoter optimization of polymerase genomes and NS genome replacement

Abstract

We developed an A/PR/8/34 (PR8) virus-based reverse genetics system in which six internal genes of PR8 and attenuated hemagglutinin and intact neuraminidase genes of field avian influenza viruses (AIVs) were used for generating highly productive recombinant vaccine strains. The 6+2 recombinant vaccine strains can induce protective humoral immunity against intended field AIVs; however, the epitopes of B and T cells encoded by internal genes may be important for heterosubtypic protection. Therefore, it is advantageous to use homologous internal genes of field AIVs for recombinant vaccine strains. However, rescue of recombinant viruses having whole internal genes of field AIVs using the PR8-based reverse genetics system was unsuccessful in some cases. Although partial replacement of an internal gene has been successful for generating highly productive and recombinant viruses that are non-pathogenic in mammals, complete replacement of internal genes may be more favorable. In this study, we successfully generated complete recombinant H9N2 AIVs possessing 8 genomes of H9N2 AIVs by the 3′-end promoter optimization of polymerase genomes, and the NS genome. All the generated recombinant viruses were highly productive but some of them showed high virus titers in embryonated chicken eggs. Additionally, we found that the same mutations of NS1 gene determined the pathogenicity of AIVs in chicken embryos as well as in mammals. Thus, 3′-end promoter optimization, and highly productive and mammalian nonpathogenic internal genes may be useful to develop vaccines against AIVs.

1.1. Introduction

Influenza A viruses (IAVs) have eight negative-sense, single-stranded RNA segments that encode polymerases (PB2, PB1, and PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural (NS) proteins (McCAULEY and Mahy, 1983). Non-coding regions (NCRs) are present at the 5′- and 3′-ends of the viral genomic RNA (vRNA), and the first 12 and 13 nucleotides at each end are highly conserved and play important roles in viral replication (Neumann et al., 2004). The fourth nucleotide at the 3′-end of vRNA is known as a promoter and an origin of replication, and genome segments with uridine at the fourth nucleotide of the promoter/origin (U4) have shown higher replication and transcription levels than those with cytidine (C4) (Jiang et al., 2010; Lee et al., 2017b; Lee et al., 2003).

Hoffmann's reverse genetics vector system is based on the high growth A/PR/8/34 (PR8) genome segments and possesses C4 in the polymerase genome segments (PB2, PB1, and PA) and U4 in the others (HA, NP, NA, M, and NS) (Hoffmann et al., 2002a; Hoffmann et al., 2001a). Therefore, recombinant viruses generated through this system have the same promoter constellation; however, the same constellation in different IAVs can cause different phenotypic results (Jiang et al., 2010; Lee et al., 2017b; Ping et al., 2015). Moreover, internal genes of PR8 are mostly used for recombinant vaccines because they are easily adaptable, its sequence is fully verified, and it shows high growth in embryonated chicken eggs (ECEs) (Webby et al., 2004b; Wood and Robertson, 2004). However, the use of internal genes homologous to those of field viruses is favorable to improve the heterosubtypic protection efficacy of vaccine strains because internal genes

are important for stimulating humoral and cellular immunity (Fujimoto et al., 2016; Townsend et al., 1984).

A/chicken/Korea/01310/2001(H9N2) (01310) was passaged 20 times (01310 CE20) through ECEs, and 01310 CE20 has been used as a low-pathogenic avian influenza (LPAI) vaccine strain in Korea (Choi et al., 2008a). However, rescue of a complete recombinant virus possessing 8 genomic segments of 01310 CE20 could not be achieved using Hoffmann's vector system. PB2 gene of 01310 CE20 is a prototypic avian gene with decreased integrity of the polymerase trimer and may cause reduced viral replication and pathogenicity in mammalian hosts (Kim et al., 2014a; Lee et al., 2017a). A human cell line, 293T, has been used for generating recombinant viruses by reverse genetics, but the host barrier may hinder replication of prototypic avian genomes thus resulting in unsuccessful virus rescue. Another Korea H9N2 strain, A/chicken/Korea/KBNP-0028/2000(H9N2) (0028), was also passaged 20 times in ECEs to increase productivity (Kwon et al., 2009). PR8-derived recombinant viruses possessing the PA or NS gene of 0028 did not cause a loss in body weight but could replicate in the lungs of BALB/c mice (Kim et al., 2014a; Kim et al., 2015a). Therefore, investigating the effect of less competent PA and NS genes on the rescue of recombinant 0028 strains in 293T cells may be valuable. Furthermore, 0028 did not cause mortality even after 72 h post inoculation via the allantoic cavity unlike 01310 CE20, and may improve the productivity of vaccine strains by increasing the virus titer and amount of harvested allantoic fluid (Kwon et al., 2009). WHO and the World Organization for Animal Health (OIE) also recommend low embryonic pathogenicity of vaccine strains (Dobbelaer et al., 2005; OIE, 2009). Therefore, investigations regarding

the molecular determinants affecting embryonic pathogenicity may be valuable to improve vaccine productivity.

In this study, we hypothesized that optimal combinations of C4 and U4 in polymerase genomes might compensate for less competent avian polymerases and facilitate the rescue and replication of complete recombinant H9N2 viruses possessing 6 internal genes of their own. We successfully rescued complete recombinant H9N2 viruses that are more replicative in ECEs. We also demonstrated the common roles of NS1 gene and its mutations in embryonic as well as mammalian pathogenicity.

1.2. Materials and Methods

Viruses, cells, eggs, and plasmids

The 01310 CE20 and 0028 (CE20) strains were propagated in 10-day old SPF ECEs (Charles River Laboratories, North Franklin, USA). The 01310 CE22 and 0028 CE21 strains have the same sequences as 01310 CE20 and 0028 CE20, and are used in this study. 293T, A549, and MDCK cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). The 293T and MDCK cells were maintained in DMEM (Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies), and A549 cells were maintained in DMEM/F12 supplemented with 10% FBS. To generate recombinant viruses, a Hoffmann vector system was used as described previously (Hoffmann et al., 2002a; Hoffmann et al., 2001a). Recombinant viruses were generated and passaged twice in 10-day old SPF ECEs and then stored at -80°C until experimental use.

Cloning and site directed mutagenesis of genes

Eight genome segments of 01310 CE22 and 0028 CE21 were amplified using Hoffmann's universal primer sets and cloned into Hoffmann's bi-directional transcription vector, pHW2000, as reported previously (Hoffmann et al., 2002a; Hoffmann et al., 2001a). The nucleotide sequences of the inserts were confirmed by sequencing with cmv-SF and bGH-SR primers listed in Table 1.1 (Macrogen Co, Seoul, Korea). The fourth nucleotide

in the 3′-ends of PB2, PB1, and PA genomes was mutated from C4 to U4 (C4U) by site-directed mutagenesis using the Muta-direct site directed mutagenesis kit (iNtRON Biotechnology, Seongnam-si, Korea). The mutagenesis primer sets used in this study are listed in Table 1.1. The mutated 0028 NS genome possessing a single amino acid mutation in NS1 [G139D, G139N, S151T, or PL motif mutation (GSEV to EPEV)] was previously cloned into the pHW2000 vector (Kim et al., 2015a); we used the same plasmids after confirming the sequence of the inserts (Macrogen Co, korea).

Table 1.1. Primer sets used for sequencing and mutagenesis of polymerase gene promoters

Primer	Sequence (5′–3′)
cmv-SF	TAAGCAGAGCTCTCTGGCTA
bGH-SR	TGGTGGCGTTTTTGGGGACA
mPB2-F	AGTTGGGGGGGA *AGCAAAA* GCAGGTCAAATA
mPB2-R	TATTTGACCTGC *TTTTGCT* TCCCCCCCAACT
mPB1-F	AGTTGGGGGGG *AGCAAAA* GCAGGCAAACCA
mPB1-R	TGGTTTGCCTGC *TTTTGCT* CCCCCCCAACT
mPA-F	GAAGTTGGGGGGG *AGCAAAA* GCAGGTACTGATC
mPA-R	GATCAGTACCTGC *TTTTGCT* CCCCCCCAACTTC

Generation of recombinant viruses by reverse genetics

Eight plasmids possessing eight genomes were transfected into 293T cells for generating recombinant viruses. One day before transfection, 293T cells were cultured in 6-well plates (5×10^5 cells/well) and 300 ng of each plasmid was transfected together into the cells using Lipofectamine and PLUS reagent (Life Technologies) in 1 mL of Opti-MEM (Life Technologies). After overnight incubation, 1 mL of Opti-MEM and 0.5 μg/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA) were added. After another overnight incubation, 0.2 mL of the harvested culture medium was inoculated into 10-day old SPF ECEs through the allantoic cavity. Allantoic fluid was harvested from inoculated ECEs after incubation at 37°C for 72h and checked for the presence and titer of recombinant viruses using a 96-well plate hemagglutination test with 1% (v/v) chicken red blood cells according to the WHO Manual on Animal Influenza Diagnosis and Surveillance. Transfection was repeated three times to generate recombinant viruses independently.

Titration of viruses

The suspension containing recombinant viruses (E1) was diluted 100-fold and inoculated into three 10-day old ECEs for virus propagation (E2). The harvested allantoic fluid was aliquoted and maintained at -80ºC until use. To measure the virus titer of allantoic fluid, each sample was serially diluted 10-fold and 0.1 mL of each dilution from 10^{-6} to 10^{-9} was inoculated into five 10-day old ECEs. The 50% chicken embryo infectious dose (EID_{50}/mL) was calculated using the Spearman-Karber method (Hamilton et al., 1977).

Mini-genome assay

Promoter activity of recombinant viruses was determined using a minigenome assay as described previously (Lee et al., 2017a). Briefly, 293T cells in 12-well plates were co-transfected with polymerase and NP genes of 01310 CE22, 0028 CE21, or PR8 together with pHW-NP-Luc and the Renilla luciferase plasmid pRL-TK (Promega, USA). After 24 h, luminescence was measured using the Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's instructions, on a TECAN Infinite200 pro machine (Tecan Benelux, Giessen, Netherlands). The results are shown as the average from three experiments, and the standard deviation.

Replication efficiency of recombinant viruses in MDCK cells

To compare the replication efficiency of recombinant viruses in MDCK cells, 10^7 EID50/0.1 mL of each recombinant virus was diluted 10-fold and inoculated into confluent MDCK cells in a 96-well plate (1×10^5 cells/well; 3 repeats for each diluted virus). After 72 h of incubation in a CO_2 incubator at 37°C, the 50% tissue culture infectious dose ($TCID_{50}$/mL) was calculated using the Spearman-Karber method (Hamilton et al., 1977).

Growth kinetics of recombinant 01310 CE22 viruses in A549 cells

The replication efficiency of AIVs in A549 cells is too low, and replication of the recombinant viruses was investigated by a growth kinetics study. Confluent A549 cells in a 6-well plate were infected with 1 multiplicity of infection of recombinant 01310 viruses. After 1 h of incubation, viruses were washed away with PBS, and 1 mL of fresh DMEM and 0.2 μg/mL TPCK-trypsin were added. During 3 days incubation in a CO_2 incubator at 37°C, the supernatant was harvested at 24, 48, and 72 h post-infection (hpi) and $TCID_{50}$ was measured.

Effect of NS genome on the pathogenicity and productivity of AIVs in ECEs

The pathogenicity of the recombinant virus was evaluated by measuring the mean death time (MDT) of chicken embryos (Hanson and Brandly, 1955). Each virus was diluted to 100 EID_{50}/0.1 mL and inoculated into ten 10-day old ECEs via the allantoic cavity. During incubation at 37°C, the eggs were candled twice a day and the death time of embryos was recorded. The MDT of each virus was calculated by average death time of all infected ECEs. To compare the productivity of recombinant viruses, the same experiment was performed as above except with 3 days of incubation and chilling of dead ECEs at 4°C. The volume and hemagglutination titer (HAT) of the harvested allantoic fluid were then measured and multiplied to calculate the total HAT of each virus.

Statistical analysis

Polymerase activity and virus titer were compared using one-way analysis-of-variance (IBM SPSS Statistics ver. 23; IBM, USA). Results were considered statistically significant if $p < 0.05$ and $p < 0.001$.

1.3. Results

Generation of recombinant 01310 CE22 and 0028 CE21 viruses by C4U mutation of promoter/origin in polymerase genomes

The PR8-derived 01310 CE22 virus (r310-PR8) possessing 6 internal genes of PR8 was generated in every transfection trial; however, we could not generate r310-C4 possessing 6 internal genes of 01310 CE22. The C4U mutations PB2 and/or PB1 (r310-U4-PB21, r310-U4-PB2, and r310-U4-PB1), PB1/PA (r310-U4-PB1A), and PB2/PB1/PA (r310-U4) facilitated virus rescue. However, the U4 promoter mutation in only PA and in both PB2 and PA (r310-U4-PA and r310-U4-PB2A) did not facilitate virus rescue (Table 1.2). In contrast, recombinant 0028 CE21 viruses (r0028-C4, r0028-U4-PB2, r0028-U4-PB1, r0028-U4-PA, r0028-U4-PB21, r0028-U4-PB1A, r0028-U4-PB2A, and r0028-U4) were successfully generated in all kinds of promoter combinations together with PR8-derived 0028 CE21 virus (r0028-PR8) (Table 1.3).

Replication efficiency of recombinant 01310 CE22 and 0028 CE21 viruses in ECEs

Titers of the generated recombinant viruses were measured in ECEs. The titers (log10 EID_{50}/mL) of recombinant 01310 CE22 viruses ranged from 8.92 ± 0.14 to 9.58 ± 0.29, and the titers of r310-U4-PB1 and r310-U4-PB21 were higher than those of the others including r310-PR8 (Table 1.2). However, an additional C4U mutation in the PA of these two viruses (r310-U4-PB1A and r310-U4) decreased their titer in ECEs (Table 1.2). The titers of recombinant 0028 CE21 viruses ranged from 8.67 ± 0.29 to 10.13 ± 0.15, and the titers of r0028-U4-PB21 and r0028-PR8 were higher than those of the others (Table 1.3). Therefore, C4U mutations in both PB2 and PB1 increased the titers of both recombinant 01310 CE22 and 0028 CE21 viruses in ECEs.

Table 1.2. Genotype, rescue, and titer of recombinant 01310 CE22 viruses

Recombinant virus	3′-end promoter[a]			Internal genes[b]	Rescue[c]	EID_{50}/mL (log_{10}) [d]
	PB2	PB1	PA			
r310-PR8	C4	C4	C4	PR8	+	8.83 ± 0.14
r310-C4	C4	C4	C4	01310 CE20	-	NT
r310-U4-PB2	U4	C4	C4	01310 CE20	+	9.00 ± 0.43
r310-U4-PB1	C4	U4	C4	01310 CE20	+	9.58 ± 0.29[e]
r310-U4-PA	C4	C4	U4	01310 CE20	-	NT
r310-U4-PB21	U4	U4	C4	01310 CE20	+	9.50 ± 0.50[e]
r310-U4-PB1A	C4	U4	U4	01310 CE20	+	9.00 ± 0.00
r310-U4-PB2A	U4	C4	U4	01310 CE20	-	NT
r310-U4	U4	U4	U4	01310 CE20	+	8.92 ± 0.14

[a] C4: the fourth nucleotide of the 3′-end promoter is cytidine, weak promoter; U4: the fourth nucleotide of the 3′-end promoter is uridine, strong promoter.

[b] The origin of six internal genes (PB2, PB1, PA, NP, M, and NS).

[c] The result of virus rescue after three times transfection; +: successful (3/3), -: unsuccessful (0/3).

[d] EID_{50}/mL, mean 50% chicken embryo infection dose/mL with standard deviation; NT, not tested.

[e] Significant difference from other recombinant viruses ($p < 0.05$).

Table 1.3. Virus rescue and titer of recombinant 0028 CE21 viruses

Recombinant virus[a]	Rescue[b]	EID_{50}/mL (log_{10})[c]
r0028-PR8	+	9.95 ± 0.14[d]
r0028-C4	+	8.67 ± 0.29
r0028-U4-PB2	+	9.08 ± 0.18
r0028-U4-PB1	+	9.10 ± 0.30
r0028-U4-PA	+	8.69 ± 0.13
r0028-U4-PB21	+	10.13 ± 0.15[d]
r0028-U4-PB1A	+	8.93 ± 0.21
r0028-U4-PB2A	+	9.25 ± 0.50
r0028-U4	+	8.75 ± 0.25

[a] C4: the fourth nucleotide of the 3′-end promoter is cytidine, weak promoter; U4: the fourth nucleotide of the 3′-end promoter is uridine, strong promoter.

[b] The origin of six internal genes (PB2, PB1, PA, NP, M, and NS).

[c] EID_{50}/mL, mean 50% chicken embryo infection dose/mL with standard deviation.

[d] Significant difference from other recombinant viruses ($p < 0.05$)

Comparison of polymerase activity between different combinations of C4U mutations in polymerase genomes

To investigate the effects of different combinations of C4U mutations in polymerase genomes on virus rescue, polymerase activity was compared using a mini-genome assay (Fig. 1.1). The polymerase activities of 01310 CE22 and 0028 CE21 mini-genomes were considerably weaker than that of PR8. It was difficult to differentiate between the activities of virus rescue-facilitating (r310-U4-PB2, r310-U4-PB1, r310-U4-PB1A, r310-U4-PB21, and r310-U4) and non-facilitating (r310-C4 and r310-U4-PB2A) C4U combinations of polymerases. Although the relative luciferase activities of both 01310 CE22 and 0028 CE21 mini-genomes were very low, all the 0028 CE21 mini-genomes showed slightly higher activities than those of the 01310 CE22 mini-genomes. Thus, the polymerase activity of 0028 CE21 may be enough to produce recombinant 0028 CE21 viruses independent of the combinations of C4U mutations.

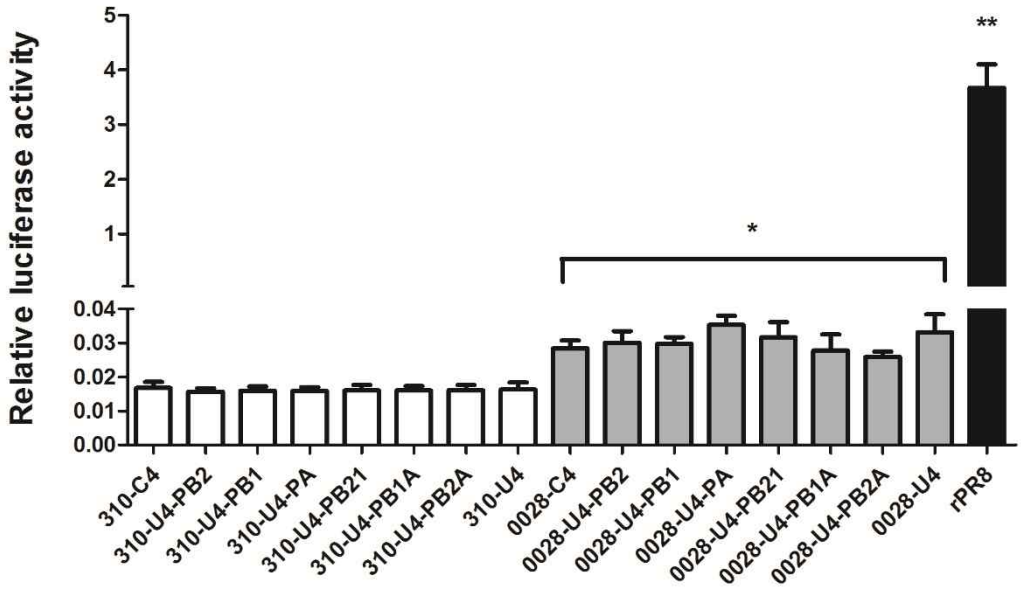

Figure 1.1. Comparison of polymerase activity from 01310 CE22 and 0028 CE21 mini-genomes with different combinations of C4U mutations in the polymerase genomes. pHW plasmids possessing polymerase genes (PB2, PB1, and PA) and NP were transfected into 293T cells with pHW-NP-Luc and the Renilla luciferase plasmid pRL-TK, and luminescence was measured. *, significant difference from the 01310 CE22 mini-genome ($p < 0.05$); **, significant difference from the 01310 CE22 and 0028 CE21 mini-genomes ($p < 0.05$).

Comparison of replication efficiency of recombinant viruses in MDCK and A549 cells

As recombinant 01310 CE22 and 0028 CE21 viruses were successfully generated from 293T cells, we evaluated their replication efficiency in mammalian cell lines, MDCK and A549 cells. Although no significant difference in virus titer was observed between the recombinant and parent viruses, recombinant 01310 CE22 viruses showed higher titers than the 01310 CE22 virus in MDCK cells (Fig. 1.2). Furthermore, the titers of recombinant 01310 CE22 viruses were higher than that of recombinant PR8. However, recombinant 0028 CE21 viruses except for r0028-PR8 showed significantly lower virus titers than recombinant 01310 CE22 and PR8 viruses. For this reason, the viral replication efficiency of recombinant 01310 CE22 viruses in A549 cells was further evaluated. All the recombinant and parent 01310 CE22 viruses could not replicate in A549 cells during 72 hpi; however, recombinant PR8 virus showed the highest titer within 48 hpi (Fig. 1.3).

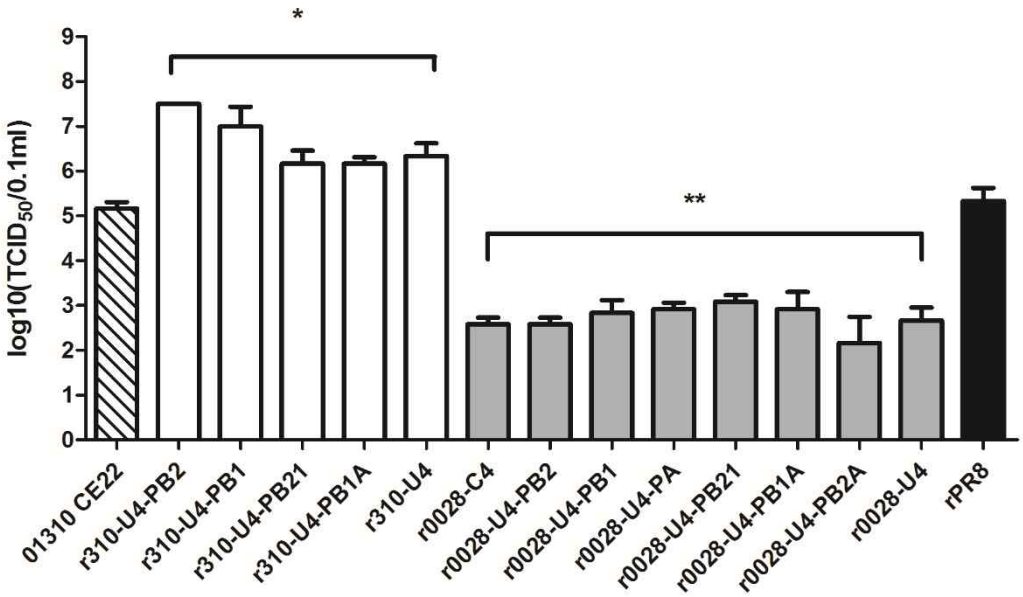

Figure 1.2. Comparison of virus titers of recombinant 01310 CE22 and 0028 CE21 viruses in MDCK cells. 1×10^7 EID_{50}/100 μl of each virus was diluted 10-fold and inoculated into confluent MDCK cells. After incubation at 37°C for 72 h, the virus titer ($TCID_{50}$/0.1 mL) was calculated. *, significant difference from parental 01310 CE22 and recombinant 0028 CE21 viruses; **, significant difference from other viruses ($p < 0.05$).

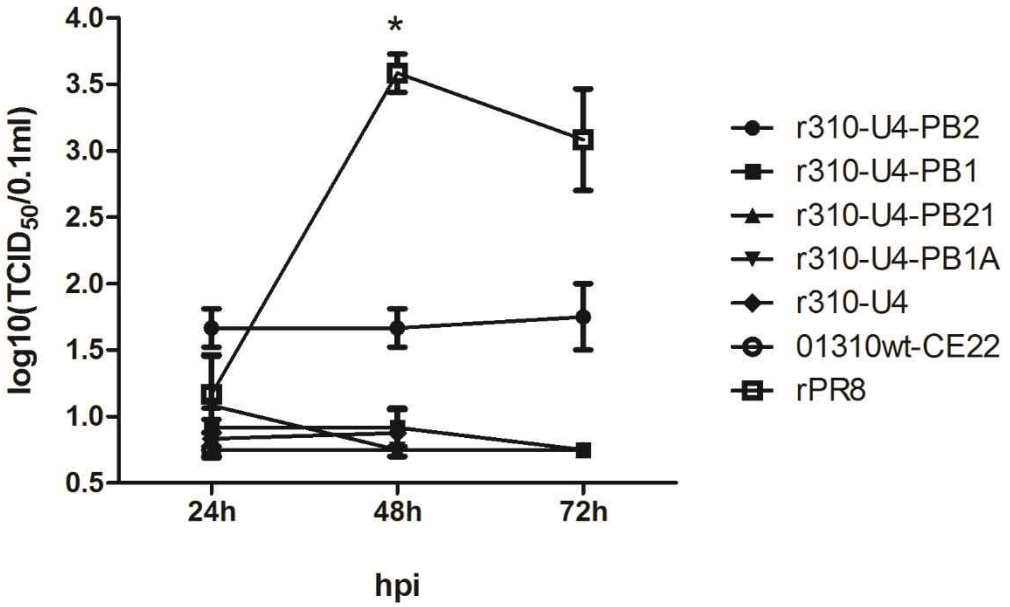

Figure 1.3. Growth kinetics of recombinant 01310 CE20 viruses in A549 cells. One multiplicity of infection (moi) of each virus was inoculated into confluent A549 cells and the supernatant was harvested at 24, 48, and 72 h post-inoculation (hpi). $TCID_{50}$ in the supernatant was determined in MDCK cells. *, significant differences from other viruses at 48 hpi ($p < 0.05$).

Effect of 0028 NS genome on the pathogenicity and productivity of recombinant 01310 CE22 viruses in ECEs

Previously, we demonstrated that mutation of mammalian non-pathogenic 0028 NS1, GSEV to EPEV, S151T, and G139D increased the mammalian pathogenicity of PR8-derived recombinant viruses possessing mutated 0028 NS1 genes, and the mammalian pathogenic mutations are shared by the 01310 NS1 gene (Kwon et al., 2009). To investigate the effect of NS genome on the embryonic pathogenicity of 01310 CE22, we generated recombinant 01310 viruses by replacing the NS genome with the parental (mammalian non-pathogenic) or mutated (mammalian pathogenic) 0028 NS genome. Recombinant viruses with the parental (r310-NS28) and mutated 0028 NS genomes (r310-NS28-EPEV, r310-NS28-S151T, r310-NS28-G139D, and r310-NS28-G139N) were also generated by C4U mutations in PB2 and PB1 genomes. The MDT of each recombinant virus was measured to compare their embryonic pathogenicity (Table 1.4). r310 was pathogenic and caused embryonic death within 48.8 ± 8 h, whereas r310-NS28 did not cause embryonic death even after 72 h incubation (94.4 ± 5). Recombinant viruses possessing EPEV (r310-NS28-EPEV), S151T (r310-NS28-S151T), G139D (r310-NS28-G139D), and G139N (r310-NS28-G139N) mutations showed a lower MDT than that with r310-NS28. Although the titers of r310-NS28-EPEV, r310-NS28-S151T, and r310-NS28-G139D were significantly less than those of r310 r310-NS28 and r310-NS28-G139N, they showed titers similar to r310 (Table 1.4). To compare the productivity of r310 and r310-NS28 in ECEs, the total HA units were calculated by multiplying the HA unit/mL and the volume of the harvested allantoic fluid (Table 1.5). The total volume and HAU of r310 was less than that

of r310-NS28. Thus, r310-NS28 was more productive than r310 in ECEs.

Table 1.4. Mean death time (MDT) and virus titer of 01310 CE22-derived recombinant viruses

Recombinant virus	MDT (Mean Death Time, h)[c]	Virus titer (log_{10} EID_{50}/mL)[d]
r310	48.8 ± 8	9.17 ± 0.29
r310-NS28	94.4 ± 5[a]	9.37 ± 0.12
r310-NS28-EPEV	61.6 ± 6[a]	8.70 ± 0.23[b]
r310-NS28-G139D	75.7 ± 1[a]	8.40 ± 0.12[b]
r310-NS28-G139N	82 ± 2.5[a]	9.33 ± 0.24
r310-NS28-S151T	67.6 ± 3[a]	8.53 ± 0.39[b]

[a, b] Significant difference from r310 ($p < 0.05$).

Table 1.5. Comparison of virus productivity between r310 and r310-NS28 in ECEs.

Harvested allantoic fluid	r310	r310-NS28
Volume (mL)[a]	11.9	13.3[d]
HAU (log 2)[b]	8.3	8.8
Total HAU[c]	3975.7	6412.8[d]

[a] Mean volume of harvested allantoic fluid from 10 ECEs.

[b] Mean hemagglutination units (HAU)/25 μL of allantoic fluid from 10 ECEs.

[c] Median total HAU of allantoic fluid from 10 ECEs.

[d] Significant difference from r310 ($p < 0.05$).

1.4. Discussion

H9N2 AIVs became endemic in Asia, the Middle East, and North Africa after severe outbreaks in poultry during the 1990s (Alexander, 2007). They caused direct infection in humans and provided internal genes to human-lethal H5N1, H5N6, H7N9, and H10N8 AIVs (Gu et al., 2014; RahimiRad et al., 2016). Vaccines against H9N2 AIVs have been developed and used in poultry farms (Choi et al., 2008a; Sun et al., 2012). However, new vaccine strains have been recommended due to the appearance of antigenic variants (Lee and Song, 2013).

A PR8-based reverse genetics vector system has been successfully used for the development of vaccines for humans and animals. As the 6 internal genes of PR8 have improved the replication efficiency of 6+2 vaccine strains in ECEs, they have been favorably used for vaccine development. However, the internal genes of PR8 contain various mammalian pathogenicity related mutations including the E627K mutation in PB2 (Kim et al., 2014a; Kim et al., 2015a; Lee et al., 2017a; Lee et al., 2017b). Therefore, the innate mammalian pathogenicity of internal genes could be inherited by the vaccine strains. Although inactivated vaccine strains are produced under controls of strict biosecurity measures, mammalian non-pathogenic vaccine strains may be more favorable (Liu et al., 2012).

Single replacement of the PB2 genomes of PR8 with 01310 CE20 increased the replication efficiency of recombinant viruses in ECEs with decreased pathogenicity in BALB/c mice (Jang et al., 2017a; Kim et al., 2014a). The heterosubtypic protection efficacy of internal proteins may encourage efforts to replace all the internal genes of PR8,

but optimal 6 avian internal gene sets supporting the generation of highly replicative and mammalian non-pathogenic recombinant viruses are not frequent (Liu et al., 2012; Shi et al., 2007).

In this study, we successfully generated a complete recombinant 01310 virus by optimization of C4U mutations in polymerases. The C4U mutation of the PB2 or PB1 genome may be the minimum essential to compensate the incompetent PB2 activity of 01310. However, C4U mutation only in the PA genome was not sufficient for virus rescue and even inhibited virus rescue when paired with a C4U mutation of the PB2 genome. PB2 is imported from the cytoplasm to the nucleus independently, but PB1 and PA form a dimer in the cytoplasm for efficient import into the nucleus. PB2 forms a trimer with the PB1/PA dimer in the nucleus (Huet et al., 2010). The PB2 of 01310 CE20 was suspected to decrease the integrity of the polymerase trimer and we speculated that the low integrity could be overcome by increased expression of PB2 and PB1 (Lee et al., 2017a).

No effect of C4U mutation in the PA genome supports the importance of PB1 in nuclear translocation and trimer formation, but the negative effect of the C4U mutation in PA together with PB2 was unexpected. Recently, PA-X generated by ribosomal frameshift of PA was reported to contain only endonuclease activity (Jagger et al., 2012). PA-X spreads in the nucleus and cytoplasm, and degrades host mRNAs (Hayashi et al., 2016). Despite the contradiction, PA-X showed generally negative effects on viral replication and pathogenicity (Gao et al., 2015; Jagger et al., 2012). Although we did not examine this in detail, we speculate that increased PA expression might also increase PA-X expression and its negative effects. Additionally, more efficient replication of r310-U4-PB21 and r0028-

U4-PB21 than that of r310-U4 and r0028-U4 in ECEs may also support the negative effect of increased PA and PA-X concentration (Ping et al., 2015). However, C4U mutations in all polymerases notably conferred positive and neutral effects on the replication efficiency of WSN and PR8 in mammalian cells, respectively (Jiang et al., 2010; Ping et al., 2015). Therefore, these effects may be strain-specific.

Although C4U mutation in polymerases of 01310 PB2 facilitated virus rescue, we could not find any difference in polymerase activity between the virus rescue-facilitating and non-facilitating mutations in 293T cells (Fig. 1.1). However, the titers of recombinant viruses possessing the virus rescue-facilitating mutations were higher than those of 01310 CE22 in MDCK cells (Fig. 1.2). Interestingly, the polymerase activities of 0028 CE21-derived mini-genomes were significantly higher than those of 01310 CE22-derived mini-genomes; however, the virus titers of 0028 CE21-derived recombinant viruses were significantly lower than those of 01310 CE22-derived recombinant viruses in MDCK cells. The relatively high polymerase activities of 0028 CE21 mini-genomes may be due to more competent PB2 compared to that of 01310 CE22 mini-genomes and the low replication efficiency of 0028 CE21-derived recombinant viruses can be explained by the low activities of PA and NS1 in mammalian hosts (Kim et al., 2014a; Kim et al., 2015a). Therefore, virus rescue and efficient replication of AIVs in different mammalian cells may be affected by multigenic traits.

MDCK cells are permissible to both avian and mammalian IAVs owing to the expression of both avian and human receptors, and the missing anti-viral activity of Mx proteins (Seitz et al., 2010). However, A549 cells express only human receptors and

possess active MxA, a potent antiviral protein (Holzinger et al., 2007). BALB/c mice have been used for the mammalian pathogenicity evaluation of AIVs but are an incomplete model owing to their lack of Mx proteins (Staeheli et al., 1988). For these reasons, A549 cells have been used for evaluating the preliminary mammalian pathogenicity of IAVs (Lee et al., 2017a; Taft et al., 2015). Therefore, we verified the preliminary mammalian pathogenicity of recombinant 01310 CE22 viruses using the A549 cell model. In contrast to recombinant PR8, all the recombinant 01310 CE22 and parent 01310 CE22 viruses did not replicate in A549 cells, and may have lower mammalian pathogenicity compared to other viruses replicating in A549 cells.

The embryonic pathogenicity of highly pathogenic (HP) AIVs is higher than that of low pathogenic (LP) AIVs due to systemic replication of HP AIVs, which is supported by multi-basic amino acids in the cleavage site of HA (Scholtissek et al., 1988). However, the reason why some LPAIVs such as 01310 CE20 show embryonic pathogenicity without multi-basic amino acids has been unclear (Choi et al., 2008a). In this study, we demonstrated the role of NS genome in the embryonic pathogenicity of AIVs. NS1 protein is a virulence factor that antagonizes host antiviral responses mediated by type I interferons (IFN) and IFN-induced proteins (Hale et al., 2008a). Several mammalian pathogenic mutations in NS1 gene were reported in a previous study (Kim et al., 2015a) and interestingly, mammalian pathogenicity related mutations in NS1 such as GSEV to EPEV, S151T, and G139D also increased the embryonic pathogenicity. To our knowledge, this is the first report demonstrating the relationship between NS1 and embryonic pathogenicity, and sharing of the same mutations for embryonic and mammalian pathogenicity .

In this study, we generated highly productive H9N2 vaccine strains by optimizing the 3′-end promoter of polymerase genomes and NS genome replacement. The optimization method and internal genes investigated in this study may be useful for generating more productive and safer vaccine strains against HPAIVs as well as antigenic variants of H9N2 AIVs.

Chapter II

Bioengineering a highly productive vaccine strain in embryonated chicken eggs and mammals from a non-pathogenic clade 2·3·4·4 H5N8 strain

Abstract

The clade 2.3.4.4 H5Nx is a highly pathogenic avian influenza (HPAI) virus, which first appeared in China and has spread worldwide since then, including Korea. It is divided into subclades a - d, but the PR8-derived recombinant clade 2.3.4.4 a viruses replicate inefficiently in embryonated chicken eggs (ECEs). High virus titer in ECEs and no mammalian pathogenicity are the most important prerequisites of efficacious and safer vaccine strains against HPAI. In this study, we have synthesized hemagglutinin (HA) and neuraminidase (NA) genes based on the consensus amino acid sequences of the clade 2.3.4.4a and b H5N8 HPAIVs, using the GISAID database. We generated PR8-derived H5N8 recombinant viruses with single point mutations in HA and NA, which are related to efficient replication in ECEs. The H103Y mutation in HA increased mammalian pathogenicity as well as virus titer in ECEs, by 10-fold. We also successfully eradicated mammalian pathogenicity in H103Y-bearing H5N8 recombinant virus by exchanging PB2 genes of PR8 and 01310 (Korean H9N2 vaccine strain). The final optimized H5N8 vaccine strain completely protected against a heterologous clade 2.3.4.4c H5N6 HPAIV in chickens, and induced hemagglutination inhibition (HI) antibody in ducks. However, the antibody titer of ducks showed age-dependent results. Thus, H103Y and 01310 PB2 gene have been successfully applied to generate a highly productive, safe, and efficacious clade 2.3.4.4 H5N8 vaccine strain in ECEs.

2.1. Introduction

Clade 2.3.4.4 H5Nx highly pathogenic avian influenza viruses (HPAIVs) with different neuraminidase (NA) subtypes have emerged in China and spread globally through multiple genetic reassortments of clade 2.3.4.4 H5N1 and other subtypes (Claes et al., 2016; Lee et al., 2015; Lee et al., 2014; Smith et al., 2015a). Clade 2.3.4.4 H5N8 HPAIV was first reported in Korea in early 2014 (Lee et al., 2014) and since then, several subclades (a to d) have been reported as of 2017 (Kwon et al., 2018). H5N8 HPAI clades 2.3.4.4a and b were detected in 2014. Clade 2.3.4.4a viruses are predominant and have caused substantial economic loss in the Korean poultry industry (Song et al., 2017). Five different reassorted H5N6 HPAI clade 2.3.4.4c viruses were isolated from poultry and wild birds in Korea in 2016. The clade 2.3.4.4b H5Nx viruses spread worldwide from 2014 to 2018 (Kwon et al., 2018; Kwon et al., 2017a; Lee et al., 2017d). Clade 2.3.4.4 HPAI viruses cause asymptomatic infection in Muscovy ducks and fatal infections in chickens and humans (Kang et al., 2017; Lee et al., 2016b; Yang et al., 2015).

A/Puerto Rico/8/1934(H1N1) (PR8)-derived clade 2.3.4.4 (6:2 reassortant) and H5N8 vaccines have been developed for poultry and humans. They have been evaluated concerning their efficacy in protecting from death caused by homologous and heterologous viruses (Jin et al., 2018; Kandeil et al., 2018; Lee et al., 2019; Santos et al., 2017). PR8-derived vaccine strains contain six internal protein coding genes of PR8 that confer high replication efficiency to embryonated chicken eggs (ECEs). The PB2 gene is one of the most important mammalian pathogenicity-determinants in AIVs and various mutations

have been identified in this gene (Subbarao et al., 1993; Taubenberger et al., 2005). The PB2 gene in PR8 has even more mammalian pathogenicity-related mutations (E627K, A199S, A674T, T271A, and A588T) than those of the 1918 pandemic H1N1 virus (E627K, A199S, and K702R). Thus, the potential mammalian pathogenicity of conventional PR8-derived vaccine strains against HPAI need to be improved (Lee et al., 2017a; Taubenberger et al., 2005). In addition, the E627K mutation negatively affects efficient replication in ECEs (Lee et al., 2017a). The PB2 gene of the A/chicken/Korea/01310/2001(H9N2) (01310) was successfully used for the generation of PR8-derived vaccine strains with high replication efficiency in ECEs and mammalian non-pathogenicity (An et al., 2019a; Choi et al., 2008b; Jang et al., 2017b; Kim et al., 2015b).

In order to increase virus titers of HPAIVs, successive virus passage through ECEs has been used and various mutations in hemagglutinin (HA) and neuraminidase (NA) have been identified (Choi et al., 2008b; Kim et al., 2013a; Kwon et al., 2009). HPAIVs replicate with high efficiency in ECEs due to polybasic amino acid residues at the cleavage site of HA. Few studies have sought to improve HA and NA genes in HPAIVs for better replication efficiency in ECEs (Stieneke-Grober et al., 1992). The H103Y mutation increases the acid stability of HA by decreasing the pH at which HA undergoes irreversible conformational changes. This mutation is regarded as a mammalian pathogenicity-related mutation, which facilitates air-borne transmission of H5N1 AIVs between mammals (de Vries et al., 2014a; Herfst et al., 2012; Linster et al., 2014; Zhang et al., 2013a). However, H103Y was also postulated to increase replication efficiency in ECEs, and a restricted role of H103Y in mammalian hosts was suspected (Kim et al., 2013a; Richard et al., 2017).

In this study, we aimed to engineer an optimized PR8-derived clade 2.3.4.4 H5N8 HPAI vaccine strain to be highly replicative in ECEs and non-pathogenic to mammalian hosts. We synthesized the consensus HA and NA genes from clade 2.3.4.4 H5Nx HPAIV genes, and inserted single point mutations into genes related to high replication efficiency (H103Y, K161E, and L317P in HA; S369N in NA) (Kim et al., 2013a). We generated PR8-derived parent and mutant H5N8 recombinant viruses with PB2 genes of PR8 or 01310, and compared the replication efficiency in ECEs and mammalian pathogenicity in mouse and mammalian cells. We also evaluated the immunogenicity of the selected vaccine strain in specific pathogen-free (SPF) chickens and commercial ducks, and the protective efficacy in SPF chickens by infecting them with a heterologous clade 2.3.4.4c H5N6 HPAIV.

2.2. Materials and Methods

Plasmids, cells, and eggs

Recombinant H5N8 viruses were generated using the Hoffmann reverse genetics vector system and the bidirectional pHW2000 vector (Hoffmann et al., 2000b). The 293T, MDCK, and A549 cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). HEK293T and MDCK cells were maintained in Dulbecco's modified Eagles' medium (DMEM) containing 10% fetal bovine serum (FBS, Life Technologies Co., Carlsbad, CA, USA) and were used for plasmid transfection and recombinant virus generation. A549 cells were maintained in DMEM/F12 supplemented with 10% FBS (Life Technologies). The recombinant viruses generated were used in experiments after three passages in 10 day-old SPF ECEs (Charles River Laboratories, North Franklin, CT. USA). All recombinant viruses were confirmed by RT-PCR and genome sequencing.

Synthesis and cloning of consensus HA and NA genomes and site-directed mutagenesis

The Global Initiative on Sharing All Influenza Data (GISAID) and Influenza Research Database (IRD) databases were used to obtain the HA and NA genome sequences of subtype H5N8 clade 2.3.4.4 HPAIVs isolated in Asia from

2014 to 2016 (n = 80). They are listed in Supplementary Table 2.S1. The BioEdit program (v7.2.5) was used for nucleotide sequence translation, amino acid comparison, and calculation of amino acid identity. The HA and NA sequences were compared and the consensus amino acid sequences were synthesized on the basis of the HA and NA genomes of A/broiler duck/Korea/Buan2/2014(H5N8) (Buan2) (Cosmo Genetech, Seoul, Korea). The multi-basic cleavage site of the HPAI clade 2.3.4.4 HA gene was substituted by a nucleotide sequence coding for ASGR in the synthetic HA genome for virulence attenuation, as previously reported (Jang et al., 2017b). Amino acid at position 499 (H5, Buan2, numbering) was mutated from asparagine (N) to threonine (T) to remove putative N-glycosylation. This likely decreases immunogenicity of the universal epitope in the stalk region of HA2 without affecting replication efficiency (Ekiert et al., 2009; Kwon, 2014). Synthetic HA and NA genomes were cloned into the pHW2000 vector using universal primer sets. Site-directed mutagenesis was performed to introduce mutations related to replication efficiency of HA (H103Y, K161E and L317P) and NA (S369N) with a commercial kit (iNtRON Biotechnology, Gyeonggi-do, Korea) as previously reported (Hoffmann et al., 2001b; Kim et al., 2013a). The mutagenesis primers used in this study are listed in Table 2.1.

Table 2.1. Primer sets used for the single point mutagenesis of the consensus H5N8 genome

Primer[a]	**Sequence (5'- 3')**
H5N8 – hmH103Y – F	TGAAGA ACTGAAATACCTATTGAGCAGAATA
H5N8 – hmH103Y – R	TATTCTGCTCAATAGGTATTTCAGTTCTTCA
H5N8 – hmK161E – F	CATACCCAACAATAGAAATAAGCTACAATAA
H5N8 – hmK161E – R	TTATTGTAGCTTATTTCTATTGTTGGGTATG
H5N8 – hmL317P – F	CTTGCGACTGGGCCCAGAAATAGTCCTC
H5N8 – hmL317P – R	GAGGACTATTTCTGGGCCCAGTCGCAAG
H5N8 – nmS369N – F	GTCGAACCTCCAGAAATGGATTTGAAATAATAAG
H5N8 – nmS369N – R	CTTATTATTTCAAATCCATTTCTGGAGGTTCGAC

[a]hm primer sets were used for HA mutation, and nm primer sets were used for NA mutation

Recombinant virus generation

PR8-derived H5N8 recombinant viruses were generated by combining eight genome segments and cloning them into a bidirectional reverse genetics vector (pHW2000) as previously described (An et al., 2019a). The PB2 genome of PR8 was replaced using the pHW2000 plasmid containing the 01310 PB2 gene, as previously described (Kim et al., 2014b). Briefly, 300 ng each of the eight plasmids was mixed with Lipofectamine 2000 and Plus reagents (Life Technologies) and transfected into 293T cells (1×10^6 cells/well in a 6-well plate). Following overnight incubation, 1 ml of Opti-MEM (Life Technologies) and 4 μg/well of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) were added and incubated for another 24 h. 200 μl of the harvested culture media were injected into 10-day-old SPF ECEs via the allantoic cavity. Inoculated ECEs were incubated for 3 days with candling twice a day, after which the allantoic fluid was harvested. The presence of recombinant virus was confirmed by the HA assay using 1% (v/v) chicken red blood cells (RBCs), according to the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance, and specific nucleotide sequences of recombinant viruses were confirmed by RT-PCR and sequencing (Kim et al., 2014b; Kim et al., 2013a).

Virus titration

The rescued viruses were inoculated into 10-day-old SPF ECEs and harvested (embryo passage 2, E2). The E2 viruses were serially diluted from 10^{-1} to 10^{-9} and injected into five 10-day-old SPF ECEs to determine viral titer. The 50% chicken embryo infectious dose (EID_{50}) was calculated using the Spearman-Karber method (Hamilton et al., 1978).

Comparative replication efficiency in ECEs and mammalian cells

Replication efficiency in ECEs was compared by inoculating 100 EID_{50}/0.1 ml of each recombinant virus (E2) into 10-day-old ECEs via the allantoic cavity route. Following incubation for 3 days, the inoculated virus was harvested and EID_{50} of each virus was measured as described above. The growth kinetics in MDCK and A549 cells were estimated by harvesting supernatants of cells inoculated with 5×10^5 EID_{50}/0.5 ml of virus in a 12-well plate, at 0, 24, 48, and 72 h post inoculation (hpi) in MDCK cells, and 51 and 71 hpi in A549 cells. The 10-fold diluent of each supernatant was inoculated into confluent MDCK cells in 96-well plates and the 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Spearman-Karber method.

Mouse pathogenicity test

The mouse pathogenicity test was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC-SNU-171214-1-1) and conducted in a biosafety level 2 facility at the Animal Center for Pharmaceutical Research of Seoul National University (Seoul, Korea) according to the national guidelines for the care and use of laboratory animals. Eight 6-week-old female BALB/c mice (KOATEC, Pyeongtaek, Korea) of each group were sedated by an intraperitoneal injection of Zoletil 50 (15 mg/kg; Virbac, Carros, France), and 10^6 EID_{50}/0.1 ml of each recombinant virus was inoculated via the intranasal route. The negative control group (mock) was inoculated with the same volume of sterile phosphate buffered saline (PBS). Three of eight mice in each group were euthanized through CO_2 asphyxiation to test virus replication and virus titers in the lungs at 3 days-post-inoculation (dpi). The remaining mice were observed for weight loss and mortality over 2 weeks. The mice were euthanized by CO_2 asphyxiation when the body weight of each mouse was reduced by more than 20%. The collected lungs were homogenized using a TissueLyzer 2 (Qiagen, Valencia, CA, USA) equipped with 5 mm diameter stainless steel beads and mixed with PBS (10 volumes of lung weight). Following centrifugation at 13,000 rpm for 10 min, 0.1 ml of each supernatant and 10-fold diluted pooled supernatants were inoculated into ECEs as described above. The presence of virus in harvested allantoic fluid was tested by the plate HA test with 1% chicken RBCs. The virus titer of pooled lung specimens was calculated as described above.

Immunogenicity of inactivated recombinant vaccine in chickens and ducks

Ten milliliters of the candidate vaccine strain in undilute allantoic fluid was inactivated with 1 ml of 1 M binary ethylenimine (BEI; final concentration 0.1 M) and incubated at 37°C for 24 h. The virus inactivation reaction was completed by adding 0.1 M sodium thiosulfate and was confirmed by inoculation into 10-day-old ECEs. The inactivated virus was mixed with ISA70 at an oil-to-virus ratio of 7:3 to make an inactivated oil emulsion vaccine. Allantoic fluid of uninfected 10-day-old ECEs was mixed with ISA70 and used as the negative control group.

The duck vaccination experiment was approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC-SNU-181205-6). Seven 1-day-old ducks and 2-week-old ducks were vaccinated with 0.5 ml of the oil-adjuvant inactivated vaccine, and serum was collected at 0, 3, and 4 weeks post vaccination (wpv).

Protection efficacy test of inactivated oil emulsion vaccine in chickens

Protection efficacy test in chickens was approved by the IACUC of Konkuk University (IACUC-KU18179) and was conducted in a biosafety level 3 facility at Konkuk University. Nine 3-week-old SPF chickens (Namduck Sanitek, Korea) in each group were vaccinated with the inactivated oil emulsion vaccine (0.5 ml/chicken) and challenged intra-nasally with the 10^6 EID of A/Mandarin duck/Korea/K16-187-3/2016 (H5N6) (K16-187-3) at 3 wpv. The serum of vaccinated chickens was collected at 0 and 3 wpv and 1 week-post-challenge (wpc) for serum antibody analysis. Oropharyngeal and cloacal swab samples were collected at 1, 3, 5, and 7 days-post-challenge (dpc) and virus shedding through oropharyngeal and cloacal routes was evaluated by RT-PCR using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) as previously described (Kwon et al., 2017b).

Serological test

Serum antibodies of vaccinated chickens and ducks were estimated by the hemagglutination inhibition (HI) assay following the WHO Manual on Animal Influenza Diagnosis and Surveillance. Chicken and duck serum samples were treated at 56°C for 30 min, and duck serum samples were mixed with three volumes of Receptor Destroying Enzyme II (RDE II; Denka Seiken Co., Ltd., Tokyo, Japan) and incubated for 24 h before

heat treatment. The treated serum was diluted 2-fold with PBS, and 25 μl of the diluted serum was mixed with the same volume of rH5N8-H103Y-310PB2 antigen with a hemagglutination titer (HAT) of 4. After incubation for 30 min at 4°C, 25 μl of 1% (v/v) chicken RBCs was added and the serum antibody titer was recorded after 40 min of incubation at 4°C.

Heat stability test

The HAT of each virus was measured before heat treatment as described above. Each virus was diluted to an HAT of 64 with PBS. Aliquots of each dilution were dispensed into three tubes and incubated for 30 min at 50, 55, and 60°C. The HAT of each virus was determined after the heat treatment.

Statistical analyses

The significance of replication efficiency and body weight changes were evaluated by one-way analysis-of-variance, and the difference of average body weight was assessed by the Student's t-test (log-rank test, 95 % confidence intervals) using SPSS statistical software (IBM, Armonk, NY, USA). A p-value < 0.05 indicated statistical significance.

2.3. Results

Genetic characteristics of synthesized consensus HA and NA genes of clade 2.3.4.4 H5N8 recombinant viruses

The variable amino acid residues in the synthesized consensus HA and NA sequences from other clade 2.3.4.4 viruses are summarized in Tables 2.2 and 2.3. The amino acid sequences of representative H5N8 Korean isolates Buan2 (clade 2.3.4.4a) and Gochang1 (clade 2.3.4.4b) from 2014, the more recent H5N8 strain from 2017, and the challenge strain (H5N6, clade 2.3.4.4c) were compared with the synthetic consensus HA and NA sequences. The sequence identity with the HA1 amino acid sequence of Buan2, Gochang1, the recent H5N8, and the challenge strains bearing the cleavage site sequences was 99.1%, 97.6%, 97.3%, and 95.9%, respectively (Table 2.2). Among the variable amino acid residues, 145 and 156 residues were the components of known epitopes and were variable in the challenge virus (145L deletion), Gochang1, and the recent H5N8 strain (A156T) (Kaverin et al., 2007; Velkov et al., 2013).

The sequence identity of Buan2, Gochang1, and the recent H5N8 strain with the NA amino acid sequence was 99.4%, 98.5%, and 98.9%, respectively (Table 2.3). The NA stalks of the viruses were compared, including the synthetic consensus NA sequence. The stalks were intact with no amino acid deletion when compared with the earliest H5N8 isolate, A/turkey/Ireland/1378/1983 (H5N8) (accession no. EPI129564).

Table 2.2. Comparison of variable amino acid sequences between clade 2.3.4.4 H5 proteins and the synthetic consensus H5 used in this study.

Variable residue [a]	Buan2	Gochang1	Recent H5N8 [b]	Challenge strain [c]	Consensus H5 [d]
Subgroup	a	b	b	c	a
10	V	I	I	V	I
16	S	S	G	S	S
30	K	E	E	E	K
110	T	S	S	N	T
130	T	I	I	T	I
140	N	D	N	N	N
145 [e]	L	L	L	- [f]	L
149	A	A	A	S	A
156 [e]	A	T	T	V	A
157	S	P	P	P	S
167	I	I	I	T	I
178	I	I	I	M	I
185	R	R	R	G	R
201	A	A	E	A	A
211	D	T	T	T	N
214	V	V	I	V	V
239	R	R	R	Q	R
285	V	V	V	M	V
298	I	V	V	I	I
336	S	N	S	S	S
% identity with the consensus HA1[g]	99.1	97.6	97.3	95.9	100.0

[a] H5 numbering including signal peptide.

[b] Recent H5N8: recently isolated H5N8 strains in Korea since 2017

[c] Challenge strain: A/Mandarin duck/Korea/K16-187-3/2016 (H5N6)

[d] Consensus H5: synthetic consensus H5 sequence used in this study

[e] Amino acid residue in reported epitope.

[f] -: deletion

[g] Homology with the consensus H5 sequence barring the cleavage site sequences

Table 2.3. Comparison of the variable amino acid sequences between natural N8 and the synthetic consensus N8 sequence used in this study.

Variable residue	Strain			
	Buan2	Gochang1	Recent H5N8	Consensus N8
8	V	M	V	V
18	V	A	V	V
30	I	T	I	I
46	N	K	K	N
136	S	A	A	S
190	A	T	T	T
264	R	Q	T	G
303	I	V	V	I
329	T	A	A	T
397	S	L	L	L
% identity with the consensus N8 sequence	99.4	98.5	98.9	100.0

Comparison of replication efficiency of H5N8 recombinant viruses in ECEs

PR8-derived parent (rH5N8) and mutant H5N8 recombinant viruses containing a single mutation in HA (rH5N8-hmH103Y, rH5N8-hmK161E, and rH5N8-hmL317P) and NA (rH5N8-nmS369N), combined mutations in HA and NA (rH5N8-wm), and single point HA mutation and 01310 PB2 (rH5N8-hmH103Y-310PB2) were successfully generated (Table 2.4). The virus titer of rH5N8 of $10^{8.3}$ EID_{50}/ml was slightly higher than that of rH5N8-hmL317P ($10^{8.0}$ EID_{50}/ml), rH5N8-nmS369N ($10^{8.1}$ EID_{50}/ml), and rH5N8-wm ($10^{7.8}$ EID_{50}/ml). However, the difference was not significant ($p > 0.05$). Similarly, rH5N8-hmK161E showed a slightly higher virus titer than that of rH5N8, which was also not significant. However, rH5N8-hmH103Y and rH5N8-hmH103Y-310PB2 showed significantly higher virus titers than that of rH5N8 ($p < 0.05$) (Table 2.4).

Table 2.4. Genome segments used for recombinant virus generation and the viral titer

Recombinant virus	Mutation		PB2	EID_{50}/ml[b]
	HA [a]	NA		
rH5N8	-[a]	-	PR8	8.3 ± 0.3
rH5N8-hmH103Y	H103Y[b]	-	PR8	9.3 ± 0.3*
rH5N8-hmK161E	K161E	-	PR8	8.8 ± 0.3
rH5N8-hmL317P	L317P	-	PR8	8.0 ± 0.4
rH5N8-nmS369N	-	S369N	PR8	8.1 ± 0.5
rH5N8-wm	H103Y, K161E, L317P	S369N	PR8	7.8 ± 0.4
rH5N8-hmH103Y-310PB2	H103Y	-	01310	9.3 ± 0.1*

[a] H3 numbering

[b] Viral titer measurement after inoculation of each virus with 100 EID_{50} into 10 do SPF ECEs

*significant difference compared to rH5N8 ($p < 0.05$).

Comparison of replication efficiency of the H5N8 recombinant viruses in mammalian cells

The replication efficiency of recombinant viruses, rH5N8, rH5N8-hmH103Y, rH5N8- rH5N8-hmH103Y-310PB2, and rPR8 in mammalian cells was compared on the basis of the growth kinetics in MDCK and A549 cells. In MDCK cells, only rH5N8-hmH103Y-310PB2 did not replicate, and rH5N8-hmH103Y replicated better than rH5N8 did, with a significantly higher virus titer. Moreover, rPR8 virus replicated efficiently in A549 cells, while rH5N8 and rH5N8-hmH103Y-310PB2 did not. Interestingly, rH5N8-hmH103Y replicated in A549 cells and showed significantly higher virus titer at 24 hpi ($10^{2.50}$ $TCID_{50}$) and 51 hpi ($10^{2.33}$ $TCID_{50}$) compared to rH5N8 ($10^{0.67}$ $TCID_{50}$) and rH5N8-hmH103Y-310PB2 (undetectable level of $TCID_{50}$) ($p < 0.05$, Fig. 2.1).

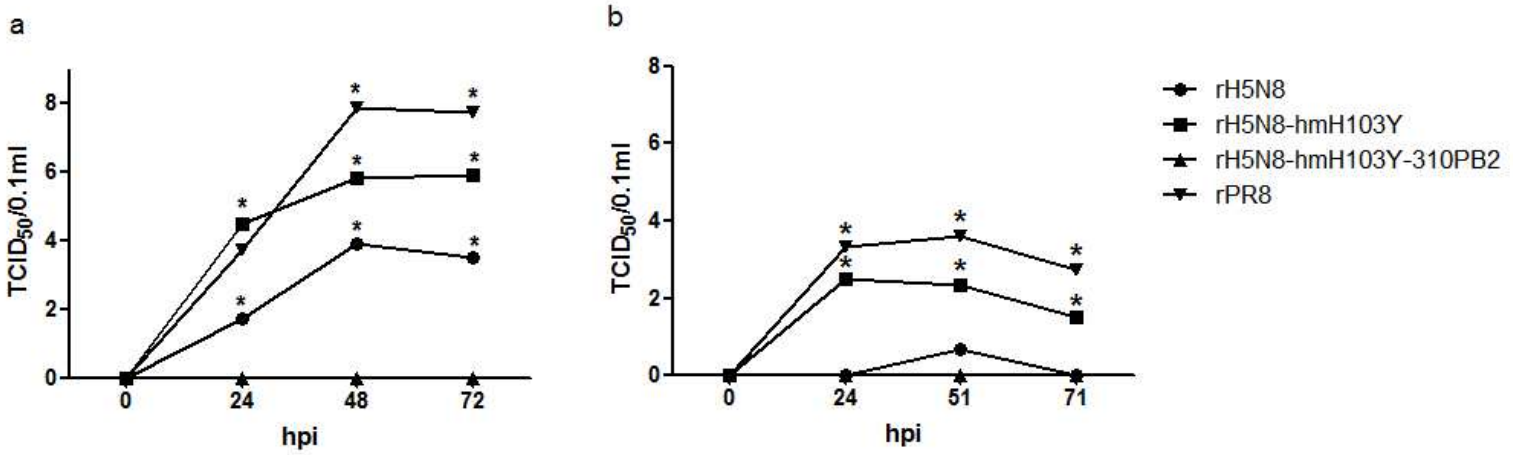

Figure 2.1. Growth kinetics of recombinant viruses in mammalian cells. Each recombinant virus (5×10^5 EID_{50}/0.5 ml) was inoculated into MDCK and A549 cells. Supernatants were harvested at 0, 24, 48, and 72 hpi in MDCK cells and at 51 and 71 hpi in A549 cells following 72 h incubation at 37°C in a CO_2 chamber. The viral titer at each time point was titrated as $TCID_{50}$ in MDCK cells. Significant difference is denoted by an asterisk ($p < 0.05$). The data was the average of three independent experiments.

Comparison of pathogenicity of H5N8 recombinant viruses in mice

Pathogenicity of rH5N8, rH5N8-hmH103Y, rH5N8-hmH103Y-310PB2, and rPR8 was compared in 6-week-old female BALB/c mice on the basis of virus titer in the lungs and changes in body weight (Table 2.5 and Fig. 2.2). All recombinant viruses, except rPR8, showed no loss in body weight, but rH5N8 and rH5N8-hmH103Y were detected in all inoculated mice at 3 dpi. The virus titer of rH5N8-hmH103Y ($10^{4.5}$ EID_{50}/0.1 ml) was significantly higher than that of rH5N8 ($10^{3.7}$ EID_{50}/0.1 ml), but rH5N8-hmH103Y-310PB2 was not detected in any of the inoculated mice.

Table 2.5. Virus isolation rate and viral titer isolated from mouse lung at 3 dpi.

Recombinant virus	Virus isolation rate	Log_{10} EID_{50}/0.1 ml[a]
rH5N8	3/3	3.70
rH5N8-hmH103Y	3/3	4.50
rH5N8-hmH103Y-310PB2	0/3	0.00
rPR8	3/3	7.25
mock	0/3	0.00

[a] EID_{50} /0.1 ml was measured with pooled lung samples.

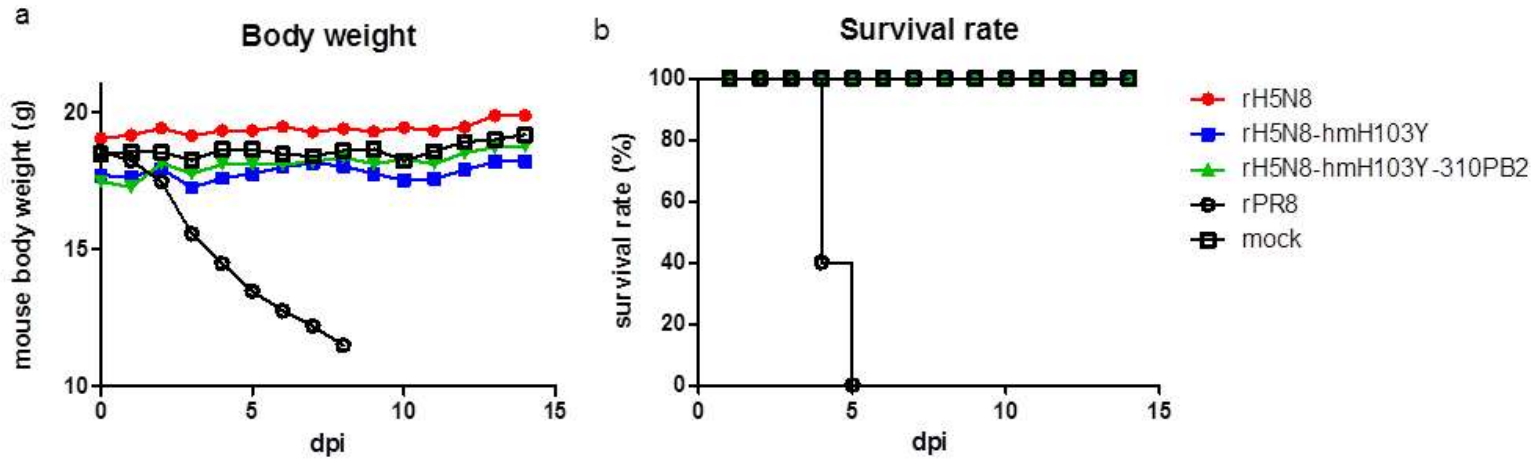

Figure 2.2. Pathogenicity of recombinant viruses in mice. Five 6-week-old BALB/c mice were challenged with 10^6 EID_{50} of each virus or PBS (mock) via the intranasal route and body weight was measured for 2 weeks. (a) Change in the body weight of mice and (b) survival rate of virus infected mice groups. Statistical significance was assessed by Student's t-test ($p < 0.05$).

Effect of H103Y mutation on heat stability of HA protein

HAT of 64 of rH5N8-hmH103Y did not decrease, but the HAT of 2 of rH5N8 decreased after heat treatment at 55°C for 30 min. Moreover, the HAT of rH5N8 decreased to an undetectable level, but rH5N8-hmH103Y showed residual hemagglutination activity (HAT of 4) after incubation at 60°C for 30 min (Fig. 2.3).

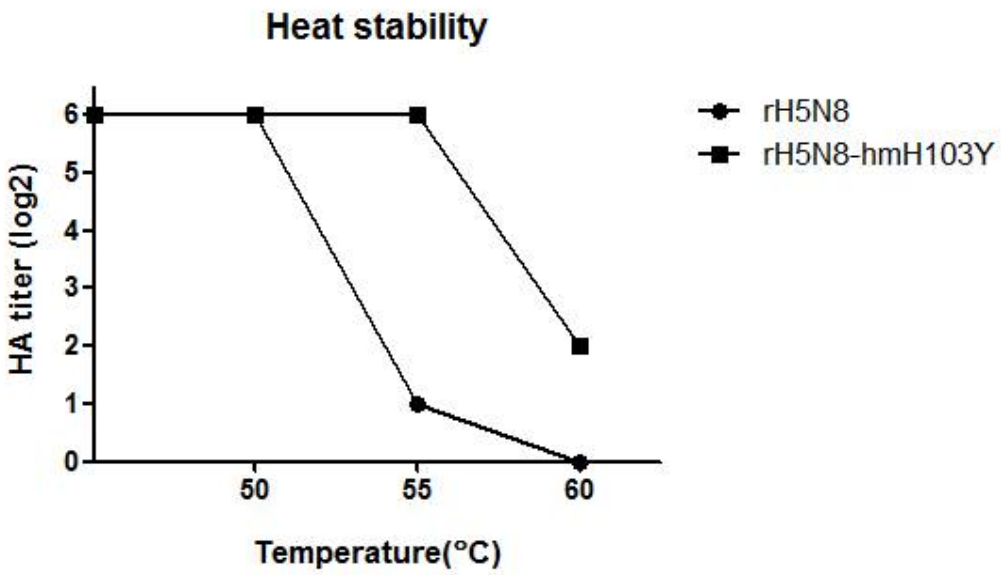

Figure 2.3. Heat stability of rH5N8 and rH5N8-hmH103Y. Each virus was diluted with PBA to an HAT of 64, and three aliquots of each virus were incubated at 50, 55, and 60°C for 30 min. After heat treatment, HAT was determined by the HA assay using 1% (v/v) chicken RBCs

Heterologous protection efficacy of inactivated oil emulsion vaccine in chickens

Chickens inoculated with the inactivated oil emulsion vaccine of rH5N8-hmH103Y-310PB2 showed geometric mean HI titers with 95 % confidence interval (CI) at 3 wpv (before challenge) of 174.2 (95% CI, 85.60 – 354.4) and at 1 wpc (after 1 week of challenge) of 94.06 (95% CI, 46.23 – 191.4). Surprisingly, the HI titer decreased after challenge, instead of increasing. The vaccinated chickens were challenged with heterologous clade 2.3.4.4c H5N6 HPAI virus, K16-187-3, at 3 wpv. Eight chickens in the mock group died at 2 dpc and one at 3 dpc. All chickens in the vaccine group were protected from mortality. However, virus shedding through the oro-pharynx and cloaca was not protected and virus was detected in the oro-pharynx (5/9) and cloaca (1/9) until the end of the experiment at 7 dpc (Table 2.6).

Table 2.6. Serum antibody titer, survival rate and viral shedding rate after wild-type H5N6 HPAI virus challenge in chickens

Inactivated vaccine stain	GMT HI titer [a]			Survival rate	Viral shedding rate							
					Oro-pharynx[b]				Cloaca			
	0	3 wpv	1 pc		1[c]	3	5	7	1	3	5	7
rH5N8-hmH103Y-310PB2	< 2[d]	174.2 (85.60 - 354.4)	94.06 (46.23 – 191.4)	9/9 (100 %)	0/9	3/9	6/9	5/9	1/9	4/9	4/9	1/9
Mock	< 2	< 2	< 2	0/9 (0 %)	9/9	1/1[e]	nt[f]	nt	7/9	1/1[e]	nt	nt

[a] Geometric mean HI titer with 95 % confident interval

[b] Viral shedding was confirmed by real-time RT-PCR with oro-pharyngeal and cloacal swab samples.

[c] dpc: day post challenge.

[d] < 2 : undectectable titer

[e] Eight and one chickens were dead at 2 and 3 dpc, respectively.

[f] nt: not tested.

Immunogenicity of inactivated oil emulsion vaccine of rH5N8-hmH103Y- 310PB2 in ducks

Ducks that were vaccinated at 1-day-of-age showed undetectable HI titers at 3 and 4 wpv. However, ducks vaccinated at 2-weeks-of-age showed significantly higher HI titers of 21.5 (95% CI, 15.3 – 30.3) at 3 wpv and 23.8 (95% CI, 9.7 – 58.2) at 4 wpv. HI antibody was not detected in any chicken and duck before vaccination in the vaccine groups and before and after vaccination in the mock groups (Table 2.6 and 2.7).

Table 2.7. Serum antibody titer of duck vaccination groups

Host	Vaccinated age	Vaccine	GMT HI titer [a]		
			0 wpv [b]	3 wpv	4 wpv
Duck	1-day-old	rH5N8-hmH103Y-310PB2	< 2[c]	< 2	< 2
		mock	< 2	< 2	< 2
	2-week-old	rH5N8-hmH103Y-310PB2	< 2	21.5 (15.3 – 30.3)	23.8 (9.7 – 58.2)
		mock	< 2	< 2	< 2

[a] Serum antibody titer was measured against 4 HAU of rH5N8 – hmH103Y– 310PB2 antigen

[b] wpv: week-post-vaccination

[c] < 2 : undectectable titer

2.4. Discussion

The HA and NA surface glycoproteins are the main targets of host humoral immunity (Potter and Oxford, 1979). Clade 2.3.4.4b is currently prevalent in wild birds and poultry, and clade 2.3.4.4c has evolved to become more pathogenic in mammals (Poen et al., 2019; Yang et al., 2015). The synthesized consensus HA gene showed higher amino acid identity of HA1 to clade 2.3.4.4a than clades 2.3.4.4b and c (Supplementary Table 2.S2). In addition, clades 2.3.4.4b and c include NA subtypes other than N8. Therefore, updating HA and NA to match the outbreak virus may be better than using only consensus HA (Giles and Ross, 2011; Kapczynski et al., 2017).

A/wild duck/Korea/SNU50-5/2011 (H5N1) (50-5) showed increased viral titer in ECEs after 20 passages and acquired multiple mutations in PA, HA, NA, M1, and M2 genes. The mutations in HA and NA genes were sufficient to increase the titer of PR8-derived recombinant viruses (Kim et al., 2013a). In this study, the single H103Y and K161E mutations increased virus titers, but the single L317P and S369N mutations and all combined mutations decreased virus titer only slightly in ECEs (Table 2.3). The H103Y mutation has been reported to enhance the air-borne transmission of H5N1 viruses in mammalian hosts by enhancing thermo- and pH stability (de Vries et al., 2014a; Herfst et al., 2012; Linster et al., 2014; Zhang et al., 2013a), but it was also related to high virus titers in ECEs (Kim et al., 2013a). AIVs and recombinant viruses were typically passaged and incubated at 37.3-37.8°C, and the pH of the allantoic fluid may be decreased at higher incubation temperature (McLean, 1945). The H103Y mutation is related to thermo- and

pH stability, which may have helped in maintaining active HA and virus infectivity during temperature and pH variations in the incubation environment of the ECEs. Lower incubation temperatures of 33-37°C achieve higher virus titers of PR8 than those achieved at 37-39°C (Miller, 1944). However, the optimal temperature for chicken embryo development is 37.8°C (Lourens et al., 2005), and the H103Y mutation may support virus replication at the optimal embryonic cell condition by buffering the negative effect of acidic condition at higher incubation temperatures. Although we did not test pH stability, the correlation of H103Y with thermo-stability was also verified in this study.

In the Y161F mutation in pandemic H1N1 (pdmH1N1) in 2009, H3N2 and H3N8 viruses increased virus titer in MDCK and ECEs and also increased thermo-stability of HA (Wen et al., 2018). The K161E mutation in this study (Kim et al., 2013a) is located three amino acids from Y161F (Wen et al., 2018), but their proximal locations may be noteworthy. However, our 161 residue is close to the HA1 epitope site group 2, and therefore, we excluded the K161E mutation for the generation of the vaccine strain (Velkov et al., 2013). T318I mutation was reported to stabilize fusion peptide and helix A resulting in thermo- and pH stability, and it is located in close proximity to the 52 residue of HA2 (de Vries et al., 2014a; Imai et al., 2012; Xiong et al., 2013). The L317P mutation occurs immediately before the 318 residue and was first observed together with the R51K mutation in HA2 of 50-5 (Kim et al., 2013a). As the R51K mutation was not included, a single L317P mutation and the combined mutations may be not enough to increase the virus titer without the epistatic R51K or in the context of different amino acids of the clade 2.3.4.4 HA (Lyons and Lauring, 2018).

Although PR8 PB2 gene already possesses multiple mammalian pathogenicity-related mutations, including the fatal E627K mutation, it has been used for the generation of vaccine strains against human fatal AIVs (Subbarao et al., 1993; Takahashi et al., 2009). Biosecurity and biosafety have been well-controlled during vaccine strain development and vaccine production, but efforts to reduce potential risks should be continued. The PB2 gene of 01310 is a prototypic PB2 gene without mammalian pathogenicity-related mutations, and also increases the viral titer of recombinant viruses in ECEs (Jang et al., 2017b; Kim et al., 2014b; Kim et al., 2015b; Lee et al., 2017a). As expected, replacement of PR8 PB2 gene with the 01310 PB2 gene (rH5N8-hmH103Y-310PB2) removed the replication capacity of rH5N8-hmH103Y in mammalian cells and the lungs of BALB/c mice, while maintaining high viral titer in ECEs. Therefore, the broad applicability of the 01310 PB2 gene to generate more productive and safer vaccines was demonstrated again (Jang et al., 2017b; Kim et al., 2015b).

Vaccination with rH5N8-hmH103Y-310PB2 completely protected against the lethal challenge of heterologous clade 2.3.4.4c viruses, but did not prevent virus shedding. Several clade 2.3.4.4 vaccine strains have been evaluated. None has shown any defense from virus shedding or high mortality after challenge (mostly heterologous) (Jin et al., 2018; Kandeil et al., 2018; Kapczynski et al., 2017). The virus shedding after vaccination observed in this study may be related to an antigenic mismatch between the vaccine and challenge strains, relatively low amino acid identity (95.9%), and heterologous NA (N8 vs. N6) (Kapczynski et al., 2017; Takahashi et al., 2009). All the reported clade 2.3.4.4 vaccine strains possess an HA that is identical to the corresponding parent field strains, except at

the cleavage site. However, information on vaccine virus titers was not available. As different experimental conditions may affect vaccine efficacy including inactivation methods, vaccine strain titer and inoculation volume per dose, we could not directly compare the relative efficacy of our vaccine to others. However, our vaccine formulation showed higher mean HI titer at 3 wpv than previous reports (Jin et al., 2018; Kapczynski et al., 2017). In addition, the mean HI titer did not change after challenge at 1 wpc. Therefore, the humoral immunity induced by single inoculation of rH5N8-hmH103Y-310PB2 may be sufficient to be no longer activated by the challenge virus (Kapczynski et al., 2017).

Ducks are more resistant than chickens to HPAI viruses and excrete virus asymptomatically (Alexander et al., 1986; van den Brand et al., 2018). Since asymptomatic ducks can transmit HPAI viruses to chickens, vaccination of ducks can be a strategy to control HPAI infection in domestic poultry. However, ducks show poor antibody responses to influenza viruses and the immune systems of ducks and chickens react differently to AIV infection (Kida et al., 1980; Kuchipudi et al., 2014; Middleton et al., 2007; Smith et al., 2015b; Webster et al., 2006). Earlier induction of acquired humoral immunity in ducks is desirable, but different and age-dependent humoral immunity of ducks was apparent in this study. Although our vaccine formulation induced HI antibody in 2 w-o ducks, protective behavior of the HI antibody titers could not be predicted (Ohmit et al., 2011; Pantin-Jackwood and Suarez, 2013). Since efficacious live vector vaccines are available, prime-boost strategy by using rH5N8-hmH103Y-310PB2 needs to be verified in the future protection efficacy study (Kapczynski et al., 2017; Pantin-Jackwood and Suarez, 2013).

In this study, we successfully bioengineered a PR8-derived highly productive in ECEs and mammalian-nonpathogenic clade 2.3.4.4 H5N8 recombinant vaccine strain by introducing H103Y mutation and replacing PR8 and 01310 PB2 genes. The established molecular formulation to bioengineer better vaccine strains may be useful for vaccine development.

2.5. Supplementary Materials

Table 2.S1. Accession number and name of H5N8 HPAI clade 2·3·4·4 viruses (2014 ~ 2016 isolates in Asia) used in consensus HA genome synthesis

Segment ID	Segment	Country	Isolation name
EPI509698	HA	Korea	A/breeder duck/Korea/Gochang1/2014
EPI 509704	HA	Korea	A/broiler duck/Korea/Buan2/2014
EPI 509709	HA	Korea	A/baikal teal/Korea/Donglim3/2014
EPI 517161	HA	Korea	A/mallard/Korea/W452/2014
EPI 543002	HA	China	A/duck/Beijing/FS01/2014
EPI 548485	HA	Japan	A/duck/Chiba/26-372-48/2014
EPI 553208	HA	Japan	A/crane/Kagoshima/KU1/2014
EPI 553343	HA	Japan	A/chicken/Miyazaki/7/2014
EPI 561187	HA	Korea	A/waterfowl/Korea/S005/2014
EPI 561336	HA	Korea	A/broiler duck/Korea/H48/2014
EPI 561508	HA	Korea	A/bean goose/Korea/H53/2014
EPI 561556	HA	Korea	A/Coot/Korea/H81/2014
EPI 561577	HA	Korea	A/breeder chicken/Korea/H122/2014
EPI 561626	HA	Korea	A/mallard/Korea/H207/2014
EPI 561633	HA	Korea	A/white-fronted goose/Korea/H231/2014
EPI 561646	HA	Korea	A/breeder duck/Korea/H249/2014
EPI 561667	HA	Korea	A/Korean native chicken/Korea/H257/2014
EPI 561674	HA	Korea	A/bean goose/Korea/H328/2014
EPI 561681	HA	Korea	A/tundra swan/Korea/H411/2014
EPI 561690	HA	Korea	A/common teal/Korea/H455-30/2014
EPI 561697	HA	Korea	A/spot-billed duck/Korea/H455-42/2014
EPI 573197	HA	Korea	A/chicken/Korea/H881/2014
EPI 573204	HA	Korea	A/goose/Korea/H1296/2014
EPI 573208	HA	Korea	A/gadwall/Korea/H1351/2014
EPI 573236	HA	Korea	A/spot-billed duck/Korea/H1981/2014
EPI 573638	HA	Japan	A/crane/Kagoshima/KU13/2014(H5N8)
EPI 583680	HA	Japan	A/mallard duck/Kagoshima/KU116/2015(H5N8)

Segment ID	Segment	Country	Isolation name
EPI 588952	HA	Taiwan	A/goose/Taiwan/a015/2015
EPI 588976	HA	Taiwan	A/duck/Taiwan/a068/2015
EPI 595055	HA	Korea	A/common teal/Korea/KU-12/2015
EPI 595066	HA	Korea	A/mallard/Korea/KU3-2/2015
EPI 595107	HA	Korea	A/mandarin duck/Korea/K14-367-1/2014
EPI 595146	HA	Korea	A/greater white-fronted goose/Korea/K14-374-1/2014
EPI 646105	HA	China	A/goose/Jiangsu/WX202/2014
EPI 659623	HA	China	A/goose/Shandong/GD-GS/2014(H5N8)
EPI 662624	HA	Japan	A/chicken/Saga/1-1/2015
EPI 662632	HA	Japan	A/chicken/Okayama/1-2/2015
EPI 662640	HA	Japan	A/chicken/Yamaguchi/6/2014
EPI 662648	HA	Japan	A/chicken/Miyazaki/2-4/2014
EPI 672795	HA	China	A/duck/Guangdong/s14044/2014
EPI 675774	HA	China	A/duck/Liaoning/S1001/2014
EPI 681297	HA	China	A/duck/Zhejiang/925019/2014
EPI 681300	HA	China	A/goose/Zhejiang/925104/2014
EPI 690792	HA	China	A/goose/Jiangsu/QD5/2014
EPI 690800	HA	China	A/goose/Shandong/WFSG1/2014
EPI 690808	HA	China	A/goose/Yangzhou/0420/2014
EPI 703602	HA	China	A/duck/Eastern China/S0215/2014
EPI 703607	HA	China	A/goose/Eastern China/S0408/2014
EPI 718280	HA	Korea	A/ostrich/Korea/H829/2014
EPI 762150	HA	China	A/duck/Eastern China/JY/2014
EPI 774410	HA	China	A/Bar-headed Goose/Qinghai/BTY18-LU/2016
EPI 774426	HA	China	A/Brown-headed Gull/Qinghai/ZTO1-B/2016
EPI 774506	HA	China	A/Great Black-headed Gull/Qinghai/YO1-B/2016
EPI 837541	HA	Korea	A/breeder duck/Korea/H2086/2015
EPI 837546	HA	Korea	A/broiler duck/Korea/H2278/2015
EPI 837550	HA	Korea	A/mallard/Korea/H2321/2015
EPI 837555	HA	Korea	A/breeder chicken/Korea/H2496/2015

Segment ID	Segment	Country	Isolation name
EPI 837558	HA	Korea	A/chicken/Korea/H2553/2015
EPI 837561	HA	Korea	A/korean native chicken/Korea/H2598/2015
EPI 837565	HA	Korea	A/duck/Korea/H2628/2015
EPI 845903	HA	Japan	A/mandarin duck/Gifu/2112D001/2014
EPI 845956	HA	Japan	A/tundra swan/Tottori/C6nk/2014
EPI 858836	HA	India	A/duck/India/10CA01/2016
EPI 858844	HA	India	A/painted stork/India/10CA03/2016
EPI 888264	HA	Korea	A/domestic mallard/Korea/LBM176/2014
EPI 926621	HA	Kazakhstan	A/graylag goose/Kazakhstan/KR/2016
EPI 961933	HA	Taiwan	A/chicken/Taiwan/x37/2016
EPI 974364	HA	Iran	A/chicken/Iran/Tehran-F-2/2016
EPI 1070026	HA	China	A/wildfowl/Shandong/SD04/2016
EPI 1159809	HA	China	A/Von Schrenck's bittern/Jiangxi/Y9/2014
EPI 1159817	HA	China	A/Cygnus atratus/Hubei/2Z2-O/2016
EPI 1176517	HA	Israel	A/turkey/Israel/1045/2016
EPI 1176519	HA	Israel	A/chicken/Israel/1048/2016
EPI 1176524	HA	Israel	A/cormorant/Israel/1035/2016
EPI 1176525	HA	Israel	A/great egret/Israel/1088/2016
EPI 1176529	HA	Israel	A/grey goose/Israel/986/2016
EPI 1176530	HA	Israel	A/peregrine falcon/Israel/1086/2016

Table 2.S2. Amino acid homology (%) of consensus H5, Buan2, and Gochang1 to clade 2·3·4·4 viruses

Strain name	**Segment ID**	**subtype**	**subgroup**	**consensus H5N8**	**buan2** (clade2.3.4.4a)	**gochang1** (clade2.3.4.4b)
A/duck/Beijing/FS01/2014	EPI543002	H5N8	clade 2·3·4·4a	99.11	99.70	97.04
A/goose/Taiwan/01019/2015	EPI750122	H5N8	clade 2·3·4·4a	97.63	98.52	95.56
A/crane/Kagoshima/KU21/2014	EPI573646	H5N8	clade 2·3·4·4a	97.93	98.82	95.86
A/goose/Zhejiang/925037/2014	EPI681299	H5N8	clade 2·3·4·4b	97.63	97.04	98.82
A/duck/Wenzhou/YHQL22/2014	EPI682915	H5N6	clade 2·3·4·4b	97.34	96.75	99.70
A/Bar-headed Goose/Qinghai/BTY1-B/2016	EPI774113	H5N8	clade 2·3·4·4b	97.63	97.04	98.82
A/duck/India/10CA01/2016	EPI858836	H5N8	clade 2·3·4·4b	96.45	95.86	97.63
A/chicken/Korea/H903/2017	EPI952639	H5N8	clade 2·3·4·4b	96.75	96.15	97.93
A/black swan/Germany-BW/R1364/2017	EPI988345	H5N8	clade 2·3·4·4b	96.45	95.86	97.63
A/grey heron/W779/2017	EPI1034974	H5N8	clade 2·3·4·4b	97.34	96.75	97.93
A/Mute swan/Hungary/3137/2017	EPI954837	H5N8	clade 2·3·4·4b	97.04	96.45	98.22
A/spoonbill/Taiwan/DB645/2017	EPI1119065	H5N6	clade 2·3·4·4b	95.86	95.27	97.34
A/chicken/Greece/39_2017b/2017	EPI1122893	H5N6	clade 2·3·4·4b	96.15	95.56	97.34
A/duck/Korea/HD1/2017	EPI1123317	H5N6	clade 2·3·4·4b	96.15	95.56	97.34
A/Great Black-backed Gull/Netherlands/1/2017	EPI1131088	H5N6	clade 2·3·4·4b	95.86	95.56	97.04
A/Mallard/Korea/K17-1825/2017	EPI1190339	H5N6	clade 2·3·4·4b	96.75	96.15	97.34
A/mute swan/Shimane/3211A001/2017	EPI1127538	H5N6	clade 2·3·4·4b	96.15	95.56	97.34
A/chicken/Guangxi/04.10 NM140/2015	EPI661903	H5N6	clade 2·3·4·4c	96.15	96.75	96.15
A/Mandarin_duck/Korea/K16-187-3/2016	EPI861484	H5N6	clade 2·3·4·4c	95.56	96.15	95.86
A/common teal/Korea/W559/2017	EPI1006737	H5N6	clade 2·3·4·4c	95.56	95.86	95.56
A/chicken/Gifu/1-10C/2017	EPI885308	H5N6	clade 2·3·4·4c	95.56	95.86	95.27

Chapter III

Novel Mutations Evading Avian Immunity around the Receptor Binding Site of the Clade 2.3.2.1c Hemagglutinin Gene Reduce Viral Thermostability and Mammalian Pathogenicity

Abstract

Since 2007, highly pathogenic clade 2.3.2 H5N1 avian influenza A (A(H5N1)) viruses have evolved to clade 2.3.2.1a, b, and c; currently only 2.3.2.1c A(H5N1) viruses circulate in wild birds and poultry. During antigenic evolution, clade 2.3.2.1a and c A(H5N1) viruses acquired both S144N and V223I mutations around the receptor binding site of hemagglutinin (HA), with S144N generating an N-glycosylation sequon. We introduced single or combined reverse mutations, N144S and/or I223V, into the HA gene of the clade 2.3.2.1c A(H5N1) virus and generated PR8-derived, 2 + 6 recombinant A(H5N1) viruses. When we compared replication efficiency in embryonated chicken eggs, mammalian cells, and mice, the recombinant virus containing both N144S and I223V mutations showed increased replication efficiency in avian and mammalian hosts and pathogenicity in mice. The N144S mutation significantly decreased avian receptor affinity and egg white inhibition, but not all mutations increased mammalian receptor affinity. Interestingly, the combined reverse mutations dramatically increased the thermostability of HA. Therefore, the adaptive mutations possibly acquired to evade avian immunity may decrease viral thermostability as well as mammalian pathogenicity.

Keywords**:** *clade 2.3.2.1c H5N1 virus; immunity evasion; HA trimer stability; thermostability; mammalian pathogenicity*

3.1. Introduction

Highly pathogenic H5N1 avian influenza A [HP A(H5N1)] viruses are fatal to poultry and cause high human fatality after dead-end transmission from infected poultry (Lai et al., 2016; Sonnberg et al., 2013). HP A(H5N1) viruses spread from Asia to Africa and Europe by migratory birds, and antigenic evolution has continued under immune pressure by vaccination and natural infection in Asia (Le and Nguyen, 2014; Smith et al., 2006b; Sonnberg et al., 2013). The ancestral HP A(H5N1) virus A/goose/Guangdong/1/96 (clade 0) has evolved into multiple clades from clade 1 to 9 (Smith et al., 2015a). Some clade 2.3.2 viruses evolved into clade 2.3.2.1 and further diversified into 2.3.2.1a, b and c in 2009 (Smith et al., 2009b; World Health Organization/World Organisation for Animal and Agriculture Organization, 2014). Clade 2.3.2.1c viruses have spread from Far East and South East Asian countries to Dubai, Bulgaria, Romania, and Nigeria and have become enzootic in Asian countries (Bi et al., 2016; Laleye et al., 2018a; Naguib et al., 2015; Nguyen et al., 2017).

Hemagglutinin (HA) is a surface glycoprotein exposed on the outside of virus particles and forms a noncovalent homotrimer composed of a distal globular head and proximal stalk (Wilson et al., 1981). The receptor binding site (RBS) on the globular head of HA is a shallow pocket-like structure consisting of three secondary structure elements (130-loop, 190-helix and 220-loop) and a base (Y98, W153, H183 and Y195 in H3 numbering) (Wilson et al., 1981). HA binds to cell surface receptors to infect the host cell, and avian and human influenza A viruses (IAVs) preferentially bind to sialic acid α2,3-linked (α2,3 SA) and α2,6-linked (α2,6 SA) to galactose in avian and mammalian receptors,

respectively. However, mutations in the RBS of avian IAVs can change receptor affinity from affinity only to α2,3 SA to both α2,3 SA and α2,6 SA or only α2,6 SA to overcome host barriers, resulting in interspecies transmission and adaptation (Imai et al., 2012; Mair et al., 2014; Weis et al., 1988). Several mutations that increase pathogenicity and affinity to mammalian receptors have been reported in the 220-loop of H5 subtype HA (Q226L, G228S, *etc*.) (Zhang et al., 2013b).

The globular head of HA is a major target of humoral immunity and is a hotspot of cumulative missense mutations to escape host immune responses (Kaverin et al., 2007; Kaverin et al., 2002; Swayne and Kapczynski, 2008; Wiley and Skehel, 1987). Epitope mapping and escape mutant studies with mouse monoclonal antibodies have revealed antigenic variations of H5N1 IAVs (Kaverin et al., 2007; Kaverin et al., 2002). The evasion mutations identified in H5 were distributed in epitope sites A (140-145 residues, H3 numbering) and B (155-166, H3 numbering) of H3 and Sa of H1 (129-133, H3 numbering) (Caton et al., 1982; Kaverin et al., 2007; Kaverin et al., 2002; Wiley et al., 1981). Acquisition of N-glycosylation sequon (NGS) in epitope not only shields the epitopes from antibody binding but also affects the binding affinity of HA to mammalian receptors (Wang et al., 2010; Zhang et al., 2013b).

The HA trimer is stabilized by polar and nonpolar interactions between the three stems and intermolecular salt bridges between the globular heads at low pH (Copeland et al., 1986; DuBois et al., 2011; Rachakonda et al., 2007). The H103Y and T318I mutations increase low pH stability and thermostability, as well as droplet transmission of H5N1 viruses between ferrets (Imai et al., 2012; Linster et al., 2014; Xiong et al., 2013; Zhang et

al., 2013b). Therefore, multiple mutations of HA acquired stepwise that play roles in receptor affinity, immunity evasion and structural/functional stability may cooperatively affect the mammalian pathogenicity of avian IAVs.

Therefore, in this study, we compared the HA amino acid sequences of clade 2.3.2 and clade 2.3.2.1a, b and c viruses and found two cumulative mutations (S144N and V223I) around the RBS. Most clade 2.3.2.1a and c viruses acquired both mutations. Previously, the PR8-based recombinant virus with HA and NA of a clade 2.3.2.1c HP A(H5N1) virus isolated in Korea, A/mandarin duck/Korea/K10-483/2010 (K10-483), did not replicate well in embryonated chicken eggs (ECEs) and had low pathogenicity in mice (Jang et al., 2017b; Lee et al., 2011). To understand the effects of the mutations on replication efficiency in ECEs and mammalian cells and on pathogenicity in mice, we generated PR8-derived mutant viruses and compared their biological characteristics. In addition, we compared the effects of the mutations on egg white resistance and thermostability of HA.

3.2. Materials and Methods

Viruses, Plasmids, Cells, and Eggs

The attenuated HA (mutation from multi-basic RERRRKR to mono-basic ASGR) and NA genome segments of a clade 2.3.2.1c HP A(H5N1) virus, A/mandarin duck/Korea/K10-483/2010 (K10-483), were previously cloned into a bidirectional reverse genetics vector, pHW2000, and 6 other internal genomes of A/Puerto Rico/8/34 (H1N1) (PR8) cloned into pHW2000 were used (Hoffmann et al., 2002b; Hoffmann et al., 2001b; Jang et al., 2017b). The amino acid sequences of HA and NA of K10-483 did not have known mutations affecting the biological traits tested in this study. 293T, MDCK and A549 cells were purchased from Korean Collection for Type Cultures (KCTC, Daejeon, Korea). 293T and MDCK cells were maintained in DMEM supplemented with 10% FBS (Life Technologies Co., CA, USA), and A549 cells were maintained in DMEM/F12 supplemented with 10% FBS. Virus was propagated with 10-day-old SPF embryonated chicken eggs (ECEs, Charles River Lab., CT, USA).

Data Mining and Analysis of HA Genes and In Silico Analysis of HA Trimer Structure and N-Glycan Profiles

The HA gene sequences of clade 2.3.2, clade 2.3.2.1, and clades 2.3.2.1a, b, and c HP A(H5N1) viruses were collected from the Influenza Virus Database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database) and Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org/) (n =

647) on April 14, 2018. The collected nucleotide sequences were translated and compared with the BioEdit program (ver. 7.2.5). Additionally, HA sequences of all A(H5) viruses were collected (n = 4189), and amino acid sequences and frequencies of 144NGS and 158NGS and residue 223 (V or I) were analyzed. In addition, HA genes (n = 513) of HP A(H5N1) strains from laboratory-confirmed human cases were analyzed as above. Residues 144N and 223 V/I were localized and analyzed for intermolecular interactions with other residues in the 3D structure of the H5 trimer (modified 4juk.pdb and 6e7g.pdb) using PyMOL (Molecular Graphis System version 2.3.1, Delano Scientific LLC, South San Francisco, CA, USA). The N-glycosylation prediction at position 144 was performed by the NetNGlyc program (DTU Bioinformatics, Lyngby, Denmark).

Site-Directed Mutagenesis and Generation of Viruses by Reverse Genetics

To generate mutated H5N1 recombinant viruses, the cloned HA genome of K10-483 was mutated with a Muta-direct Site Directed Mutagenesis Kit (iNtRON, Gyeonggi, Korea) and specific primer sets (Table 3.1). Hoffmann's reverse genetics system with a few modifications was used for recombinant virus generation (Hoffmann et al., 2002b). Briefly, 300 ng of each of the 8 plasmids were transfected together into confluent 293T cells in 6-well plates (10^6 cells/well) with Lipofectamine 2000 and PLUS reagents (Life Technologies Co.). After overnight incubation, 1 mL of Opti-MEM (Life Technologies Co.) and 1 μg/mL of TPCK-treated trypsin (Sigma-Aldrich, MO, USA) were added to transfected cells. The supernatant was harvested after another overnight incubation, and

200 μL of the supernatant was inoculated into 10-day-old ECEs. The presence of the recombinant viruses in allantoic fluids was checked by HA assay according to the WHO Manual on Animal Influenza Diagnosis and Surveillance and the genome segments were confirmed by RT-PCR and sequencing as previously described (Kim et al., 2013a).

Table 3.1. Primers used in this study for site-directed mutagenesis.

Primer	Sequence (5′–3′)
N144S-F	GTTCATACCAGGGAAGTTCCTCCTTCTTCAGAAATG
N144S-R	CATTTCTGAAGAAGGAGGAACTTCCCTGGTATGAAC
I223V-F	CACTAGATCCAAAGTAAACGGGCAAAGTGGC
I223V-R	GCCACTTTGCCCGTTTACTTTGGATCTAGTG

Comparative Replication Efficiency in ECEs and Growth Kinetics in MDCK and A549 Cells

The generated recombinant viruses (E1) were passaged in 10-day-old SPF ECEs, and the titers of the recombinant viruses (E2) were measured to obtain the EID_{50}. Each virus was diluted 10-fold and inoculated into ECEs. Virus replication was confirmed by plate hemagglutination test with 1.0% chicken RBCs, and the EID_{50} was calculated by the Spearman-Karber method (Hamilton et al., 1978). To compare the replication efficiency of the recombinant viruses, the same amount of viruses were used to infect ECEs and mammalian cells. One hundred EID_{50} of recombinant virus (E2) were inoculated into 10-day-old SPF ECEs, and after 3 days, the EID_{50} of harvested allantoic fluid was measured as above. In MDCK and A549 cells, 10^5 EID_{50} of each virus were used to infect confluent MDCK and A549 cells in 12-well plates. The supernatant of the infected cells was harvested at 0, 12, 24, 48, and 72 hours post inoculation (hpi), and 10-fold diluted supernatant was used to inoculate confluent MDCK cells in a 96-well plate to measure the 50% tissue culture infectious dose ($TCID_{50}$) at each time point. Virus replication was confirmed by a hemagglutination assay, and $TCID_{50}$ was calculated using the Spearman-Karber method as above.

Mouse Pathogenicity Test

The mouse pathogenicity test was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC-SNU-171214-1-1). The approved experiment was performed in a biosafety level 2 facility at the Animal Center

for Pharmaceutical Research of Seoul National University (Seoul, Korea) according to the national guidelines for the care and use of laboratory animals. Six-week-old female BALB/c mice (*n* = 8) (KOATEC, Pyeongtaek, Korea) were anesthetized by Zoletil 50 (15 mg/kg, IP) (Virbac, Carros, France), and 10^6 EID_{50}/50 μL of each recombinant virus was inoculated intranasally. Five mice from each group were weighed for 2 weeks, and three mice were euthanized to obtain lung samples at 3 days post inoculation (dpi). During the experiment, mice with 20% or more body weight loss were euthanized. The sampled lungs were ground with TissueLyzer 2 and 5 mm stainless steel beads (Qiagen, CA, USA) and suspended in PBS. The virus titer (EID_{50}) was measured as previously described (Lee et al., 2018b).

Solid-Phase Receptor Binding Assays

To evaluate the receptor binding affinity of recombinant viruses, a solid-phase assay was used as previously described with some modifications (Lee et al., 2018a; Matrosovich and Gambaryan, 2012). In short, 96-well enzyme-linked immunosorbent assay plates (SPL, Gyeonggi, Korea) coated with 10 μg/ml fetuin (Sigma-Aldrich) were bound with the recombinant viruses overnight. After washing the virus-bound plates 3 times with PBS + 0.05% Tween 20 (PBST), the plates were blocked with 0.1% desialylated BSA + 10 μM oseltamivir (Sigma-Aldrich) for 1 h at 4°C. The blocked plates were washed 3 times again with PBST, and the biotinylated sialylglycopolymers (Neu5Acα2-3Galb1-4GlcNAcb-PAA-biotin, 3'SLN-PAA, and Neu5Acα2-6GalNAca-PAA-biotin, 6'SLN-PAA) (Glycotech Corporation, MD, USA) were serially diluted and added to the plates for

1 h at 4°C. Then, the plates were washed 3 times with PBST and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher Scientific, MA, USA) for 1 h at 4°C. Finally, HRP was developed with TMB substrate (SurModics, MN, USA), and the chromogenic reaction was stopped by adding 0.1 M sulfuric acid. The absorbance at 450 nm was measured by a microplate reader (TECAN, Männedorf, Switzerland).

HA and Hemagglutination Inhibition (HI) Tests

The HA test and HI test with chicken RBCs and guinea-pig RBCs were performed according to the WHO manual for the laboratory diagnosis and virological surveillance of influenza. The recombinant virus was serially diluted 2-fold in 96-well plates, and chicken RBCs (1%) or guinea-pig RBCs (1%) were added. After 40 min of incubation at 4°C, the hemagglutination unit (HAU) of each virus was recorded. Chicken RBCs have similar amounts of sialic acids bound to galactose by α2,3 linkage (SAα2,3Gal) and sialic acid linked to galactose by α2,6 linkage (SAα2,6Gal), and guinea pig RBCs have more SAα2,6Gal than SAα2,3Gal (Ito et al., 1997). The HI test of recombinant viruses was conducted with chicken egg white to compare the resistance of the recombinant viruses against egg white. Chicken egg white was serially diluted as in the HA test, and 4 HAU of each virus were added to each well and incubated for 30 min at 4°C. Then, chicken RBCs or guinea pig RBCs were added, and the HI titer was recorded after 40 min of incubation at 4°C. All experiments were repeated three times independently.

Heat Stability Test

Recombinant viruses were diluted to the same HA titer (24) and aliquoted for heat treatment. Each aliquot was incubated at 60 °C for 0, 5, 15, and 30 min, and the HA titer was measured.

SDS-PAGE and Western Blotting

To confirm the 144N glycosylation, 4 μL of each recombinant virus (CE3) treated or untreated with PNGase F enzyme (New England Biolabs, Ipswich, MA, USA) was mixed with Protein 5X Sample Buffer (ELPIS BIOTECH, Daejeon, Korea) to denature it for 5 min at 95 °C, and SDS-PAGE was performed using NuPAGE 4%–12% Bis-Tris Protein Gels (Life Technologies Co.). The proteins were transferred to a nitrocellulose membrane (Life Technologies Co.), and the membrane was incubated with anti-H5N1 virus (A/Vietnam/1194/2004), HA rabbit IgG (Sino Biological Inc., Beijing, China), followed by incubation with horseradish peroxidase conjugated-goat anti-rabbit IgG (Bethyl Laboratories Inc., Montgomery, AL, USA). Then, HA proteins were visualized with BioFX TMB One Component HRP Membrane Substrate (SurModics IVD, INC., Eden Prairie, MN, USA) and sulfuric acid stop solution (Sigma-Aldrich).

Statistical Analysis

All data were analyzed with IBM SPSS Statistics version 23 (IBM., Armonk, NY, USA). The statistical significance of viral titers in ECEs, growth kinetics in cells, and receptor binding affinity were evaluated by one-way analysis of variance ($p < 0.05$).

Survival rates were compared by Kaplan–Meier survival analysis, and the differences in frequency were assessed by chi-square test ($p < 0.05$).

3.3. Results

Comparison of Amino Acid Sequences of Clade 2.3.2, Clade 2.3.2.1, and Clade 2.3.2.1a, b, and c Proteins

All the amino acid sequences of HA proteins of clade 2.3.2 and clade 2.3.2.1 strains in database were compared, and we found variations at 144NGS and 158NGS and at amino acid residue 223 around the RBS (Table 3.2). We summarized the genetic profiles of representative early strains of each clade in Table 3.2. The early strains of clade 2.3.2 and clade 2.3.2.1b contained neither 144NGS nor 158NGS, or only 144NGS with 223V in common, but clade 2.3.2.1a (none or only 144NGS) and clade 2.3.2.1c (only 144NGS) did have in common the V223I mutation. The frequencies of 144NGS and 158NGS were similar in strains of clade 2.3.2, but most of the 2.3.2.1a, b and c viruses had 144NGS rather than 158NGS (data not shown).

Table 3.2. Comparison of 144NGS, 158NGS, and the residue 223 amino acid of hemagglutinin (HA) proteins from early clade 2.3.2, clade 2.3.2.1, clades 2.3.2.1.a, b, and c highly pathogenic (HP) A(H5N1) viruses.

Clade	Strain	Accession No.	144NGS	158NGS	223	Reference
2.3.2	A/duck/China/E319-2/03	AY518362	– [b]	–	V	(Lee et al., 2007a)
2.3.2.1	A/chicken/Hunan/3/07	GU182142	–	–	V	Direct submission to GenBank
	A/common buzzard/Hong Kong/9213/07	CY036221	+ [c]	–	V	(Smith et al., 2009a)
2.3.2.1a	A/environment/Chang Sha/25/2009	JN543378	–	–	I	Direct submission to GenBank
2.3.2.1b	A/chicken/Guangxi/S2039/09	KT762439	+	–	V	(Feng et al., 2016)
2.3.2.1c	A/great crested-grebe/Qinghai/1/2009	CY063318	+	–	I	(Li et al., 2011)
	A/ruddy shelduck/Mongolia/X42/09	HM006736	+	–	I	(Kang et al., 2011)

[a] H3 numbering

[b] -, absence

[c] +, presence.

Analysis of 144NGS, 158NGS, and Residue 223 Profiles among H5 Sequences in the Database

According to the mutation profiles (MPs) of 144NGS, 158NGS, and residue 223, the H5 sequences deposited in the Influenza Virus Database (n = 4189) were classified into 7 MPs (Table 3.3). MP1-1 characterized by the presence of 223V without either 144NGS or 158NGS was the most frequent (56.7%, wild-type), and MP2-1, containing 158NGS and 223V, was the second most frequent (26.8%). MP3-2, containing 144NGS and 223I, similar to clades 2.3.2.1a and c, was 11.4%. MP1-1 was significantly more frequent than other MPs ($p < 0.05$) (Table 3.3). We classified the 144–146 and 158–160 amino acid sequences according to the required number of point mutations to become putative NGS (precursor NGS) (Table 3.S1). The frequency of 144NGS + 1 was 16.5% and was less frequent than 158NGS+1 (44.9%). The frequency of 144NGS + 2 was 61.1% and was more frequent than 158NGS + 2 (24.5%). To confirm the preferred selection of 158NGS to 144NGS, we counted the total number (188) of HA genes with both amino acid sequences converting to 144NGS (144NGS + 1) or 158NGS (158NGS + 1) by a single point mutation. In addition, we counted the numbers of genes harboring 144NGS or 158NGS with 158NGS + 1 (117) or 144NGS + 1 (406), respectively. The frequency of 144NGS/158NGS+1 was 16.5% (117/711, the total number is the sum of 188, 117, and 406), and 2.4% (17/711) and 14.1% (100/711) possessed V and I at residue 223, respectively. The frequency of 144NGS+1/158NGS was 57.1% (406/711), and 54.6% (388/711) and 2.5% (18/711) possessed V and I at residue 223, respectively (Table 3.2). Therefore, 158NGS was significantly more frequent than 144NGS, and 223V was more

frequent than 223I in the 144NGS + 1/158NGS group ($p < 0.05$). However, 223I was more frequent than 223V in the 144NGS/158NGS+1 group ($p < 0.05$).

Table 3.3. Mutation profiles (MPs) of 144NGS, 158NGS, and residue 223 in HA genes of A(H5) viruses.

MP	144NGS	158NGS	223	n (4189)	Frequency (%)	n (711) [e]	Frequency (%)
1-1	− [a]	−	V	2376	56.7 [b]		
1-2	−	−	I/others	115 (I(40), others (75))	1.0 (I)		
2-1	−	+ [a]	V	1122	26.8 [c]	388	54.6 [c,f]
2-2	−	+	I/L/R	18/1/1	0.4 (I)	18	2.5
3-1	+	−	V	73	1.7	17	2.4
3-2	+	-	I	478	11.4 [d]	100	14.1 [c]
4	+	+	V/I	3/2	0.1		

[a] +/− used for marking presence/absence of N-glycan in HA

[b] Significant difference from other MPs ($p < 0.05$)

[c] Significant difference from MP2-2 ($p < 0.05$)

[d] Significant difference from MP3-1 ($p < 0.05$)

[e] Total number of 144NGS/158NGS + 1, 144NGS + 1/158NGS, and 144NGS + 1/158NGS + 1

[f] Significant difference from MP3-1 and MP3-2 ($p < 0.05$).

Generation of PR8-derived clade 2.3.2.1 H5N1 recombinant viruses and comparison of viral replication efficiency in ECEs.

PR8-derived H5N1 recombinant viruses containing single (N144S or I223V) and combined (N144S and I223V) mutations were generated (Table 3.4). The replication efficiency of the recombinant viruses was compared in terms of 50% chicken embryo infection dose (EID_{50}). The virus titer of rH5N1-N144S-I223V (109.05±0.18 EID_{50}/ml) was significantly higher than those of rH5N1-N144S (108.20±0.17 EID_{50}/ml) and rH5N1-V223I (107.48±0.23 EID_{50}/ml) ($p < 0.05$) but insignificantly higher than that of rH5N1 (Table 3.4).

Table 3.4. Gene constellation and viral titers in ECEs of the H5N1 recombinant viruses.

Recombinant virus	**HA**	**NA**	**Internal genes**	**EID_{50} / 0.1 ml (log10)**
rH5N1	K10-483	K10-483	PR8	7.43 ± 0.28
rH5N1-N144S	K10-483-N144S	K10-483	PR8	6.48 ± 0.23
rH5N1-I223V	K10-483-I223V	K10-483	PR8	7.20 ± 0.17
rH5N1-N144S-I223V	K10-483-N144S-I223V	K10-483	PR8	8.05 ± 0.18[a]

[a] Significant difference from rH5N1-N144S and rH5N1-I223V ($p < 0.05$).

Comparison of replication efficiency in mammalian cells and mouse pathogenicity of the H5N1 recombinant viruses.

The replication efficiency of H5N1 recombinant viruses was compared in MDCK and A549 cells. The viral titers in MDCK cells were similar among the recombinant viruses at 24 and 48 hpi, but rH5N1-N144S-I223V showed a significantly higher titer than other H5N1 recombinant viruses at 72 hpi (Fig. 3.1A). In A549 cells, rH5N1-N144S-I223V showed significantly higher viral titers at 48 and 72 hpi (Fig. 3.1B).

All the H5N1 recombinant viruses replicated in the lungs of infected BALB/c mice at 3 dpi, but rH5N1-N144S-I223V showed a significantly higher virus titer than other H5N1 recombinant viruses (Table 3.5). In addition, rH5N1-N144S-I223V infection resulted in apparent body weight loss in all mice, and the mice died (100% mortality) within 4 dpi (Fig. 3.2). However, rH5N1-N144S, rH5N1-I223V and rH5N1 did not cause significant body weight loss during the observation period.

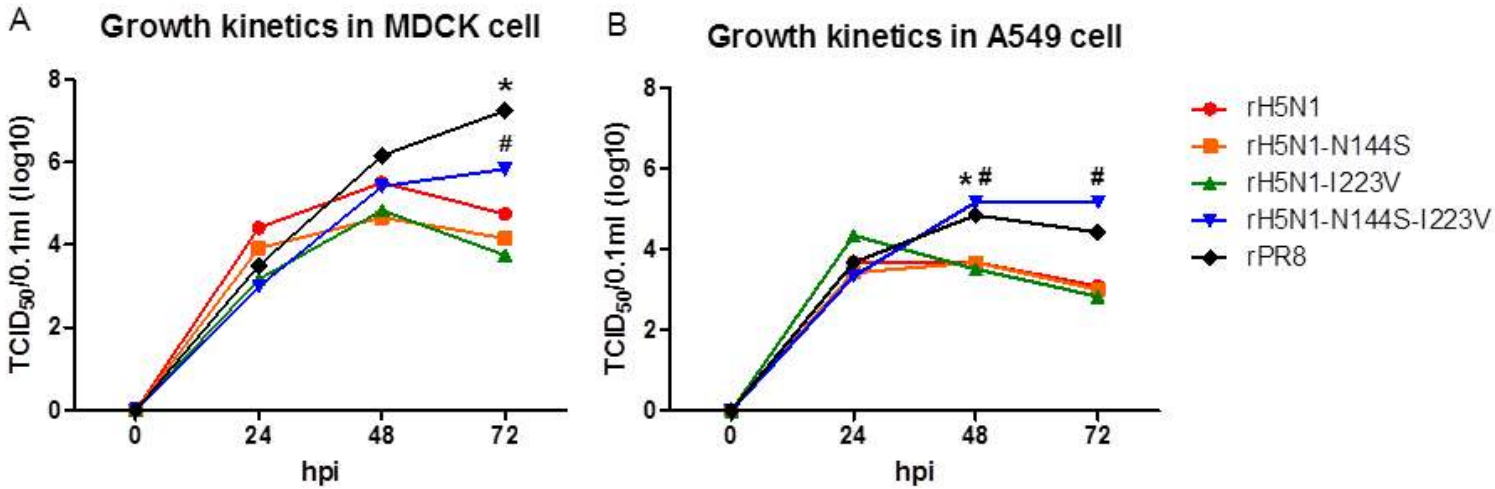

Figure 3.1. Growth kinetics of recombinant H5N1 viruses in MDCK and A549 cells. Each recombinant virus was diluted to 105 EID_{50}/0.1 mL, and 0.5 mL of diluents were inoculated into confluent MDCK and A549 cells in 6-well plates for 1 h. After 1 h, the inoculated virus was removed, and 1 mL of fresh medium was added. During 72 h of incubation, the supernatant was harvested at 0, 24, 48 and 72 hpi, and TCID50/0.1 mL of each time point was measured in MDCK cells. The TCID50/0.1 mL values are the average of three independent experiments. #, *, significant differences of rH5N1-N144S-I223V(#) and rPR8(*) in comparison with other viruses ($p < 0.05$).

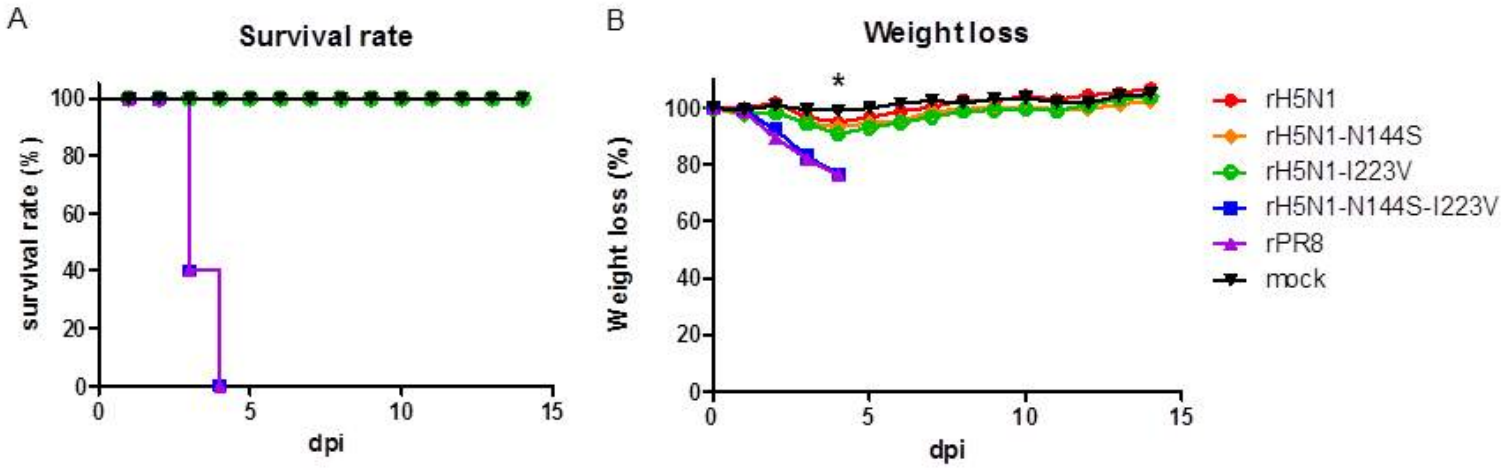

Figure 3.2. Mouse pathogenicity of recombinant H5N1 viruses. Mortality and weight loss of mouse experimental groups infected with recombinant H5N1 viruses. Five 6-week-old female BALB/c mice per group were inoculated with 106 EID_{50} of virus or an equivalent volume PBS (mock) intranasally. Weight loss was monitored for 2 weeks, and mice with more than 20% weight loss were euthanized. The weight loss was calculated based on the body weight measured at 0 dpi, and the data are the average of each group; *, significant difference of the rH5N1–I223V and rH5N1-N144S groups compared to the mock groups ($p < 0.05$).

Table 3.5. Replication efficiency of the H5N1 recombinant viruses in mice.

Recombinant virus	**Virus isolation rate**	**EID_{50}/0.1ml (log10)[a]**
rH5N1	3/3	5.75
rH5N1-N144S	3/3	5.25
rH5N1-I223V	3/3	5.00
rH5N1-N144S-I223V	3/3	7.75[b]
rPR8	3/3	8.00
Mock (PBS)	0/3	ND[c]

[a] EID_{50}/0.1ml was average of three independent replicate experiments

[b] Significant difference from other H5N1 recombinant viruses ($p < 0.05$)

[c] Not detected

Comparison of binding affinity of the H5N1 recombinant viruses to avian (3'-SLN) and mammalian (6'-SLN) receptors and egg white.

We compared the receptor binding affinity of recombinant viruses to 3'-SLN and 6'-SLN (Fig. 3.3). All the recombinant viruses bound to 3'-SLN more strongly than to 6'-SLN. rH5N1 and rH5N1-N144S-I223V showed significantly higher binding affinities than other viruses, and rH5N1-N144S had the weakest binding affinity to the avian receptor (Fig. 3.3). When chicken RBCs were used to measure HI titers in egg white for the H5N1 recombinant viruses, only rH5N1-N144S showed slightly lower HI titers than other viruses (64 vs. 128, Table 3.6). However, the difference was much higher when we used guinea pig RBCs (<8 vs. 64). Therefore, rH5N1-N144S was significantly less inhibited by egg white, and 223I may play a role in the resistance to egg white.

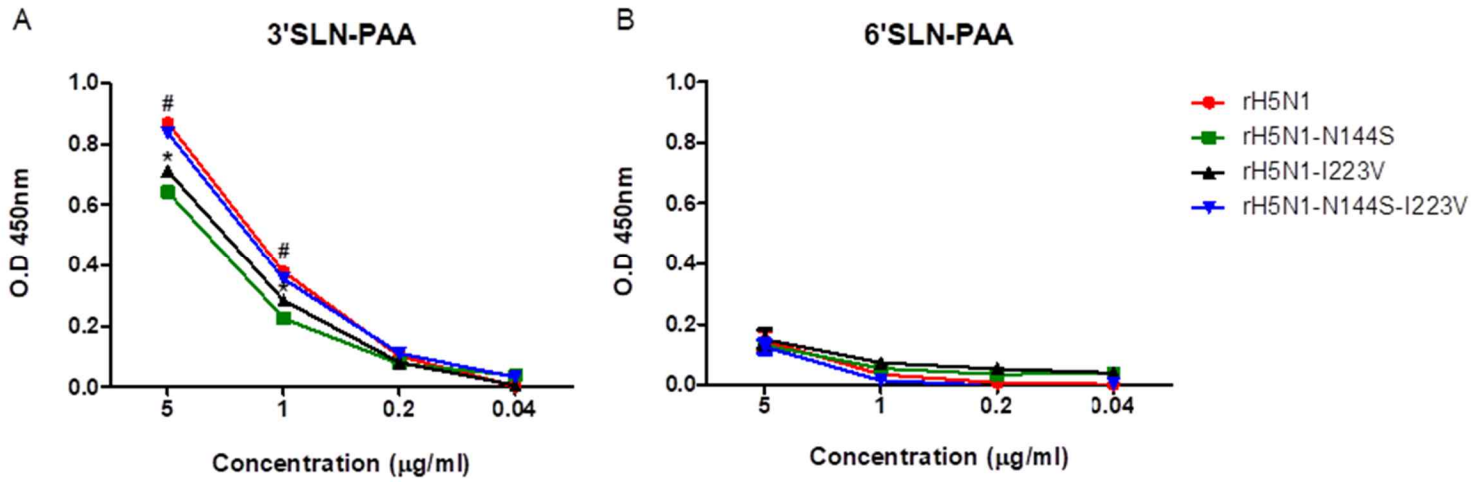

Figure 3.3. Receptor binding affinity of recombinant H5N1 viruses. The two types of serially diluted biotinylated sialylglycopolymers (Neu5Acα2-3Galb1-4GlcNAcb-PAA-biotin [3'SLN-PAA] and Neu5Acα2-6GalNAca-PAA-biotin [6'SLN-PAA]) were incubated with the same concentration (105 EID_{50}) of recombinant viruses. After development with HRP-conjugated streptavidin and TMB substrate, the reaction was stopped by adding stop solution, and the absorbance at 450 nm was measured. (a) Receptor binding affinity of recombinant viruses to 3'SLN-PAA and (b) Receptor binding affinity of recombinant viruses to 6'SLN-PAA. The absorbance data are the average of three independent experiments, #, significant difference of rH5N1 and rH5N1-N144S-I223V compared to the other viruses, *, significant difference compare to rH5N1–N144S ($p < 0.05$).

Table 3.6. Comparison of HI titers of egg white against the H5N1 recombinant viruses.

Recombinant virus	HI titer of chicken egg white	
	Chicken RBC (1%)	Guinea-pig RBC (1%)
rH5N1	128	64
rH5N1-N144S	64	< 8
rH5N1-I223V	128	64
rH5N1-N144S-I223V	128	64

Comparison of thermostability of the H5N1 recombinant viruses.

The HA titers of the H5N1 recombinant viruses decreased to zero within 5 (rH5N1-N144S), 15 (rH5N1) and 30 min (rH5N1-I223V) after heat treatment, but that of rH5N1-N144S-I223V was maintained even after heat treatment for 30 min (Fig. 3.4). Therefore, the V223I mutation may decrease HA thermostability.

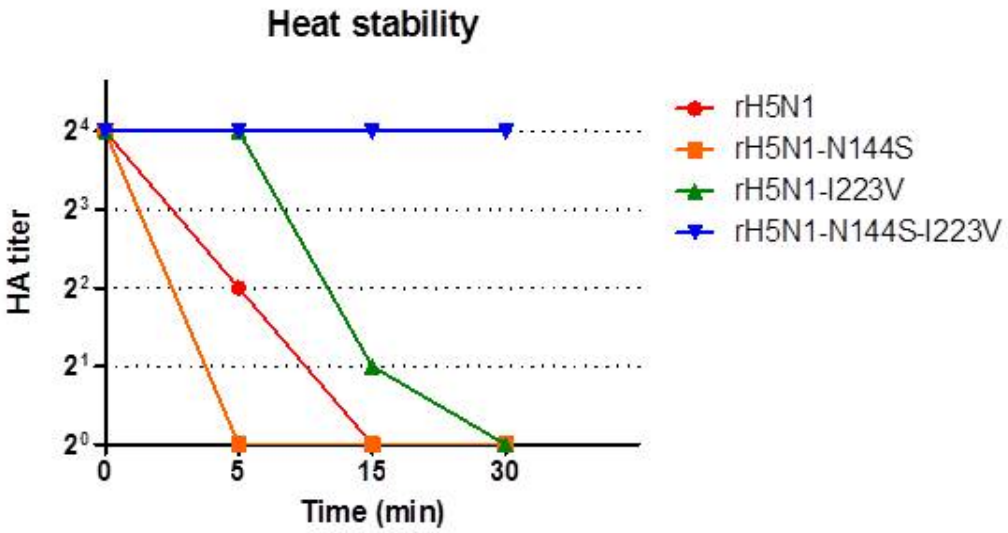

Figure 3.4. Heat stability of recombinant H5N1 viruses. Each of the recombinant viruses was diluted to a 2^4 HA titer, and aliquots were incubated at 60 °C for 0, 5, 15 and 30 min. After heat treatment, the HA titer of each aliquot was measured by HA assay with 1% chicken RBCs.

Intra- and intermolecular interactions of residue 223 in the HA trimer and confirmation of the 144N-glycosylation.

Residue 223 is located in the 220-loop, and both 223I and 223V interact intramolecularly with 226Q via hydrogen bonding. Interestingly, residue 223 is located close to 207S of another neighboring HA monomer. Considering the larger side chain of I than V, the V223I mutation may affect the integrity of the HA trimer (Fig. 3.5). So far, 144NGS is considered to be a real N-glycosylation site, and our western blotting data also agree with previous report (Fig. 3.6) (Herve et al., 2015).

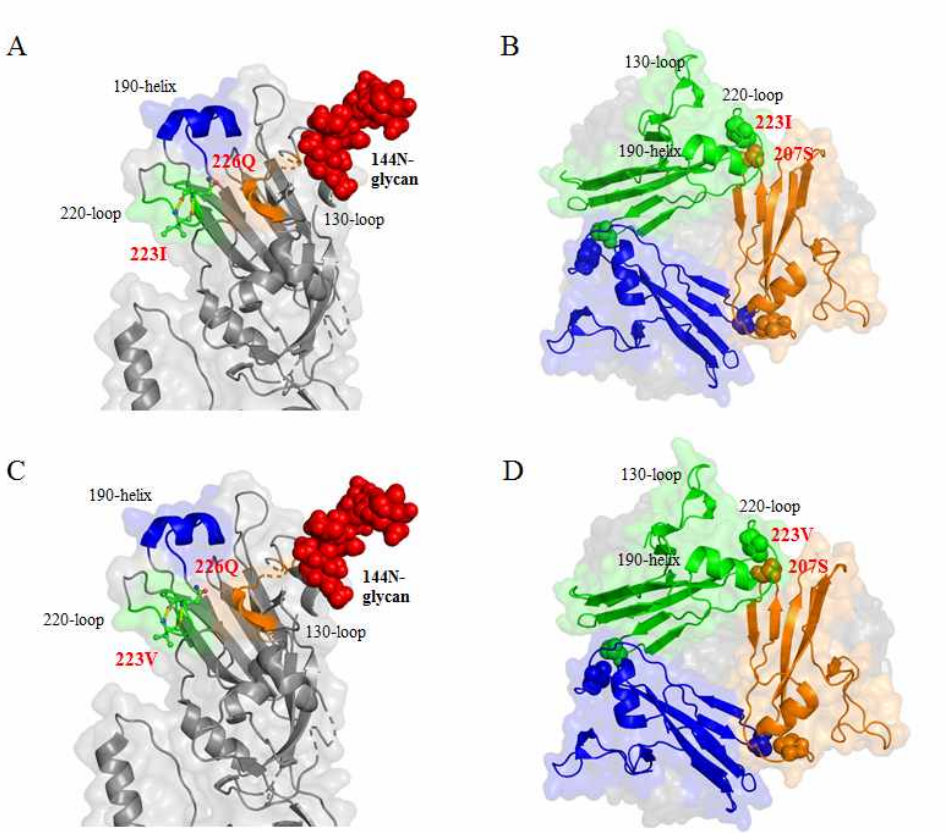

Figure 3.5. Location and intermolecular interaction of 144N and 223V/I residues in the 3D structure of the HA trimer. HA and HA trimer structure were modified from 4juk.pdb and 6e7g.pdb using PyMOL. (a) 223I and (c) 223V were located in the 220-loop of the receptor binding site (RBS) of the globular head. Position 223 was close to position 226Q, and both 223I and 223V were interacting with 226Q by hydrogen bond (dotted line). (a), (c) 144N followed by 145S and 146S was located near the RBS, and 144N glycosylation was formed by N-X-S/T. (b) 223I and (d) 223V were close to the 207S of another HA monomer, and 223I had more side chains extruding and was much closer to 207S than 223V.

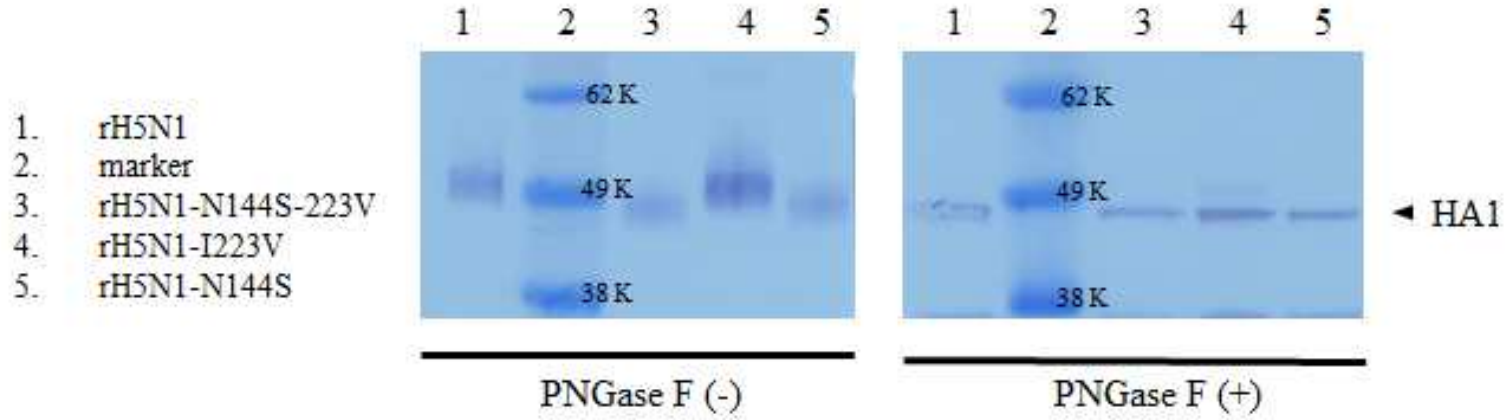

Figure 3.6. Verification of 144N-glycosylation by western blotting. Recombinant viruses untreated and treated with PNGase F enzyme were denatured and separated by SDS-PAGE. Transferred membranes were incubated with rabbit anti-influenza A H5N1 (A/Vietnam/1194/2004) HA IgG, followed by goat anti-rabbit IgG HRP-conjugated secondary antibody. Then, HRP was developed by TMB substrate. HA proteins of rH5N1 and rH5N1-I223V had 144N-glycan, and they had higher molecular weight than rH5N1-N144S and rH5N1-N144S-I223V in the absence of PNGase F enzyme treatment (-). However, the difference was disappeared after treatment of PNGase F enzyme (+).

3.4. Discussion

Clade 2.3.2 viruses were isolated from ducks and wild birds in mainland China and Hong Kong in 2004, but they might have been already present in mainland China in 2003 due to virus isolation from Muscovy ducks smuggled from China to Taiwan (Group, 2012; Lee et al., 2007b; Smith et al., 2009b). New clade 2.3.2.1 viruses derived from clade 2.3.2 viruses appeared in 2007, and clade 2.3.2.1a, b and c viruses appeared in 2009 (Group, 2012; World Health Organization/World Organisation for Animal and Agriculture Organization, 2014). In Viet Nam clade 2.3.2.1a, b and c viruses used to cocirculate, but clade 2.3.2.1b viruses have disappeared (Nguyen et al., 2017). Clade 2.3.2.1 viruses acquired 144NGS, and this mutation was conserved in most of the clade 2.3.2.1a, b and c viruses. Considering the significantly higher frequency of 158NGS than 144NGS among A(H5) viruses having the same possibility of acquiring either 144NGS or 158NGS (Table 3.S1), the acquisition of 144NGS may reflect the presence of certain selection pressure or other circumstances.

HA glycosylation to evade humoral immunity may be the most effective but final choice because it reduces viral fitness (Das et al., 2011). The variability of NGS precursors and their higher frequencies than NGS at 144-146 (86.5% vs. 13.5%) and 158-160 (71.9% vs. 28.1%) may be in line with the above notion (Table 3.S1). 144N-glycan reduced the replication efficiency in ECEs and the a2,3 SA affinity of rH5N1-I223V in comparison with rH5N1-N144S-I223V. Therefore, 144N-glycan in H5 reduced viral fitness, similar to the findings in a previous report in H1 (Das et al., 2011). However, circumstantial evidence

explaining why clade 2.3.2.1 viruses chose 144NGS instead of the more prevalent and preferable 158NGS needs to be discussed further.

Humoral immunity induced by vaccination may facilitate the appearance of mutants evading vaccine immunity (Lee et al., 2004). The vaccine program was implemented with A/chicken/Mexico/232-CPA/1994 (H5N2) in HK during 2002-2003 and with A/turkey/England/N28/1973 (H5N2) during 2004-2006 in mainland China. Monovalent [Re-1 (clade 1) during 2004-2008, Re-4 (clade 7.2) during 2006-2012, Re-5 (clade 2.3.4) during 2008-2012, Re-6 (clade 2.3.2) in 2012] and bivalent (Re-1/Re-4 during 2007-2008, Re-4/Re-5 during 2008-2012 and Re-4/Re-6 in 2012) inactivated PR8-derived recombinant vaccines have been used (Chen and Bu, 2009; Ellis et al., 2004). Among them, Re-1, Re-4 and Re-5 were the major vaccines in mainland China during the evolution period (2005-2009) of clade 2.3.2 to clade 2.3.2.1a, b and c, and Re-4 and Re-5 contained only 158NGS, and other viruses, except Re-6 (only 144NGS), contained neither 144NGS nor 158NGS (Li et al., 2014). Therefore, vaccine-induced antibodies might have targeted the shielded epitope site B (group 2, 155-166) rather than epitope site A (group 1, 140-145), and mutant viruses acquiring 144NGS to shield epitope site B might have been selected (Herve et al., 2015). Re-6 with 144NGS might have been effective against clade 2.3.2.1a, b and c viruses not only due to antigenic similarity but also due to the restricted acquisition of additional 158NGS. The very low frequency of A(H5) viruses possessing both 144NGS and 158NGS (0.1%) may reflect the inferior competitiveness of such HA in nature (Table 3.3). In Viet Nam where clade 2.3.4 and clade 2.3.2.1a, b and c viruses had cocirculated, Re-1 during 2005-2010 and Re-5 (clade 2.3.4) since 2011 had been used for

vaccinations (WHO, 2011). Clade 2.3.4 viruses declined after vaccination, but clade 2.3.2.1c viruses became enzootic, possibly due to antigenic mismatch and the shielding effect of 144N-glycan (FAO, 2011; Nguyen et al., 2017; Nguyen et al., 2019).

The V223I mutation is unlikely to stand alone without 144NGS due to its very low frequency in nature, and stepwise acquisitions of 144NGS and V223I mutations during clade 2.3.2.1a, b and c diversification from clade 2.3.2.1 are noteworthy (Table 3.3). Similarly, cooperating mutations with N-glycans, K147 with 144NGS and N227S with 158NGS, have been reported (Kim et al., 2013b; Wang et al., 2010). The biological effects and roles of the V223I mutation are unclear, but a single V223I mutation decreased the virus replication efficiency of rH5N1-N144S in ECEs. Additionally, rH5N1-N144S showed resistance to egg white due to a relatively steep decrease in HI titer and significantly lower a2,3 SA affinity than those of the other viruses in this study (Table 3.6, Fig. 3.3). Ovomucin in egg white is an effector molecule of innate immunity present on the surface of mucous membrane, and a different mutation reducing the inhibition has been reported (Lanni and Beard, 1948; Lee et al., 2018a). Therefore, characterization of egg white resistance may be useful to understand the evolutionary status of IAVs. Mutations such as S223N (S224N according to our H3 numbering), N224K, G225D, Q226L, G228S, and S227N directly or indirectly increase a2,6 SA affinity, and their side chains are located in or near the RBS. However, the side chain of residue 223 is located at the interface of the globular heads of the HA trimer (Fig. 3.5). The amino acid residues located between the interfaces of the HA trimer affect structure and pH stability, and the electrostatic intermolecular interaction between T212 and N216 and the increased rigidity of the S221P

mutation stabilized the HA trimer to increase pH stability (DuBois et al., 2011; Rachakonda et al., 2007). The lower thermostability of rH5N1-N144S than rH5N1 and rH5N1-I223V may imply a negative effect of the intermolecular interaction between 223I and 207S on HA thermostability (Fig. 3.5). V/I223 did not interact with S207 via hydrogen bonding, and the bulkier side chain of I may cause steric hindrance at the interface. Although we did not test pH stability, a pH stability-related mutation (H103Y) showed increased thermostability by stabilizing the HA trimer in previous reports (An et al., 2019b; de Vries et al., 2014b). Therefore, the V223I mutation acquired in addition to S144N during adaptation in vaccinated poultry might have improved viral fitness in terms of a2,3 SA receptor affinity at the cost of decreased HA trimer stability.

The mammalian pathogenicity of HP A(H5N1) viruses is a multigenic trait, and the human pathogenicity of clade 2.3.2.1 viruses has been regarded as lower than that of other clades (Creanga et al., 2017). In our previous studies, a PR8-derived clade 2.3.2.1c recombinant vaccine strain showed less pathogenicity in mice than another PR8-derived recombinant virus containing HA and NA genes from low pathogenic (LP) A(H5N1) virus (Jang et al., 2017b; Kim et al., 2015b). Although the amino acid sequences of the two HA proteins are only 89% identical, the HA of LP A(H5N1) virus has V223 and neither 144NGS nor 158NGS. The higher mouse pathogenicity and replication efficiency of rH5N1-N144S-V223I than other H5N1 recombinant viruses in MDCK and A549 cells may indicate a reduction in mammalian pathogenicity during poultry adaptation to evade immune responses. Poorly glycosylated HA was recognized by an ER stress pathway and induced strong lung injury (Hrincius et al., 2015). The relatively high mammalian

pathogenicity of viruses without 144NGS and 158NGS has been verified (Wang et al., 2010). As we did not directly compare the pathogenicity of 144NGS- or 158NGS-bearing recombinant viruses, we cannot conclude which virus is more pathogenic in humans. However, a significantly higher frequency of 158NGS- (70.0%) than 144NGS-bearing (1.6%) HA in human cases compared with the frequencies of single 158NGS- (27.2%) or 144NGS-bearing (13.1%) HA in nature may reflect a higher risk of 158NGS than 144NGS in human infection (Table 3.S2).

In conclusion, intensive inoculation of certain types of vaccines may distort natural evolutionary pathways, and the acquired novel adaptive mutations may reduce viral fitness by destabilizing the HA trimer as well as affect mammalian pathogenicity. Our results may provide clues for future studies to develop more effective vaccine strains and programs that reduce the appearance of antigenic or more human-pathogenic variants.

3.5. Supplementary Materials

Table 3.S1. Classification and frequency of 144NGS, 158NGS, and their precursors of A(H5) viruses.

No. of mutations to become 144NGS (4075)				No. of mutations to become 158NGS (4065)				
0 (551)	+1(658)	+2 (2489)	+3 (377)	0 (1141)	+1 (1824)	+2 (996)	+3 (104)	+4 (2)
NSS (551)	KSS (550)	RSS (1812)	GPS (130)	NNT (490)	NNA(927)	DNA(841)	DNV(56)	ENV(1)
	NPS (36)	TPS (292)	MPS (92)	NDT (49)	NDA(596)	NNV(54)	GNA(29)	GNE(1)
	SSS (55)	SPS (115)	RPS (68)	NST (563)	NSA(217)	DDA(21)	DGV(6)	
	TSS (10)	ASS (74)	VPS (39)	NSS (30)	DNT(53)	DSA(18)	DDV(4)	
	SHS (1)	KPS (73)	APS (20)	NNS (5)	NAA(9)	SNA(18)	DNM(3)	
		QSS (51)	EPS (19)	NGT (2)	NGA(8)	NNE(16)	QNA(2)	
		GSS (15)	RSA (6)	NDS (1)	DST(3)	NDV(10)	INV(1)	
		MSS (14)	ASA (1)	NHT (1)	NNI(2)	SDA(6)	DNE(1)	
		GLS (12)	KPF (1)	NTT (1)	NSI(2)	DSS(3)	ANA(1)	
		ESS (8)	RSF (1)		NDK(1)	GST(2)	GDA(1)	
		ISS (7)			NDP(1)	NDE(1)		
		RTS (6)			NNK(1)	NNQ(1)		
		RAS (3)			NNM(1)	DTA(1)		
		VSS (2)			DNS(1)	ENA(1)		
		DPS (1)			SNS(1)	SNT(1)		
		ELS (1)			SST(1)	YNA(1)		
		GRS (1)						
		MLS (1)				KNN(1)		
		RFS (1)						
% of 144NGS	% of precursor 144NGS			% of 158NGS	% of precursor 158NGS			
13.5% (551/4075)	86.5% (3524/4075)			28.1% (1141/4065)	71.9% (2924/4065)			

Table 3.S2. Frequency of 144NGS and 158NGS in HA of A(H5) viruses from laboratory-confirmed human cases.

NGS	Human (n=513)	Avian (n=4,189)
144NGS	1.6%	13.1%[a]
158NGS	70.0%[b]	27.2%
144NGS/158NGS	0.4%	0.1%
None	28.1%	57.7%[c]

[a] significant difference with 144NGS in human ($p < 0.05$).

[b] significant difference with 158NGS in avian ($p < 0.05$).

[c] significant difference with none in human ($p < 0.05$).

General Conclusion

In these studies, several genetic modifications were adopted without affecting antigenicity of viruses to improve recombinant avian influenza vaccine strains using reverse genetics system and antigenic evolution of clade 2.3.2.1c H5N1 HPAIV related with mass vaccination in poultry was studied.

Generation of the H9N2 LPAI vaccine strain, 01310, using reverse genetics was hard, but it was successfully achieved by optimization of 3'-end promoter of polymerase genes. High replication of PB2 and PB1 by C4U mutation compensated low integrity of polymerase complex and facilitate viral recue, but C4U mutation in PA also increased PA-X expression which was negative effects on viral replication and didn't facilitate viral rescue. However, another H9N2 LPAIV, 0028, was generated without C4U mutation in polymerase gene and effect of C4U mutations was strain-specific. To eliminate embryonic pathogenicity of 01310, recombinant 01310 virus having mammalian non-pathogenic NS gene of 0028 was generated through 3'-end promoter modification. The recombinant 01310 with NS (0028) showed decreased embryonic pathogenicity and increased productivity in ECEs, and several mammalian pathogenic mutation found in NS gene (G139D, S151T, GSEV to EPEV) were also pathogenic factor in chicken embryo. The 3'-end promoter optimization and mammalian non-pathogenic genes can be a strategy to improve safety and productivity of vaccine strains generated by reverse genetics system.

In recombinant clade 2.3.4.4 H5N8 vaccine, H103Y single mutation in HA protein increased viral replication in ECEs and recombination of prototypic PB2 gene of

01310 virus eliminated mammalian pathogenicity. The H103Y mutation was related with structure stability of HA protein and H103Y mutant had heat-stability which was advantageous for storage and transport of vaccine in countries with poor cold chain systems. Two useful methods for effective vaccine production in this study were found to have same effect on LPAI H5N1 and clade 2.3.2.1c H5N1 viruses, and they can be usefully applied to other H5 vaccine development. However, a single dose of the recombinant H5N8 vaccine didn't prevent viral shedding even with high serum antibody titer, and it is necessary to improve the antigenicity of the recombinant H5N8 vaccine strain to prevent viral shedding.

At last, two adaptive mutations appeared during viral evolution of clade 2.3.2.1 H5N1 HPAIVs were found, and analyzed their effect in viral characteristics by reverse mutation of clade 2.3.2.1c HA protein and generation of recombinant viruses. Considering most of HPAI vaccine strains used in HPAI endemic countries had 158N-glycosylation, the 144N-glycosylation in clade 2.3.2.1 was result of antigenic evolution for shielding epitopes. After then, V223I mutation was accumulated to adapt to the natural host because N-glycosylation decreased binding affinity of HA protein and required compensation mutations. Clade 2.3.2.1c H5N1 could remained and spread broadly by two adaptive mutations, but it lost structural stability of HA protein and high replication efficiency in mammalian host. Thus, intensive vaccination with certain subtypes can diversified HPAIV strains having distinct antigenicity, and antigenic evolution pattern of clade 2.3.2.1c in this study may be helpful to developing HPAI vaccine strains and vaccination programs.

Through several strategies found in these studies, avian influenza vaccine strain

with improved productivity and biosafety using reverse genetics system was developed, and mammalian non-pathogenic evolutionary pattern can be helpful to develop HPAI vaccination program. In order to produce vaccines with increased immunogenicity, further studies on inactivated vaccines with internal genes of avian influenza A virus, and not PR8, and whether this will improve cellular immunity are required.

References

Alexander, D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine 25, 5637-5644.

Alexander, D.J., Parsons, G., Manvell, R.J., 1986. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. Avian Pathol 15, 647-662.

An, S.-H., Lee, C.-Y., Choi, J.-G., Lee, Y.-J., Kim, J.-H., Kwon, H.-J., 2019a. Generation of highly productive and mammalian nonpathogenic recombinant H9N2 avian influenza viruses by optimization of 3'end promoter and NS genome. Vet Microbiol 228, 213–218.

An, S.H., Lee, C.Y., Hong, S.M., Choi, J.G., Lee, Y.J., Jeong, J.H., Kim, J.B., Song, C.S., Kim, J.H., Kwon, H.J., 2019b. Bioengineering a highly productive vaccine strain in embryonated chicken eggs and mammals from a non-pathogenic clade 2.3.4.4 H5N8 strain. Vaccine.

Beard, C.W., 2003. Immunization approaches to avian influenza. Avian Diseases 47, 172-177.

Bender, B.S., Croghan, T., Zhang, L., Small, P., 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. Journal of Experimental Medicine 175, 1143-1145.

Bi, Y., Chen, J., Zhang, Z., Li, M., Cai, T., Sharshov, K., Susloparov, I., Shestopalov, A., Wong, G., He, Y., Xing, Z., Sun, J., Liu, D., Liu, Y., Liu, L., Liu, W., Lei, F., Shi,

W., Gao, G.F., 2016. Highly pathogenic avian influenza H5N1 Clade 2.3.2.1c virus in migratory birds, 2014-2015. Virol Sin 31, 300-305.

Boulo, S., Akarsu, H., Ruigrok, R.W., Baudin, F., 2007. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. Virus research 124, 12-21.

Brown, D.M., Dilzer, A.M., Meents, D.L., Swain, S.L., 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. The journal of immunology 177, 2888-2898.

Bublot, M., Swayne, D., Selleck, P., Montiel, E., Pritchard, N., Lee, M. 2005. TROVAC AIH5, AN AVIAN INFLUENZA FOWLPOX VECTOR VACCINE, AS AN ALTERNATIVE VACCINE FOR HATCHERIES. In: Meeting Abstract, 68.

Carrat, F., Flahault, A., 2007. Influenza vaccine: the challenge of antigenic drift. Vaccine 25, 6852-6862.

Caton, A.J., Brownlee, G.G., Yewdell, J.W., Gerhard, W., 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31, 417-427.

Cauthen, A.N., Swayne, D.E., Sekellick, M.J., Marcus, P.I., Suarez, D.L., 2007. Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. Journal of virology 81, 1838-1847.

Chambers, T.M., Kawaoka, Y., Webster, R.G., 1988. Protection of chickens from lethal influenza infection by vaccinia-expressed hemagglutinin. Virology 167, 414-421.

Chen, H., Bu, Z., 2009. Development and application of avian influenza vaccines in China. Curr Top Microbiol Immunol 333, 153-162.

Choi, J.G., Lee, Y.J., Kim, Y.J., Lee, E.K., Jeong, O.M., Sung, H.W., Kim, J.H., Kwon, J.H., 2008a. An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. Journal of veterinary science 9, 67-74.

Choi, J.G., Lee, Y.J., Kim, Y.J., Lee, E.K., Jeong, O.M., Sung, H.W., Kim, J.H., Kwon, J.H., 2008b. An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. J Vet Sci 9, 67–74.

Choi, J.G., Lee, Y.J., Kim, Y.J., Lee, E.K., Jeong, O.M., Sung, H.W., Kim, J.H., Kwon, J.H.J.J.o.v.s., 2008c. An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. 9, 67-74.

Claes, F., Morzaria, S.P., Donis, R.O., 2016. Emergence and dissemination of clade 2.3.4.4 H5Nx influenza viruses-how is the Asian HPAI H5 lineage maintained. Curr Opin Virol 16, 158-163.

Copeland, C.S., Doms, R.W., Bolzau, E.M., Webster, R.G., Helenius, A., 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J Cell Biol 103, 1179-1191.

Crawford, J., Wilkinson, B., Vosnesensky, A., Smith, G., Garcia, M., Stone, H., Perdue, M.L., 1999. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine 17, 2265-2274.

Creanga, A., Hang, N.L.K., Cuong, V.D., Nguyen, H.T., Phuong, H.V.M., Thanh, L.T., Thach, N.C., Hien, P.T., Tung, N., Jang, Y., Balish, A., Dang, N.H., Duong, M.T., Huong, N.T., Hoa, D.N., Tho, N.D., Klimov, A., Kapella, B.K., Gubareva, L., Kile, J.C., Hien, N.T., Mai, L.Q., Davis, C.T., 2017. Highly Pathogenic Avian Influenza

A(H5N1) Viruses at the Animal-Human Interface in Vietnam, 2003-2010. J Infect Dis 216, S529-S538.

Crotty, S., 2011. Follicular helper CD4 T cells (Tfh). Annual review of immunology 29, 621-663.

Das, S.R., Hensley, S.E., David, A., Schmidt, L., Gibbs, J.S., Puigbo, P., Ince, W.L., Bennink, J.R., Yewdell, J.W., 2011. Fitness costs limit influenza A virus hemagglutinin glycosylation as an immune evasion strategy. Proc Natl Acad Sci U S A 108, E1417-1422.

de Vries, R.P., Zhu, X., McBride, R., Rigter, A., Hanson, A., Zhong, G., Hatta, M., Xu, R., Yu, W., Kawaoka, Y., 2014a. Hemagglutinin receptor specificity and structural analyses of respiratory droplet-transmissible H5N1 viruses. Journal of virology 88, 768–773.

de Vries, R.P., Zhu, X., McBride, R., Rigter, A., Hanson, A., Zhong, G., Hatta, M., Xu, R., Yu, W., Kawaoka, Y., de Haan, C.A., Wilson, I.A., Paulson, J.C., 2014b. Hemagglutinin receptor specificity and structural analyses of respiratory droplet-transmissible H5N1 viruses. J Virol 88, 768-773.

Dias, A., Bouvier, D., Crépin, T., McCarthy, A.A., Hart, D.J., Baudin, F., Cusack, S., Ruigrok, R.W., 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458, 914.

Dobbelaer, R., Levandowsky, R., Wood, J.A., 2005. 3. Recommendations for the production and control of influenza vaccine (inactivated). WHO technical report series.

DuBois, R.M., Zaraket, H., Reddivari, M., Heath, R.J., White, S.W., Russell, C.J., 2011. Acid stability of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity. PLoS Pathog 7, e1002398.

Eisfeld, A.J., Neumann, G., Kawaoka, Y., 2015. At the centre: influenza A virus ribonucleoproteins. Nat Rev Microbiol 13, 28-41.

Ekiert, D.C., Bhabha, G., Elsliger, M.-A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., Wilson, I.A., 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246-251.

Ellis, T.M., Leung, C.Y., Chow, M.K., Bissett, L.A., Wong, W., Guan, Y., Malik Peiris, J.S., 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol 33, 405-412.

Epstein, S.L., Kong, W.-p., Misplon, J.A., Lo, C.-Y., Tumpey, T.M., Xu, L., Nabel, G.J., 2005. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 23, 5404-5410.

FAO 2011. FAOAIDE news, 6.

Feng, X., Wang, Z., Shi, J., Deng, G., Kong, H., Tao, S., Li, C., Liu, L., Guan, Y., Chen, H.J.J.o.v., 2016. Glycine at position 622 in PB1 contributes to the virulence of H5N1 avian influenza virus in mice. 90, 1872-1879.

Fujimoto, Y., Tomioka, Y., Takakuwa, H., Uechi, G., Yabuta, T., Ozaki, K., Suyama, H., Yamamoto, S., Morimatsu, M., Mai le, Q., Yamashiro, T., Ito, T., Otsuki, K., Ono, E., 2016. Cross-protective potential of anti-nucleoprotein human monoclonal antibodies against lethal influenza A virus infection. J Gen Virol 97, 2104-2116.

Gamblin, S.J., Skehel, J.J., 2010. Influenza hemagglutinin and neuraminidase membrane glycoproteins. Journal of Biological Chemistry 285, 28403-28409.

Gao, H., Sun, Y., Hu, J., Qi, L., Wang, J., Xiong, X., Wang, Y., He, Q., Lin, Y., Kong, W., Seng, L.G., Sun, H., Pu, J., Chang, K.C., Liu, X., Liu, J., 2015. The contribution of PA-X to the virulence of pandemic 2009 H1N1 and highly pathogenic H5N1 avian influenza viruses. Sci Rep 5, 8262.

Ge, J., Deng, G., Wen, Z., Tian, G., Wang, Y., Shi, J., Wang, X., Li, Y., Hu, S., Jiang, Y., 2007. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. Journal of virology 81, 150-158.

GeurtsvanKessel, C., Lambrecht, B., 2008. Division of labor between dendritic cell subsets of the lung. Mucosal immunology 1, 442.

Giles, B.M., Ross, T.M., 2011. A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets. Vaccine 29, 3043–3054.

Gotch, F., McMichael, A., Smith, G., Moss, B., 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. Journal of Experimental Medicine 165, 408-416.

Group, W.H.O.W.O.f.A.H.F.A.O.H.N.E.W., 2014. Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses. Influenza other respiratory viruses 8, 384-388.

Group, W.O.F.H.N.E.W., 2012. Continued evolution of highly pathogenic avian influenza

A (H5N1): updated nomenclature. Influenza Other Respir Viruses 6, 1-5.

Gu, M., Chen, H., Li, Q., Huang, J., Zhao, M., Gu, X., Jiang, K., Wang, X., Peng, D., Liu, X., 2014. Enzootic genotype S of H9N2 avian influenza viruses donates internal genes to emerging zoonotic influenza viruses in China. Veterinary microbiology 174, 309-315.

Hale, B.G., Randall, R.E., Ortin, J., Jackson, D., 2008a. The multifunctional NS1 protein of influenza A viruses. J Gen Virol 89, 2359-2376.

Hale, B.G., Randall, R.E., Ortín, J., Jackson, D., 2008b. The multifunctional NS1 protein of influenza A viruses. Journal of general virology.

Hamada, H., Bassity, E., Flies, A., Strutt, T.M., de Luz Garcia-Hernandez, M., McKinstry, K.K., Zou, T., Swain, S.L., Dutton, R.W., 2013. Multiple redundant effector mechanisms of CD8+ T cells protect against influenza infection. The Journal of Immunology 190, 296-306.

Hamilton, M., Russo, R., Thurston, R.J.E.S., Technology, 1978. Trimmed Spearman-Karber method for estimating median lethal concentrations in bioassays. Environmental Science Technology 12, 417-417.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environmental Science & Technology 11, 714-719.

Hanson, R., Brandly, C., 1955. Identification of vaccine strains of Newcastle disease virus. Science 122, 156-157.

Hatta, M., Gao, P., Halfmann, P., Kawaoka, Y., 2001. Molecular basis for high virulence

of Hong Kong H5N1 influenza A viruses. Science 293, 1840-1842.

Hayashi, T., Chaimayo, C., McGuinness, J., Takimoto, T., 2016. Critical Role of the PA-X C-Terminal Domain of Influenza A Virus in Its Subcellular Localization and Shutoff Activity. J Virol 90, 7131-7141.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529.

Herfst, S., Schrauwen, E.J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V.J., Sorrell, E.M., Bestebroer, T.M., Burke, D.F., Smith, D.J., Rimmelzwaan, G.F., Osterhaus, A.D., Fouchier, R.A., 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336, 1534-1541.

Herve, P.L., Lorin, V., Jouvion, G., Da Costa, B., Escriou, N., 2015. Addition of N-glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity. Virology 486, 134-145.

Hikono, H., Kohlmeier, J.E., Ely, K.H., Scott, I., Roberts, A.D., Blackman, M.A., Woodland, D.L., 2006. T-cell memory and recall responses to respiratory virus infections. Immunological reviews 211, 119-132.

Hoffmann, E., Krauss, S., Perez, D., Webby, R., Webster, R.G., 2002a. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 20, 3165-3170.

Hoffmann, E., Krauss, S., Perez, D., Webby, R., Webster, R.G.J.V., 2002b. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 20, 3165-3170.

Hoffmann, E., Neumann, G., Hobom, G., Webster, R.G., Kawaoka, Y., 2000a. "Ambisense" approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. Virology 267, 310-317.

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000b. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97, 6108-6113.

Hoffmann, E., Stech, J., Guan, Y., Webster, R., Perez, D., 2001a. Universal primer set for the full-length amplification of all influenza A viruses. Archives of virology 146, 2275-2289.

Hoffmann, E., Stech, J., Guan, Y., Webster, R., Perez, D.J.A.o.v., 2001b. Universal primer set for the full-length amplification of all influenza A viruses. Archives of virology 146, 2275-2289.

Holzinger, D., Jorns, C., Stertz, S., Boisson-Dupuis, S., Thimme, R., Weidmann, M., Casanova, J.-L., Haller, O., Kochs, G., 2007. Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling. Journal of virology 81, 7776-7785.

Hrincius, E.R., Liedmann, S., Finkelstein, D., Vogel, P., Gansebom, S., Samarasinghe, A.E., You, D., Cormier, S.A., McCullers, J.A., 2015. Acute Lung Injury Results from Innate Sensing of Viruses by an ER Stress Pathway. Cell Rep 11, 1591-1603.

Huet, S., Avilov, S.V., Ferbitz, L., Daigle, N., Cusack, S., Ellenberg, J., 2010. Nuclear import and assembly of influenza A virus RNA polymerase studied in live cells by fluorescence cross-correlation spectroscopy. Journal of virology 84, 1254-1264.

Imai, M., Watanabe, T., Hatta, M., Das, S.C., Ozawa, M., Shinya, K., Zhong, G., Hanson, A., Katsura, H., Watanabe, S., Li, C., Kawakami, E., Yamada, S., Kiso, M., Suzuki, Y., Maher, E.A., Neumann, G., Kawaoka, Y., 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486, 420-428.

Ito, T., Suzuki, Y., Mitnaul, L., Vines, A., Kida, H., Kawaoka, Y.J.V., 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology 227, 493-499.

Jagger, B., Wise, H., Kash, J., Walters, K.-A., Wills, N., Xiao, Y.-L., Dunfee, R., Schwartzman, L., Ozinsky, A., Bell, G., 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337, 199-204.

Jang, J.-W., Lee, C.-Y., Kim, I.-h., Choi, J.-G., Lee, Y.-J., Yuk, S.-S., Lee, J.-H., Song, C.-S., Kim, J.-H., Kwon, H.-J., 2017a. Optimized clade 2.3. 2.1 c H5N1 recombinant-vaccine strains against highly pathogenic avian influenza. Journal of veterinary science 18, 299-306.

Jang, J.W., Lee, C.Y., Kim, I.H., Choi, J.G., Lee, Y.J., Yuk, S.S., Lee, J.H., Song, C.S., Kim, J.H., Kwon, H.J., 2017b. Optimized clade 2.3.2.1c H5N1 recombinant-vaccine strains against highly pathogenic avian influenza. J Vet Sci 18, 299-306.

Jansen, T., Hofmans, M.P., Theelen, M.J., Schijns, V.E., 2005. Structure–activity relations of water-in-oil vaccine formulations and induced antigen-specific antibody responses. Vaccine 23, 1053-1060.

Jayasekera, J.P., Vinuesa, C.G., Karupiah, G., King, N.J., 2006. Enhanced antiviral antibody secretion and attenuated immunopathology during influenza virus infection in nitric oxide synthase-2-deficient mice. Journal of general virology 87, 3361-3371.

Jiang, H., Zhang, S., Wang, Q., Wang, J., Geng, L., Toyoda, T., 2010. Influenza virus genome C4 promoter/origin attenuates its transcription and replication activity by the low polymerase recognition activity. Virology 408, 190-196.

Jin, M., Jang, Y., Seo, T., Seo, S.H., 2018. Inactivated H5 Antigens of H5N8 Protect Chickens from Lethal Infections by the Highly Pathogenic H5N8 and H5N6 Avian Influenza Viruses. J Vet Res 62, 413-420.

Johansson, B., Bucher, D., Kilbourne, E., 1989. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. Journal of virology 63, 1239-1246.

Johansson, B.E., Pokorny, B.A., Tiso, V.A., 2002. Supplementation of conventional trivalent influenza vaccine with purified viral N1 and N2 neuraminidases induces a balanced immune response without antigenic competition. Vaccine 20, 1670-1674.

Kandeil, A., Sabir, J.S.M., Abdelaal, A., Mattar, E.H., El-Taweel, A.N., Sabir, M.J., Khalil, A.A., Webby, R., Kayali, G., Ali, M.A., 2018. Efficacy of commercial vaccines against newly emerging avian influenza H5N8 virus in Egypt. Sci Rep 8, 9697.

Kang, H.-M., Batchuluun, D., Kim, M.-C., Choi, J.-G., Erdene-Ochir, T.-O., Paek, M.-R., Sugir, T., Sodnomdarjaa, R., Kwon, J.-H., Lee, Y.-J.J.V.m., 2011. Genetic analyses

of H5N1 avian influenza virus in Mongolia, 2009 and its relationship with those of eastern Asia. 147, 170-175.

Kang, H.M., Lee, E.K., Song, B.M., Heo, G.B., Jung, J., Jang, I., Bae, Y.C., Jung, S.C., Lee, Y.J., 2017. Experimental infection of mandarin duck with highly pathogenic avian influenza A (H5N8 and H5N1) viruses. Vet Microbiol 198, 59-63.

Kapczynski, D.R., Pantin-Jackwood, M.J., Spackman, E., Chrzastek, K., Suarez, D.L., Swayne, D.E., 2017. Homologous and heterologous antigenic matched vaccines containing different H5 hemagglutinins provide variable protection of chickens from the 2014 U.S. H5N8 and H5N2 clade 2.3.4.4 highly pathogenic avian influenza viruses. Vaccine 35, 6345–6353.

Kaverin, N.V., Rudneva, I.A., Govorkova, E.A., Timofeeva, T.A., Shilov, A.A., Kochergin-Nikitsky, K.S., Krylov, P.S., Webster, R.G., 2007. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J Virol 81, 12911-12917.

Kaverin, N.V., Rudneva, I.A., Ilyushina, N.A., Varich, N.L., Lipatov, A.S., Smirnov, Y.A., Govorkova, E.A., Gitelman, A.K., Lvov, D.K., Webster, R.G., 2002. Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. J Gen Virol 83, 2497-2505.

Kida, H., Yanagawa, R., Matsuoka, Y., 1980. Duck influenza lacking evidence of disease signs and immune response. Infection immunity 30, 547–553.

Kim, H.R., Park, C.K., Lee, Y.J., Woo, G.H., Lee, K.K., Oem, J.K., Kim, S.H., Jean, Y.H., Bae, Y.C., Yoon, S.S., Roh, I.S., Jeong, O.M., Kim, H.Y., Choi, J.S., Byun, J.W.,

Song, Y.K., Kwon, J.H., Joo, Y.S., 2010. An outbreak of highly pathogenic H5N1 avian influenza in Korea, 2008. Vet Microbiol 141, 362-366.

Kim, I.-H., Choi, J.-G., Lee, Y.-J., Kwon, H.-J., Kim, J.-H., 2014a. Effects of different polymerases of avian influenza viruses on the growth and pathogenicity of A/Puerto Rico/8/1934 (H1N1)-derived reassorted viruses. Veterinary microbiology 168, 41-49.

Kim, I.H., Choi, J.G., Lee, Y.J., Kwon, H.J., Kim, J.H., 2014b. Effects of different polymerases of avian influenza viruses on the growth and pathogenicity of A/Puerto Rico/8/1934 (H1N1)-derived reassorted viruses. Vet Microbiol 168, 41-49.

Kim, I.H., Kwon, H.J., Choi, J.G., Kang, H.M., Lee, Y.J., Kim, J.H., 2013a. Characterization of mutations associated with the adaptation of a low-pathogenic H5N1 avian influenza virus to chicken embryos. Vet Microbiol 162, 471-478.

Kim, I.H., Kwon, H.J., Lee, S.H., Kim, D.Y., Kim, J.H., 2015a. Effects of different NS genes of avian influenza viruses and amino acid changes on pathogenicity of recombinant A/Puerto Rico/8/34 viruses. Vet Microbiol 175, 17-25.

Kim, I.H., Kwon, H.J., Park, J.K., Song, C.S., Kim, J.H., 2015b. Optimal attenuation of a PR8-derived mouse pathogenic H5N1 recombinant virus for testing antigenicity and protective efficacy in mice. Vaccine 33, 6314-6319.

Kim, J.I., Lee, I., Park, S., Hwang, M.W., Bae, J.Y., Lee, S., Heo, J., Park, M.S., Garcia-Sastre, A., Park, M.S., 2013b. Genetic requirement for hemagglutinin glycosylation and its implications for influenza A H1N1 virus evolution. J Virol

87, 7539-7549.

Kuchipudi, S.V., Tellabati, M., Sebastian, S., Londt, B.Z., Jansen, C., Vervelde, L., Brookes, S.M., Brown, I.H., Dunham, S.P., Chang, K.-C., 2014. Highly pathogenic avian influenza virus infection in chickens but not ducks is associated with elevated host immune and pro-inflammatory responses. Veterinary research 45, 118.

Kwon, H.-J., Cho, S.-H., Ahn, Y.-J., Kim, J.-H., Yoo, H.-S., Kim, S.-J., 2009. Characterization of a chicken embryo-adapted H9N2 subtype avian influenza virus. Open Vet Sci J 3, 9-16.

Kwon, H.J.K., J.H.; Choi, J.G.; Lee, Y.J. 2014. A METHOD OF PREPARING HA2 COMMON EPITOPE OF INFLUENZA VIRUS WITH IMPROVED IMMUNOGENICITY (Republic of Korea, Seoul National University).

Kwon, J.H., Jeong, S., Lee, D.H., Swayne, D.E., Kim, Y.J., Lee, S.H., Noh, J.Y., Erdene-Ochir, T.O., Jeong, J.H., Song, C.S., 2018. New Reassortant Clade 2.3.4.4b Avian Influenza A(H5N6) Virus in Wild Birds, South Korea, 2017-18. Emerg Infect Dis 24, 1953-1955.

Kwon, J.H., Lee, D.H., Swayne, D.E., Noh, J.Y., Yuk, S.S., Erdene-Ochir, T.O., Hong, W.T., Jeong, J.H., Jeong, S., Gwon, G.B., Lee, S., Song, C.S., 2017a. Reassortant Clade 2.3.4.4 Avian Influenza A(H5N6) Virus in a Wild Mandarin Duck, South Korea, 2016. Emerg Infect Dis 23, 822-826.

Kwon, J.H., Noh, Y.K., Lee, D.H., Yuk, S.S., Erdene-Ochir, T.O., Noh, J.Y., Hong, W.T., Jeong, J.H., Jeong, S., Gwon, G.B., Song, C.S., Nahm, S.S., 2017b. Experimental infection with highly pathogenic H5N8 avian influenza viruses in the Mandarin

duck (Aix galericulata) and domestic pigeon (Columba livia domestica). Vet Microbiol 203, 95-102.

Lai, S., Qin, Y., Cowling, B.J., Ren, X., Wardrop, N.A., Gilbert, M., Tsang, T.K., Wu, P., Feng, L., Jiang, H., Peng, Z., Zheng, J., Liao, Q., Li, S., Horby, P.W., Farrar, J.J., Gao, G.F., Tatem, A.J., Yu, H., 2016. Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997–2015: a systematic review of individual case data. The Lancet Infectious Diseases 16, e108-e118.

Laleye, A., Joannis, T., Shittu, I., Meseko, C., Zamperin, G., Milani, A., Zecchin, B., Fusaro, A., Monne, I., Abolnik, C., 2018a. A two-year monitoring period of the genetic properties of clade 2.3.2.1c H5N1 viruses in Nigeria reveals the emergence and co-circulation of distinct genotypes. Infect Genet Evol 57, 98-105.

Laleye, A., Joannis, T., Shittu, I., Meseko, C., Zamperin, G., Milani, A., Zecchin, B., Fusaro, A., Monne, I., Abolnik, C., 2018b. A two-year monitoring period of the genetic properties of clade 2.3. 2.1 c H5N1 viruses in Nigeria reveals the emergence and co-circulation of distinct genotypes. nfection, Genetics Evolution 57, 98-105.

Lanni, F., Beard, J., 1948. Inhibition by egg-white of hemagglutination by swine influenza virus. Proceedings of the Society for Experimental Biology and Medicine 68, 312-313.

Le, T.H., Nguyen, N.T., 2014. Evolutionary dynamics of highly pathogenic avian influenza A/H5N1 HA clades and vaccine implementation in Vietnam. Clin Exp Vaccine Res 3, 117-127.

Lee, C.-W., Suarez, D.L., 2005. Avian influenza virus: prospects for prevention and control by vaccination. Animal health research reviews 6, 1-15.

Lee, C.W., Senne, D.A., Suarez, D.L., 2004. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 78, 8372-8381.

Lee, C.W., Suarez, D.L., Tumpey, T.M., Sung, H.W., Kwon, Y.K., Lee, Y.J., Choi, J.G., Joh, S.J., Kim, M.C., Lee, E.K., Park, J.M., Lu, X., Katz, J.M., Spackman, E., Swayne, D.E., Kim, J.H., 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J Virol 79, 3692-3702.

Lee, C.Y., An, S.H., Choi, J.G., Lee, Y.J., Kim, J.H., Kwon, H.J., 2018a. Acquisition of Innate Inhibitor Resistance and Mammalian Pathogenicity During Egg Adaptation by the H9N2 Avian Influenza Virus. Front Microbiol 9, 1939.

Lee, C.Y., An, S.H., Kim, I., Choi, J.G., Lee, Y.J., Kim, J.H., Kwon, H.J., 2018b. Novel mutations in avian PA in combination with an adaptive mutation in PR8 NP exacerbate the virulence of PR8-derived recombinant influenza A viruses in mice. Vet Microbiol 221, 114-121.

Lee, C.Y., An, S.H., Kim, I., Go, D.M., Kim, D.Y., Choi, J.G., Lee, Y.J., Kim, J.H., Kwon, H.J., 2017a. Prerequisites for the acquisition of mammalian pathogenicity by influenza A virus with a prototypic avian PB2 gene. Sci Rep 7, 10205.

Lee, C.Y., Kwon, H.J., Nguyen, T.T., Kim, I., Jang, H.K., Kim, J.H., 2017b. Effect of the fourth nucleotide at the 3' end of neuraminidase and matrix viral genomic RNA on the pathogenicity of influenza virus A/PR/8/34. J Vet Sci 18, 307-313.

Lee, D.-H., Bahl, J., Torchetti, M.K., Killian, M.L., Ip, H.S., DeLiberto, T.J., Swayne, D.E.,

2016a. Highly pathogenic avian influenza viruses and generation of novel reassortants, United States, 2014–2015. Emerging infectious diseases 22, 1283.

Lee, D.-H., Park, J.-K., Youn, H.-N., Lee, Y.-N., Lim, T.-H., Kim, M.-S., Lee, J.-B., Park, S.-Y., Choi, I.-S., Song, C.-S., 2011. Surveillance and isolation of HPAI H5N1 from wild Mandarin Ducks (Aix galericulata). Journal of wildlife diseases 47, 994-998.

Lee, D.-H., Song, C.-S., 2013. H9N2 avian influenza virus in Korea: evolution and vaccination. Clinical and experimental vaccine research 2, 26-33.

Lee, D.H., Bertran, K., Kwon, J.H., Swayne, D.E., 2017c. Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. J Vet Sci 18, 269-280.

Lee, D.H., Kwon, J.H., Noh, J.Y., Park, J.K., Yuk, S.S., Erdene-Ochir, T.O., Lee, J.B., Park, S.Y., Choi, I.S., Lee, S.W., Song, C.S., 2016b. Pathogenicity of the Korean H5N8 highly pathogenic avian influenza virus in commercial domestic poultry species. Avian Pathol 45, 208-211.

Lee, D.H., Torchetti, M.K., Winker, K., Ip, H.S., Song, C.S., Swayne, D.E., 2015. Intercontinental Spread of Asian-Origin H5N8 to North America through Beringia by Migratory Birds. J Virol 89, 6521-6524.

Lee, E.K., Song, B.M., Lee, Y.N., Heo, G.B., Bae, Y.C., Joh, S.J., Park, S.C., Choi, K.S., Lee, H.J., Jang, I., Kang, M.S., Jeong, O.M., Choi, B.K., Lee, S.M., Jeong, S.C., Park, B.K., Lee, H.S., Lee, Y.J., 2017d. Multiple novel H5N6 highly pathogenic avian influenza viruses, South Korea, 2016. Infect Genet Evol 51, 21-23.

Lee, M., Deng, M., Lin, Y., Chang, C., Shieh, H.K., Shiau, J., Huang, C., 2007a. Characterization of an H5N1 avian influenza virus from Taiwan. J Veterinary microbiology 124, 193-201.

Lee, M.K., Bae, S.H., Park, C.J., Cheong, H.K., Cheong, C., Choi, B.S., 2003. A single-nucleotide natural variation (U4 to C4) in an influenza A virus promoter exhibits a large structural change: implications for differential viral RNA synthesis by RNA-dependent RNA polymerase. Nucleic acids research 31, 1216-1223.

Lee, M.S., Deng, M.C., Lin, Y.J., Chang, C.Y., Shieh, H.K., Shiau, J.Z., Huang, C.C., 2007b. Characterization of an H5N1 avian influenza virus from Taiwan. Vet Microbiol 124, 193-201.

Lee, M.S., Jang, E.Y., Cho, J., Kim, K., Lee, C.H., Yi, H., 2019. Development and comparison of two H5N8 influenza A vaccine candidate strains. Arch Virol 164, 127–136.

Lee, Y.-J., Choi, Y.-K., Kim, Y.-J., Song, M.-S., Jeong, O.-M., Lee, E.-K., Jeon, W.-J., Jeong, W., Joh, S.-J., Choi, K.-s.J.E.i.d., 2008. Highly pathogenic avian influenza virus (H5N1) in domestic poultry and relationship with migratory birds, South Korea. Emerging infectious diseases 14, 487.

Lee, Y.-J., Kang, H.-M., Lee, E.-K., Song, B.-M., Jeong, J., Kwon, Y.-K., Kim, H.-R., Lee, K.-J., Hong, M.-S., Jang, I.J.E.i.d., 2014. Novel reassortant influenza A (H5N8) viruses, South Korea, 2014. 20, 1087.

Lee, Y.-J., Shin, J.-Y., Song, M.-S., Lee, Y.-M., Choi, J.-G., Lee, E.-K., Jeong, O.-M., Sung, H.-W., Jae-Hong, K., Kwon, Y.-K., 2007c. Continuing evolution of H9 influenza

viruses in Korean poultry. Virology 359, 313-323.

Li, C., Bu, Z., Chen, H., 2014. Avian influenza vaccines against H5N1 'bird flu'. Trends Biotechnol 32, 147-156.

Li, K., Guan, Y., Wang, J., Smith, G., Xu, K., Duan, L., Rahardjo, A., Puthavathana, P., Buranathai, C., Nguyen, T., 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430, 209.

Li, Y., Liu, L., Zhang, Y., Duan, Z., Tian, G., Zeng, X., Shi, J., Zhang, L., Chen, H.J.E.i.d., 2011. New avian influenza virus (H5N1) in wild birds, Qinghai, China. 17, 265.

Lin, J., Zhang, J., Dong, X., Fang, H., Chen, J., Su, N., Gao, Q., Zhang, Z., Liu, Y., Wang, Z., 2006. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. The Lancet 368, 991-997.

Linster, M., van Boheemen, S., de Graaf, M., Schrauwen, E.J.A., Lexmond, P., Manz, B., Bestebroer, T.M., Baumann, J., van Riel, D., Rimmelzwaan, G.F., Osterhaus, A., Matrosovich, M., Fouchier, R.A.M., Herfst, S., 2014. Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. Cell 157, 329-339.

Liu, M., Liu, C.-G., Zhang, Y., Shi, W.-L., Wang, W., Liu, Y.-Y., 2012. Efficacy of a high-yield attenuated vaccine strain wholly derived from avian influenza viruses by use of reverse genetics. Veterinary microbiology 161, 43-48.

Lourens, A., van den Brand, H., Meijerhof, R., Kemp, B., 2005. Effect of eggshell temperature during incubation on embryo development, hatchability, and

posthatch development. Poult Sci 84, 914-–910.

Lycett, S.J., Bodewes, R., Pohlmann, A., Banks, J., Bányai, K., Boni, M.F., Bouwstra, R., Breed, A.C., Brown, I.H., Chen, H., 2016. Role for migratory wild birds in the global spread of avian influenza H5N8. Science 354.

Lyons, D.M., Lauring, A.S., 2018. Mutation and epistasis in influenza virus evolution. Viruses 10.

Mair, C.M., Ludwig, K., Herrmann, A., Sieben, C., 2014. Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection. Biochim Biophys Acta 1838, 1153-1168.

Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T.I., Bushkin, Y., Davis, D.M., Strominger, J.L., Yewdell, J.W., Porgador, A., 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 409, 1055.

Masopust, D., Vezys, V., Marzo, A.L., Lefrançois, L., 2001. Preferential localization of effector memory cells in nonlymphoid tissue. Science 291, 2413-2417.

Matrosovich, M.N., Gambaryan, A.S. 2012. Solid-phase assays of receptor-binding specificity, In: Influenza Virus. Springer, 71-94.

McCAULEY, J.W., Mahy, B., 1983. Structure and function of the influenza virus genome. Biochemical Journal 211, 281.

McLean, I.W.C., G.R.Jr.; Taylor, A.R.; Beard, D.; Beard, J.W., 1945. pH of the chorio-allantoic fluid of chick embryos infected with influenza virus B. Proc Soc Exp Biol Med 59, 192–195.

Middleton, D., Bingham, J., Selleck, P., Lowther, S., Gleeson, L., Lehrbach, P., Robinson, S., Rodenberg, J., Kumar, M., Andrew, M., 2007. Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. Virology 359, 66-71.

Miller, G.L., 1944. A Study of conditions for the optimum production of RR8 influenza virus in chick embryos. J Exp Med 79, 173–183.

Munster, V.J., Wallensten, A., Baas, C., Rimmelzwaan, G.F., Schutten, M., Olsen, B., Osterhaus, A.D., Fouchier, R.A., 2005. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. J Emerging infectious diseases 11, 1545.

Naguib, M.M., Kinne, J., Chen, H., Chan, K.H., Joseph, S., Wong, P.C., Woo, P.C., Wernery, R., Beer, M., Wernery, U., Harder, T.C., 2015. Outbreaks of highly pathogenic avian influenza H5N1 clade 2.3.2.1c in hunting falcons and kept wild birds in Dubai implicate intercontinental virus spread. J Gen Virol 96, 3212-3212.

Neirynck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W.M., Fiers, W., 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nature medicine 5, 1157.

Neumann, G., Brownlee, G., Fodor, E., Kawaoka, Y. 2004. Orthomyxovirus replication, transcription, and polyadenylation, In: Biology of Negative Strand Rna Viruses: the Power of Reverse Genetics. Springer, 121-143.

Neumann, G., Kawaoka, Y., 2002. Generation of influenza A virus from cloned cDNAs—historical perspective and outlook for the new millenium. Reviews in medical virology 12, 13-30.

Nguyen, D.T., Jang, Y., Nguyen, T.D., Jones, J., Shepard, S.S., Yang, H., Gerloff, N., Fabrizio, T., Nguyen, L.V., Inui, K., Yang, G., Creanga, A., Wang, L., Mai, D.T., Thor, S., Stevens, J., To, T.L., Wentworth, D.E., Nguyen, T., Pham, D.V., Bryant, J.E., Davis, C.T., 2017. Shifting Clade Distribution, Reassortment, and Emergence of New Subtypes of Highly Pathogenic Avian Influenza A(H5) Viruses Collected from Vietnamese Poultry from 2012 to 2015. J Virol 91.

Nguyen, L.T., Firestone, S.M., Stevenson, M.A., Young, N.D., Sims, L.D., Chu, D.H., Nguyen, T.N., Van Nguyen, L., Thanh Le, T., Van Nguyen, H., Nguyen, H.N., Tien, T.N., Nguyen, T.D., Tran, B.N., Matsuno, K., Okamatsu, M., Kida, H., Sakoda, Y., 2019. A systematic study towards evolutionary and epidemiological dynamics of currently predominant H5 highly pathogenic avian influenza viruses in Vietnam. Sci Rep 9, 7723.

Ohmit, S.E., Petrie, J.G., Cross, R.T., Johnson, E., Monto, A.S., 2011. Influenza hemagglutination-inhibition antibody titer as a correlate of vaccine-induced protection. J Infect Dis 204, 1879–1885.

OIE, 2009. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009.

Pang, I.K., Iwasaki, A., 2011. Inflammasomes as mediators of immunity against influenza virus. Trends in immunology 32, 34-41.

Pantin-Jackwood, M.J., Suarez, D.L., 2013. Vaccination of domestic ducks against H5N1 HPAI: a review. Virus Res 178, 21-34.

Park, M.-S., Steel, J., García-Sastre, A., Swayne, D., Palese, P., 2006. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease.

Proceedings of the National Academy of Sciences 103, 8203-8208.

Parvin, R., Begum, J.A., Chowdhury, E.H., Islam, M.R., Beer, M., Harder, T., 2019. Co-subsistence of avian influenza virus subtypes of low and high pathogenicity in Bangladesh: Challenges for diagnosis, risk assessment and control. Sci Rep 9, 8306.

Paul, W.E., Seder, R.A., 1994. Lymphocyte responses and cytokines. cell 76, 241-251.

Peiris, J., Hui, K.P., Yen, H.-L., 2010. Host response to influenza virus: protection versus immunopathology. Current opinion in immunology 22, 475-481.

Peper, R.L., Van Campen, H., 1995. Tumor necrosis factor as a mediator of inflammation in influenza A viral pneumonia. Microbial pathogenesis 19, 175-183.

Ping, J., Lopes, T.J., Nidom, C.A., Ghedin, E., Macken, C.A., Fitch, A., Imai, M., Maher, E.A., Neumann, G., Kawaoka, Y., 2015. Development of high-yield influenza A virus vaccine viruses. Nat Commun 6, 8148.

Plotch, S.J., Bouloy, M., Ulmanen, I., Krug, R.M., 1981. A unique cap (m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23, 847-858.

Poen, M.J., Venkatesh, D., Bestebroer, T.M., Vuong, O., Scheuer, R.D., Oude Munnink, B.B., de Meulder, D., Richard, M., Kuiken, T., Koopmans, M.P.G., Kelder, L., Kim, Y.J., Lee, Y.J., Steensels, M., Lambrecht, B., Dan, A., Pohlmann, A., Beer, M., Savic, V., Brown, I.H., Fouchier, R.A.M., Lewis, N.S., 2019. Co-circulation of genetically distinct highly pathogenic avian influenza A clade 2.3.4.4 (H5N6) viruses in wild waterfowl and poultry in Europe and East Asia, 2017-18. Virus

Evol 5, vez004.

Potter, C., Oxford, J., 1979. Determinants of immunity to influenza infection in man. British medical bulletin 35, 69–75.

Prel, A., Gall-Reculé, G.L., Cherbonnel, M., Grasland, B., Amelot, M., Jestin, V., 2007. Assessment of the protection afforded by triple baculovirus recombinant coexpressing H5, N3, M1 proteins against a homologous H5N3 low-pathogenicity avian influenza virus challenge in Muscovy ducks. Avian diseases 51, 484-489.

Rachakonda, P.S., Veit, M., Korte, T., Ludwig, K., Bottcher, C., Huang, Q., Schmidt, M.F., Herrmann, A., 2007. The relevance of salt bridges for the stability of the influenza virus hemagglutinin. FASEB J 21, 995-1002.

RahimiRad, S., Alizadeh, A., Alizadeh, E., Hosseini, S.M., 2016. The avian influenza H9N2 at avian-human interface: A possible risk for the future pandemics. Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences 21.

Richard, M., Herfst, S., van den Brand, J.M.A., de Meulder, D., Lexmond, P., Bestebroer, T.M., Fouchier, R.A.M., 2017. Mutations driving airborne transmission of A/H5N1 virus in mammals cause substantial attenuation in chickens only when combined. Sci Rep 7, 7187.

Robinson, H., Hunt, L., Webster, R., 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine 11, 957-960.

Romagnani, S., 1994. Lymphokine production by human T cells in disease states. Annual

review of immunology 12, 227-257.

Román, E., Miller, E., Harmsen, A., Wiley, J., Von Andrian, U.H., Huston, G., Swain, S.L., 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. Journal of Experimental Medicine 196, 957-968.

Rossman, J.S., Lamb, R.A., 2011. Influenza virus assembly and budding. Virology 411, 229-236.

Sambhara, S., Kurichh, A., Miranda, R., Tumpey, T., Rowe, T., Renshaw, M., Arpino, R., Tamane, A., Kandil, A., James, O., 2001. Heterosubtypic Immunity against Human Influenza A Viruses, Including Recently Emerged Avian H5 and H9 Viruses, Induced by FLU–ISCOM Vaccine in Mice Requires both Cytotoxic T-Lymphocyte and Macrophage Function. Cellular immunology 211, 143-153.

Santos, J.J.S., Obadan, A.O., Garcia, S.C., Carnaccini, S., Kapczynski, D.R., Pantin-Jackwood, M., Suarez, D.L., Perez, D.R., 2017. Short- and long-term protective efficacy against clade 2.3.4.4 H5N2 highly pathogenic avian influenza virus following prime-boost vaccination in turkeys. Vaccine 35, 5637–5643.

Schild, G., Newman, R., Webster, R., Major, D., Hinshaw, V.S., 1980. Antigenic analysis of influenza A virus surface antigens: considerations for the nomenclature of influenza virus. J Comparative immunology, microbiology infectious diseases 3, 5-18.

Schnell, J.R., Chou, J.J., 2008. Structure and mechanism of the M2 proton channel of influenza A virus. Nature 451, 591-595.

Scholtissek, C., Müller, K., Herzog, S., Frese, K., 1988. Multiplication of influenza A

viruses with cleavable and non-cleavable haemagglutinin in chicken embryo membranes or organs, and cell cultures derived therefrom. Journal of general virology 69, 2155-2164.

Seitz, C., Frensing, T., Höper, D., Kochs, G., Reichl, U., 2010. High yields of influenza A virus in Madin–Darby canine kidney cells are promoted by an insufficient interferon-induced antiviral state. Journal of General Virology 91, 1754-1763.

Shapiro, G., Gurney, T., Krug, R., 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. Journal of virology 61, 764-773.

Shi, H., Liu, X.F., Zhang, X., Chen, S., Sun, L., Lu, J., 2007. Generation of an attenuated H5N1 avian influenza virus vaccine with all eight genes from avian viruses. Vaccine 25, 7379-7384.

Shi, Y., Wu, Y., Zhang, W., Qi, J., Gao, G.F., 2014. Enabling the 'host jump': structural determinants of receptor-binding specificity in influenza A viruses. Nat Rev Microbiol 12, 822-831.

Shu, L., Bean, W., Webster, R., 1993. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. Journal of virology 67, 2723-2729.

Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual review of biochemistry 69, 531-569.

Smith, G., Fan, X., Wang, J., Li, K., Qin, K., Zhang, J., Vijaykrishna, D., Cheung, C., Huang, K., Rayner, J., 2006a. Emergence and predominance of an H5N1 influenza

variant in China. J Proceedings of the National Academy of Sciences 103, 16936-16941.

Smith, G., Naipospos, T., Nguyen, T., De Jong, M., Vijaykrishna, D., Usman, T., Hassan, S., Nguyen, T., Dao, T., Bui, N.J.V., 2006b. Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology 350, 258-268.

Smith, G.J., Donis, R.O., World Health Organization/World Organisation for Animal, H.F., Agriculture Organization, H.E.W.G., 2015a. Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. Influenza Other Respir Viruses 9, 271-276.

Smith, G.J., Vijaykrishna, D., Ellis, T.M., Dyrting, K.C., Leung, Y.C., Bahl, J., Wong, C.W., Kai, H., Chow, M.K., Duan, L.J.E.i.d., 2009a. Characterization of avian influenza viruses A (H5N1) from wild birds, Hong Kong, 2004–2008. 15, 402.

Smith, G.J., Vijaykrishna, D., Ellis, T.M., Dyrting, K.C., Leung, Y.H., Bahl, J., Wong, C.W., Kai, H., Chow, M.K., Duan, L., Chan, A.S., Zhang, L.J., Chen, H., Luk, G.S., Peiris, J.S., Guan, Y., 2009b. Characterization of avian influenza viruses A (H5N1) from wild birds, Hong Kong, 2004-2008. Emerg Infect Dis 15, 402-407.

Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., Danner, A.F., Seiler, J.P., Digard, P., Webster, R.G., Burt, D.W., 2015b. A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. BMC Genomics 16, 574.

Song, B.M., Lee, E.K., Lee, Y.N., Heo, G.B., Lee, H.S., Lee, Y.J., 2017. Phylogeographical characterization of H5N8 viruses isolated from poultry and wild birds during 2014-2016 in South Korea. J Vet Sci 18, 89-94.

Sonnberg, S., Webby, R.J., Webster, R.G., 2013. Natural history of highly pathogenic avian influenza H5N1. Virus Res 178, 63-77.

Staeheli, P., Grob, R., Meier, E., Sutcliffe, J.G., Haller, O., 1988. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol Cell Biol 8, 4518-4523.

Stephenson, I., Nicholson, K.G., Wood, J.M., Zambon, M.C., Katz, J.M., 2004. Confronting the avian influenza threat: vaccine development for a potential pandemic. The Lancet Infectious Diseases 4, 499-509.

Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.D., Garten, W., 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J 11, 2407–2414.

Suarez, D.L., Schultz-Cherry, S., 2000. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian diseases, 861-868.

Subbarao, E.K., London, W., Murphy, B.R., 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67, 1761–1764.

Subbarao, K., Joseph, T., 2007. Scientific barriers to developing vaccines against avian influenza viruses. Nature Reviews Immunology 7, 267.

Sun, Y., Pu, J., Fan, L., Sun, H., Wang, J., Zhang, Y., Liu, L., Liu, J., 2012. Evaluation of

the protective efficacy of a commercial vaccine against different antigenic groups of H9N2 influenza viruses in chickens. Veterinary microbiology 156, 193-199.

Swain, S.L., McKinstry, K.K., Strutt, T.M., 2012. Expanding roles for CD4+ T cells in immunity to viruses. Nature Reviews Immunology 12, 136.

Swayne, D.E., Beck, J.R., Mickle, T.R., 1997. Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. Avian diseases, 910-922.

Swayne, D.E., Kapczynski, D., 2008. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunological reviews 225, 314-331.

Swayne, D.E., Perdue, M.L., Beck, J.R., Garcia, M., Suarez, D.L., 2000. Vaccines protect chickens against H5 highly pathogenic avian influenza in the face of genetic changes in field viruses over multiple years. Veterinary microbiology 74, 165-172.

Taft, A.S., Ozawa, M., Fitch, A., Depasse, J.V., Halfmann, P.J., Hill-Batorski, L., Hatta, M., Friedrich, T.C., Lopes, T.J., Maher, E.A., Ghedin, E., Macken, C.A., Neumann, G., Kawaoka, Y., 2015. Identification of mammalian-adapting mutations in the polymerase complex of an avian H5N1 influenza virus. Nat Commun 6, 7491.

Takahashi, Y., Hasegawa, H., Hara, Y., Ato, M., Ninomiya, A., Takagi, H., Odagiri, T., Sata, T., Tashiro, M., Kobayashi, K., 2009. Protective immunity afforded by inactivated H5N1 (NIBRG-14) vaccine requires antibodies against both hemagglutinin and neuraminidase in mice. J Infect Dis 199, 1629–1637.

Taubenberger, J.K., Reid, A.H., Lourens, R.M., Wang, R., Jin, G., Fanning, T.G., 2005. Characterization of the 1918 influenza virus polymerase genes. Nature 437, 889-

893.

Te Velthuis, A.J., Fodor, E., 2016. Influenza virus RNA polymerase: insights into the mechanisms of viral RNA synthesis. Nat Rev Microbiol 14, 479-493.

Team, F.G. 2014. H5N1 highly pathogenic avian influenza monthly overview—January–March 2012.

Teijaro, J.R., Verhoeven, D., Page, C.A., Turner, D., Farber, D.L., 2010. Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. Journal of virology 84, 9217-9226.

Topham, D., Tripp, R., Doherty, P., 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. The Journal of Immunology 159, 5197-5200.

Townsend, A., McMichael, A., Carter, N., Huddleston, J., Brownlee, G., 1984. Cytotoxic T cell recognition of the influenza nucleoprotein and hemagglutinin expressed in transfected mouse L cells. Cell 39, 13-25.

Tumpey, T.M., García-Sastre, A., Taubenberger, J.K., Palese, P., Swayne, D.E., Pantin-Jackwood, M.J., Schultz-Cherry, S., Solórzano, A., Van Rooijen, N., Katz, J.M., 2005. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. Journal of virology 79, 14933-14944.

van den Brand, J.M.A., Verhagen, J.H., Veldhuis Kroeze, E.J.B., van de Bildt, M.W.G., Bodewes, R., Herfst, S., Richard, M., Lexmond, P., Bestebroer, T.M., Fouchier, R.A.M., Kuiken, T., 2018. Wild ducks excrete highly pathogenic avian influenza virus H5N8 (2014-2015) without clinical or pathological evidence of disease.

Emerg Microbes Infect 7, 67.

Velkov, T., Ong, C., Baker, M.A., Kim, H., Li, J., Nation, R.L., Huang, J.X., Cooper, M.A., Rockman, S., 2013. The antigenic architecture of the hemagglutinin of influenza H5N1 viruses. Mol Immunol 56, 705-719.

Wang, W., Lu, B., Zhou, H., Suguitan, A.L., Jr., Cheng, X., Subbarao, K., Kemble, G., Jin, H., 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. J Virol 84, 6570-6577.

Watts, C., 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. Annual review of immunology 15, 821-850.

Webby, R., Perez, D., Coleman, J., Guan, Y., Knight, J., Govorkova, E., McClain-Moss, L., Peiris, J., Rehg, J., Tuomanen, E., 2004a. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. The Lancet 363, 1099-1103.

Webby, R.J., Perez, D.R., Coleman, J.S., Guan, Y., Knight, J.H., Govorkova, E.A., McClain-Moss, L.R., Peiris, J.S., Rehg, J.E., Tuomanen, E.I., Webster, R.G., 2004b. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. The Lancet 363, 1099-1103.

Webster, R., Air, G., Metzger, D., Colman, P., Varghese, J., Baker, A., Laver, W., 1987. Antigenic structure and variation in an influenza virus N9 neuraminidase. Journal of virology 61, 2910-2916.

Webster, R., Reay, P., Laver, W., 1988. Protection against lethal influenza with neuraminidase. Virology 164, 230-237.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. J Microbiology molecular biology reviews 56, 152-179.

Webster, R.G., Webby, R.J., Hoffmann, E., Rodenberg, J., Kumar, M., Chu, H.J., Seiler, P., Krauss, S., Songserm, T., 2006. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology 351, 303-311.

Weis, W., Brown, J., Cusack, S., Paulson, J., Skehel, J., Wiley, D.J.N., 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature 333, 426.

Wen, F., Li, L., Zhao, N., Chiang, M.J., Xie, H., Cooley, J., Webby, R., Wang, P.G., Wan, X.F., 2018. A Y161F Hemagglutinin Substitution Increases Thermostability and Improves Yields of 2009 H1N1 Influenza A Virus in Cells. J Virol 92.

WHO, F.O. 2011. FAO-OIE-WHO Technical Update: Current evolution of avian influenza H5N1 viruses, 1-6.

Wiley, D.C., Skehel, J.J., 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annual review of biochemistry 56, 365-394.

Wiley, D.C., Wilson, I.A., Skehel, J.J., 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 289, 373-378.

Wilson, I., Skehel, J., Wiley, D.J.N., 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature 289, 366.

Wood, J.M., Robertson, J.S., 2004. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nature Reviews Microbiology 2, 842.

World Health Organization/World Organisation for Animal, H.F., Agriculture Organization, H.N.E.W.G., 2014. Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses. Influenza Other Respir Viruses 8, 384-388.

Wu, Y., Wu, Y., Tefsen, B., Shi, Y., Gao, G.F., 2014. Bat-derived influenza-like viruses H17N10 and H18N11. Trends in microbiology 22, 183-191.

Xiong, X., Coombs, P.J., Martin, S.R., Liu, J., Xiao, H., McCauley, J.W., Locher, K., Walker, P.A., Collins, P.J., Kawaoka, Y., Skehel, J.J., Gamblin, S.J., 2013. Receptor binding by a ferret-transmissible H5 avian influenza virus. Nature 497, 392-396.

Yang, Z.F., Mok, C.K., Peiris, J.S., Zhong, N.S., 2015. Human infection with a novel avian influenza A(H5N6) virus. N Engl J Med 373, 487–489.

Zhang, W., Shi, Y., Lu, X., Shu, Y., Qi, J., Gao, G.F., 2013a. An airborne transmissible avian influenza H5 hemagglutinin seen at the atomic level. Science 340, 1463–1467.

Zhang, W., Shi, Y., Lu, X., Shu, Y., Qi, J., Gao, G.F.J.S., 2013b. An airborne transmissible avian influenza H5 hemagglutinin seen at the atomic level. 340, 1463-1467.

국 문 초 록

고생산성, 포유류 무병원성 재조합 조류인플루엔자 백신주 개발

안 세 희

(지도교수: 김재홍, D.V.M, Ph.D.)

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

(수의미생물학)

조류 인플루엔자 바이러스는 물새류에서 강한 전염성을 지닌 병원체이지만, 변이의 축적을 통해 종간 장벽을 넘어 포유류에 감염될 수 있다. 저병원성조류인플루엔자 바이러스 (Low pathogenic avian influenza viruses, LPAIVs)는 호흡기 증상과 산란율 저하 등 약한 임상 증상을 유발하지만 고병원성조류인플루엔자 바이러스 (highly pathogenic avian influenza viruses, HPAIVs)는 닭과

칠면조에서 심한 임상 증상을 일으키며, H5 아형의 HPAIV는 가금과 야생조류에서 대유행을 초래할 수 있다. 국내에서는 1999년 H9N2 LPAI의 재발생 이후 지속적으로 피해를 일으켰으며, 불활화 백신이 개발되어 농장에 성공적으로 적용되었다. 2003년 이후로는 7번의 H5 HPAIV가 발생하였으며, 살처분 정책이 시행되고 있으나 최근에는 긴급 백신 생산을 위한 백신주 배양액을 비축하고 있다.

불활화 조류인플루엔자 백신을 위한 백신주는 발육란에서 야외주를 적응시키거나 역유전학기술을 이용하여 재조합 바이러스로 작출되고 있다. Hoffmann의 역유전학 벡터시스템은 야외주의 hemagglutinin (HA)와 neuraminidase (NA)와 A/Puerto Rico/8/1934 (PR8) 바이러스의 내부 유전자들(PB2, PB1/PB1-F2, PA/PA-X, NP, M1/M2, and N2/NEP genes)을 이용한 2 + 6 역유전학 체계를 가지고 있으며, 사람과 동물의 백신주 제작에 널리 사용되고 있다. PR8의 내부 유전자들이 생산성 좋은 재조합 바이러스 제작에 필요하지만 PB2, PA, NS1 유전자에는 포유류 병원성과 관련된 중요한 돌연변이들이 존재하고 있다. 또한, 일부 PR8 기반 재조합 백신주들은 발육란에서 불활화 백신주로 사용할 정도의 증식성을 보이지 않는다. 더 효과적인 백신주를 만들기 위해서는 야외주의 내부 유전자에 존재하는 T cell과 B cell epitope들을 맞춰주는 것이 좋지만, 야외주의 8개 게놈을 모두 갖는 재조합 바이러스의 작출은 늘 성공적인 것은 아니다.

상업용 백신주인 A/chicken/Korea/310_E20//2001(H9N2) (01310)은 발육란

에서 20번의 계대를 통해 확립되었으며, 높은 증식성과 동시에 높은 계태아 병원성을 획득했다. 높은 병원성은 계태아의 조기 폐사를 유발하며 요막액의 수득량을 감소시켜 바이러스의 생산성을 떨어뜨린다. 01310 PB2 유전자는 포유류 병원성이 없는 원시유전자(prototypic gene)로 293T 세포주에서의 01310의 8개 게놈을 갖는 재조합 바이러스의 작출은 불가능했다. Hoffmann의 벡터시스템은 바이러스 게놈 RNA의 3'-말단에 존재하는 프로모터 조성이 polymerase (PB1, PB2, PA) 게놈은 약한 프로모터 (4번째 염기가 cytidine, C4)이고, 그 외의 게놈은 강한 프로모터 (4번째 염기가 Uridine, U4) 이다. 01310의 작출이 어려운 문제를 해결하기 위해서 polymerase 유전자의 promoter를 C4에서 U4로 변경하여 293T 세포주에서의 약한 PB2의 활성을 보완한 결과 성공적으로 바이러스를 작출하였다. 또한, 01310의 NS1/NEP 유전자를 계태아에 병원성이 없는 A/chicken/Korea/KBNP-0028/2000 (H9N2) (0028) 바이러스에서 유래한 포유류 무병원성 NS1/NEP 유전자로 교체한 재조합 01310 바이러스 (r310-NS28)를 작출하였다. r310-NS28은 증가한 계태아 평균폐사시간을 보여 계태아 병원성이 감소했고, 요막액 수득량 증가로 인한 총 항원량 증가로 생산성이 향상되었다. 포유류 병원성과 관련된 G139D/N, S151T, GSEV에서 EPEV로의 돌연변이들은 0028의 NS1/NEP 유전자에 이식한 경우 계태아 병원성이 증가하였다.

Clade 2.3.4.4 H5Nx HPAIVs는 전세계적으로 확산되었으며, clade 2.3.4.4a H5N8 바이러스는 국내 비상용 백신주로 선발되었다. 그러나 기존방식의 PR8 기반 재조합 H5N8 백신주는 발육란에서 낮은 증식성을 보였고, 마우스의 폐

에서 증식할 수 있어 잠재적인 병원성을 보유하고 있다. 이러한 문제들을 해결하기 위해 우선 선행연구를 통해 발육란 고증식성과 관련된 돌연변이(HA 유전자의 H103Y, K161E, L317P 변이와 NA 유전자에서 S369N 변이)를 갖는 재조합 H5N8 바이러스들을 작출하여 발육란에서의 증식성을 비교하였다. 그 결과 H103Y 변이만이 유의적으로 바이러스 역가를 증가시켰으나 포유류 병원성 또한 증가시켰다. H103Y는 HA의 내산성과 내열성을 증가시키며 조류인플루엔자 바이러스의 포유류 호흡기 전염성을 증가시키는 것으로 알려져 있다. 그러나 PR8의 PB2 유전자를 01310의 유전자로 교체한 결과 증가된 포유류 병원성이 성공적으로 제거되었다. 이렇게 최적화된 재조합 H5N8 바이러스로 사독오일백신을 제조하여 닭에 접종한 결과 이종인 clade 2.3.4.4 H5N6 HPAIV 공격 접종 시 폐사를 완벽하게 방어하였고, 오리에서 주령에 비례하는 체액성 면역을 유도하였다.

HPAIVs에 대한 지속적인 백신 접종은 항원성 변이주 출현을 촉발한다는 사실이 보고되어 있으므로 미래의 HPAIV 백신주 개발과 백신 프로그램을 위한 전략을 탐색하고자 H5N1 HPAIV의 진화 과정을 분석하였다. Clade 2.3.2.1c H5N1 HPAIVs는 백신으로 형성된 면역 하에서 clade 2.3.2로부터 진화해 왔으므로 바이러스 분리 시기에 따른 HA 단백질 아미노산 서열(n=647)의 변화를 분석하였다. 그 결과 Clade 2.3.2.1c H5N1 HPAIVs의 HA 단백질은 진화 과정에서 수용체 결합 부위 근처에서 S144N과 V223I 돌연변이를 순서대로 획득하였다. S144N 돌연변이로 144N-linked glycosylation site (NGS)가 새로 생성되는

데 데이터베이스의 H5 아형의 HA 단백질 서열을 분석한 결과 158NGS 보다 144NGS의 빈도가 유의적으로 높으므로 자연계에서는 144NGS가 덜 선호되는 것으로 추정하였다. 144N과 158N은 서로 다른 에피톱에 위치하며 이들에 당쇄 결합이 일어나는 경우 인근 에피톱을 가려 체액성 면역을 회피하게 된다. 따라서 144NGS의 획득은 백신 면역 회피 과정에서 나타난 인공적인 결과로 추정된다. 단일 역변이 (N144S 또는 I223V)와 조합 역변이 (N144S와 I223V)를 적용한 재조합 clade 2.3.2.1c H5N1 바이러스를 제작하여 특성을 분석하였다. 그 결과 단일 역변이를 갖는 재조합 바이러스들은 낮은 바이러스 역가와 조류 수용체 결합력을 보였으나, 조합 역변이는 높은 발육란과 포유류세포주에서의 증식성과 마우스 병원성을 보였다. V223I 돌연변이는 HA 단백질의 내열성을 매우 감소시켰는데 223번 아미노산의 위치상 heterotrimer간 상호작용을 불안정화 시키기 때문인 것으로 추정되었다. 따라서 clade 2.3.2.1c H5N1 HPAIVs는 조류 면역을 회피하고자 진화하였으나 그 대가로 바이러스의 fitness와 포유류 병원성을 잃은 것으로 판단된다.

본 연구에서는 재조합 백신주의 효능과 병원성에 영향을 주는 Hoffmann 벡터시스템의 내재적 결함과 한계를 바이러스 게놈 3’-말단의 promoter 변경과 조류인플루엔자 바이러스 유래의 저병원성 PB2, NS1/NEP 유전자로 PR8의 해당 유전자를 대체함으로써 개선하였다. 닭과 포유류에서 공통적으로 관찰되는 NS1과 H103Y의 기능은 닭이 조류인플루엔자 바이러스의 포유류 병원성 진화에 중요하다는 것을 의미한다. 본 연구에서 개발한 발육란

고생산성, 포유류 무병원성 H9N2 및 clade 2.3.4.4 H5N8 백신주는 효과적인 백신 생산에 유용하며 clade 2.3.2.1c 바이러스 연구로부터 얻은 실험 및 생물정보학 데이터는 향후 보다 개선된 백신주 개발에 활용 할 수 있다.

주요어: 역유전학, 조류 인플루엔자 A 바이러스, 불활화 재조합 백신, 고생산성, 포유류 무병원성

학 번: 2015-21820