



HOKKAIDO UNIVERSITY

Title	Studies on the interspecies transmission of influenza virus and vaccine preparation for the emergence of H5N1 highly pathogenic avian influenza virus infection
Author(s)	七戸, 新太郎
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第11279号
Issue Date	2014-03-25
DOI	https://doi.org/10.14943/doctoral.k11279
Doc URL	https://hdl.handle.net/2115/56165
Type	doctoral thesis
File Information	Shintaro_Shichinohe.pdf



**Studies on the interspecies transmission of influenza virus
and vaccine preparation for the emergence of
H5N1 highly pathogenic avian influenza virus infection**

(インフルエンザウイルスの宿主間伝播とH 5 N 1 高病原性
鳥インフルエンザウイルスワクチンの試製に関する研究)

Shintaro Shichinohe

Contents

Preface	1
----------------------	----------

Chapter I

Selection of H3 avian influenza viruses with α 2,6Gal receptor specificity in pigs

Introduction.....	5
Materials and Methods.....	7
Results.....	11
Discussion	18
Brief Summary	20

Chapter II

Potency of an inactivated influenza vaccine prepared from a non-pathogenic H5N1 virus against a challenge with antigenically drifted highly pathogenic avian influenza viruses in chickens

Introduction.....	21
Materials and Methods.....	24
Results.....	30
Discussion	39
Brief Summary	42
Conclusion	43
Acknowledgements	45
References.....	47
和文要旨	56

Preface

Influenza A virus belongs to genus *Influenzavirus A* of family *Orthomyxoviridae* [20]. Influenza A virus is widely distributed in birds and mammals including humans. On the basis of antigenic specificity of hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are divided into sixteen HA and nine NA subtypes (H1-H16 and N1-N9, respectively). Influenza A viruses of all of HA and NA subtypes have been isolated from water birds such as migratory ducks [10]. Ducks are infected with the viruses through water-borne in the lakes and marshes where they nest in summer in the northern territories such as Siberia, Canada, and Alaska [13]. The viruses replicate in the columnar epithelial cells forming crypts in the colon of ducks that do not show clinical signs and are shed in the feces [18]. In autumn, the ducks leave their nesting lakes to the south for migration in Asia. It is known that the pathogenicity of influenza A viruses for chickens ranges from asymptomatic to symptomatic infections with low to high mortality. Highly pathogenic avian influenza (HPAI) viruses are selected when avirulent viruses of wild ducks transmit to chickens through domestic water birds and terrestrial birds, and are passaged among chicken population [13]. HA subtypes of HPAI viruses are restricted to H5 and H7, although not all viruses of these subtypes cause HPAI.

There are four instances of the emergence of new pandemic strains in humans in the last one hundred years. A new pandemic strain is defined as a virus possessing new HA and/or NA subtypes that humans have not experienced for the last several decades [13]. It is revealed that the A/Hong Kong/68 (H3N2) pandemic influenza virus emerged in 1968 via genetic reassortment in pigs concurrently infected with an H3Nx influenza virus circulating in migratory ducks through domestic ducks and the H2N2 virus that was prevailing among humans at the time. The A/Hong Kong/68 (H3N2) pandemic strain bore the PB1 and the HA genes from the H3Nx virus of duck origin and the others from a human H2N2 Asian flu virus strain. Avian influenza viruses possess HA which preferentially binds to sialic acid α 2,3-galactose sialyloligosaccharides (SA α 2,3Gal), whereas swine and human influenza viruses bind to sialic acid α 2,6-galactose sialyloligosaccharides (SA α 2,6Gal) [9, 28]. Pigs play an important role in the generation of pandemic influenza viruses since they have both SA α 2,3Gal and SA α 2,6Gal receptors and are susceptible to infection with both avian and human influenza viruses [9, 14]. Receptor specificity of influenza A viruses is the key determinant of host range. It is assumed that binding specificity to SA α 2,6Gal receptor is required for efficient human-to-human transmission. However, the mechanisms by which avian influenza viruses acquire binding specificity to SA α 2,6Gal receptor and cause pandemic influenza are not fully understood. In this study, to

assess whether viruses recognizing the SA α 2,6Gal receptor are selected, H3 avian influenza virus was consecutively passaged in pigs.

Avian influenza caused by H5N1 HPAI viruses has spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China [32, 42]. The WHO/OIE/FAO H5N1 Evolution Working Group has previously identified 20 new clades of viruses and established specific criteria for naming H5N1 clades [41]. In Japan, H5N1 HPAI viruses belonging to clade 2.3.2.1 were isolated from dead whooper swans in 2008 [24, 37], fecal samples of ducks that flew from Siberia in October 2010 [12], and from wild birds and domestic poultry in 2011 [29]. Recently, antigenic variants of H5N1 HPAI viruses have appeared in poultry in Asian countries and Egypt under immunological selection pressure [1, 4]. It is previously demonstrated that an inactivated avian influenza vaccine prepared from non-pathogenic avian influenza virus, A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/Vac-1/04), conferred protective immunity against the challenge with H5N1 HPAI viruses isolated until 2008. In this study, a vaccine from A/duck/Hokkaido/Vac-3/07 (H5N1) (Dk/Vac-3/07), which is antigenically closely related with Dk/Vac-1/04, was prepared. Growth potential of Dk/Vac-3/07 in embryonated chicken eggs was higher than that of Dk/Vac-1/04 [35].

The potency of the Vac-3 vaccine was assessed by the challenge with antigenically drifted HPAI viruses prevailing recently in Asia.

Chapter I

Selection of H3 avian influenza viruses with SA α 2,6Gal receptor specificity in pigs

Introduction

Influenza A virus is widely distributed in birds and mammals including humans and is classified into 16 HA and 9 NA subtypes [25]. Epidemiological studies have revealed that a vast influenza virus gene pool for influenza exists in migratory ducks [13, 40]. Each of the known subtypes of influenza A viruses perpetuates among migratory water birds and their nesting lake water in nature [10]. Avian influenza viruses possess HA which preferentially binds to SA α 2,3Gal, whereas human influenza viruses bind to SA α 2,6Gal [9, 28]. Each of the earliest isolates in the 1918, 1957, and 1968 pandemics preferentially recognize the SA α 2,6Gal receptor, despite the fact that those HA genes were avian virus origin [15, 27, 40]. Influenza viruses that preferentially bind to SA α 2,3Gal were isolated from pigs, although most swine influenza viruses preferentially recognize SA α 2,6Gal [16]. Experimental infection studies revealed that pigs are susceptible to avian and human influenza viruses of H1–H13 subtypes and

genetic reassortants are generated by concurrent viral infection of cells of upper respiratory tract in pigs [14]. Epithelial cells of the upper respiratory tract of pigs have both SA α 2,3Gal and SA α 2,6Gal receptors [9]. It has been, thus, proposed that pigs serve as intermediate hosts for the generation of genetic reassortants between avian and human influenza viruses. Receptor specificity of H3 influenza viruses is determined by the amino acid at positions 226 and 228 in HA [2, 28]. Amino acids 226Gln and 228Gly (226Gln/228Gly) in HA confer binding specificity to SA α 2,3Gal receptor, which are found in avian influenza virus isolates, whereas amino acids 226Leu and 228Ser (226Leu/228Ser) confer binding specificity to SA α 2,6Gal receptor, which are found in human viruses.

Pigs play an important role in the generation of pandemic viruses since they are susceptible to infection with both avian and human influenza viruses. However, the mechanisms by which avian influenza viruses acquire binding specificity to SA α 2,6Gal and cause pandemic influenza are not fully understood. In the present study, an H3 avian influenza virus was consecutively passaged in pigs to assess whether viruses recognizing the SA α 2,6Gal receptor are selected.

Materials and Methods

Viruses and cells

A/duck/Hokkaido/5/77 (H3N2) (Hok/77-P0) was propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h and infectious allantoic fluid was stored at –80°C until use. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 0.3 mg/ml L-glutamine, 10% calf serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 8 µg/ml gentamicin.

Consecutive passages of duck influenza viruses in pigs

Three-week-old crossbred (Landrace × Duroc × Yorkshire) specific pathogen-free pigs (Yamanaka chikusan, Hokkaido, Japan) were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) in a BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Before virus inoculation, serum samples were checked for the absence of antibodies against Hok/77-P0 using hemagglutination-inhibition (HI) tests. Consecutive virus passages were initiated in 2 naïve pigs by intranasal inoculation with 2 ml allantoic fluid containing Hok/77-P0. Nasal swabs were collected from these pigs into MEM every 12 h for 7 days post inoculation (dpi). The last isolated virus samples that were recovered from pigs were

propagated in MDCK cells for 48-72 h at 35°C and were intranasally re-inoculated into 2 naïve pigs. Passages were repeated 3 times. The remaining supernatants were stored at -80°C until use. The institutional animal care and use committee of the Hokkaido University authorized this animal experiment (approval number: 11-0087), and all experiments were performed according to the guidelines of this committee.

Plaque assay

Serial 10-time dilutions of virus samples were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for 1 h. Unbound viruses were removed, and cells were washed with phosphate-buffered saline (PBS, pH7.2). Cells were subsequently overlaid with MEM containing 1% Bacto-agar (Difco, Sparks, MD, USA) and 5 µg/ml trypsin acetylated (Sigma-Aldrich, St Louis, MO, USA). Forty-eight hours after incubation at 35°C, cells were stained with 0.005% neutral red. To analyze nucleotide sequences of passaged viruses, plaques showing cytopathic effects were picked up using a sterile capillary pipette and suspended in MEM.

Sequencing

Viral RNA was extracted from suspensions of picked-up plaques using Trizol-LS (Sigma). Universal primer sets for influenza A virus were used for reverse

transcription polymerase chain reaction (RT-PCR) of all eight gene segments [6]. PCR products were ligated with gene-specific primers and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA). Sequences of DNA templates were determined using the 3500 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed using GENETYX version 10 (Genetyx Corporation, Tokyo, Japan).

Deep sequencing

For bead-bound cDNA of Hok/77-P0 and variants, emulsion PCRs (emPCRs) were performed using GS Junior Titanium emPCR kit (Roche, Basel, Switzerland) according to the manufacturer's instructions, with 2 copies per bead. After bead recovery and enrichment, beads were sequenced using the GS Junior Titanium Sequencing kit (Roche) and a GS Junior Bench Top System (Roche) according to the appropriate instrument run protocol. The resulting reads were sorted and assembled using CLC Genomics Workbench software (CLC bio, Aarhus, Denmark).

Solid-phase direct binding assay

Receptor binding specificity of the viruses was assessed using a solid-phase direct binding assay with sialylglycopolymers (6'-Sialyllactose-PAA, 6'-SL and

3'-Sialyllactose-PAA, 3'-SL) (Cosmo Bio Co., Ltd., Tokyo, Japan) as previously described by Totani *et al.* (36) with some modifications. Briefly, each sialylglycopolymer was serially diluted and added to each well of a Universal-BINDTM, 96-well polystyrene stripwell microplate (Corning, NY, USA). Each well was blocked with 1% bovine serum albumin (BSA) at room temperature for 3 h. After washing with PBS containing 0.05% Tween 20 (PBST), a solution containing influenza viruses (8 HAU in PBST and 0.5% BSA) was added to each well and the plates were incubated at 4°C for 16 h. After washing with cold PBST, mouse anti-HA monoclonal antibodies were added to each well and the plates were incubated at 4°C for 2 h. The wells were subsequently washed with cold PBST and incubated with goat anti-mouse IgG-HRP conjugate (Bio-Rad, Carlsbad, CA, USA) at 4°C for 2 h. After washing with cold PBST, the substrates 0.5 mM 3,3'-tetramethylbenzidine (TMB) and 0.04% H₂O₂, were added to each well. After incubation at room temperature for 10 min, the reactions were stopped using 100 µl of 2N H₂SO₄, and absorbance at 450/630 nm was measured using a SunriseTM Microplate Reader (TECAN Group Ltd., Männedorf, Switzerland).

Results

Consecutive passages of Hok/77-P0 in pigs

Hok/77-P0 was passaged in pigs to assess whether human-type receptor binding specificity could be acquired. Virus titers in nasal swabs of infected pigs are shown in Table 1. Passaging of the virus was initiated in 2 pigs by intranasal inoculation of 2 ml allantoic fluid containing Hok/77-P0 at $10^{7.7}$ PFU. Viruses recovered from the swab from pig #1 at 5 dpi (Hok/77-P1) were used for the next passage. Hok/77-P1 was grown in MDCK cells and the yielded viruses were inoculated into 2 naïve pigs (#3 and #4) at $10^{7.6}$ PFU. Viruses were recovered from swab of pig #3 at 5 dpi (Hok/77-P2) were used for next passage. Hok/77-P2 was grown in MDCK cells and the yielded viruses were inoculated into 2 naïve pigs (#5 and #6) at $10^{7.3}$ PFU. Viruses were recovered from swab of pig #5 at 7 dpi (Hok/77-P3) were used for next passage.

Amino acid substitutions of the viruses during consecutive passages in pigs

To assess whether variants were selected in pigs, 10 plaque-picked virus clones were obtained from the nasal swabs of pigs, and amino acid sequence at the receptor binding site of HA was determined (Fig. 1). Nucleotide sequences of the HA genes

Table 1 Virus titers in nasal swabs of infected pigs

Passage No.	Animal ID	Virus titers on the dpi (log PFU/ml) ^a						
		1	2	3	4	5	6	7
Passage 1	#1	4.5/4.4	5.3/4.9	4.5/4.6	4.4/4.3	3.6/3.1*	-/-	-NT ^c
	#2	^b -/-	2.9/-	-/-	-/-	-/-	-/-	-NT
Passage 2	#3	-/4.3	3.6/3.0	2.0/1.6	3.0/3.3	2.7*/-	-/-	-NT
	#4	3.1/4.4	3.0/-	-/-	-/-	-/-	-/-	-NT
Passage 3	#5	3.5/4.4	3.0/2.1	1.5/2.7	3.1/3.5	3.7/3.0	2.3/2.9	2.7*/NT
	#6	3.8/4.6	2.2/-	-/3.1	3.1/4.0	3.2/3.0	2.5/4.1	-NT

^a Swabs were collected from pigs every 12 h (AM/PM).

^b <10^{1.0} PFU/ml.

^c Not Tested.

* The viruses were inoculated into the next pigs and amino acid substitution of HA was identified for each virus sample.

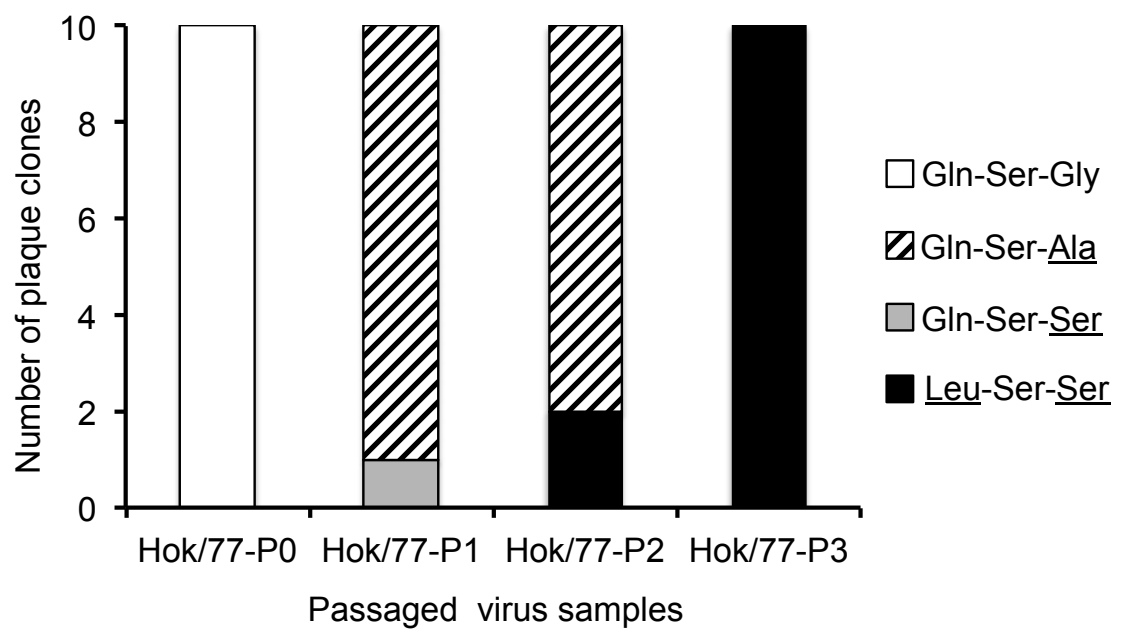


Fig. 1. The proportions of variants with the amino acid substitutions at positions 226-228 in HA. Ten plaque-picked virus clones were collected from allantoic fluid containing Hok/77-P0 and from nasal swabs containing the last-recovered viruses from each passage (indicated by asterisk in Table 1). Deduced amino acids at position 226 and 228 in HA of plaque-picked virus clones were identified.

from plaque-picked virus clones were compared with those of Hok/77-P0. All 10 plaque-picked virus clones in Hok/77-P0 had amino acid residues of 226Gln/228Gly at the HA receptor binding site. Variants with 226Gln/228Ala, 226Gln/228Ser, and 226Leu/228Ser at the receptor binding site of HA were recovered from Hok/77-P1 and Hok/77-P2. In Hok/77-P3, all variants contained 2 amino acid substitutions, 226Leu/228Ser, in HA. Seven amino acid substitutions in addition to amino acids 226 and 228 in HA were found in Cys196Tyr and Ala716Val in PB2, Asp78Glu in PB1, Asp431Asn in PA, Thr87Ile in HA2, Ala156Thr in NP, and Glu101Glu/Lys in NS1 from Hok/77-P3. To examine whether the variants were selected in MDCK cells, Hok/77-P0 was passaged 3 times in MDCK cells and 10 plaque clones in each passage were obtained from supernatants to identify deduced amino acid sequences. Each of the plaque-picked virus clones has amino acid residues at 226Gln/228Gly in HA (data not shown), indicating that the variants with 226Leu/228Ser were selected in pigs, but not in MDCK cells.

Single-nucleotide polymorphism (SNP) analysis was performed using a deep sequence method (Table 2). Deduced amino acid substitutions at positions 226 and 228 in HA were not observed in Hok/77-P0. In Hok/77-P1, substitution was observed in 78.6% at 226Gln or in 21.0% at 226Leu and in 3.5% at 228Gly, in 54.2% at 228Ala, or in 41.4% at 228Ser of the deduced amino acid sequence in HA. In Hok/77-P2,

Table 2 Quasispecies composition of Hok/77-P0 and passaged viruses at the codon sites which responsible for receptor binding in H1A

Nucleotide position and sequence ^a	Hok/77-P0	Rate (%) in passaged virus samples			Amino acid position and deduced amino acid ^c
		Hok/77-P1	Hok/77-P2	Hok/77-P3	
676-678					226
CAG	100	78.9	76.2	<2.8	Gln
. T .	^b	21.0	23.6	97.2	<u>Leu</u>
682-684					228
GGT	100	3.5	6.7	<0.4	Gly
. C .	-	54.2	68.8	<0.4	<u>Ala</u>
A . .	-	41.4	24.2	99.6	<u>Ser</u>

^a Periods indicate the same nucleotide as the sequence above.

^b Undetectable.

^c The amino acid substitutions found after passages in pigs are underlined.

substitution was observed in 76.2% at 226Gln or in 23.6% at 226Leu, and in 6.7% at 228Gly, in 68.8% at 228Ala, or in 24.2% at 228Ser of the deduced amino acid sequence in HA. Nucleotides encoding the deduced amino acids at position 226Leu and 228Ser in HA were predominantly detected in Hok/77-P3 at 97.2% and 99.6%, respectively. These results indicate that avian influenza viruses recognizing SA α 2,6Gal were selected during multiple replication in pigs.

Receptor binding specificity of the viruses passaged in pigs

To assess receptor binding specificity of variants, direct binding assays of plaque-picked virus clones to SA α 2,3Gal and SA α 2,6Gal sialylglycopolymers were performed. Hok/77-P0, which has 226Gln/228Gly residues in HA, preferentially bound to 3'-SL than 6'-SL (Fig. 2). Plaque-picked virus clones from nasal swabs of the pigs, Hok/77-P2 clone 4 (226Gln/228Ala) or Hok/77-P1 clone 7 (226Gln/228Ser), showed preferential binding specificity to 3'-SL. Plaque-picked virus clone from pig, Hok/77-P3 clone 5 (226Leu/228Ser), preferentially bound to 6'-SL. Therefore, these data confirmed that HA of Hok/77-P0 acquired SA α 2,6Gal receptor binding specificity after consecutive passages in pigs.

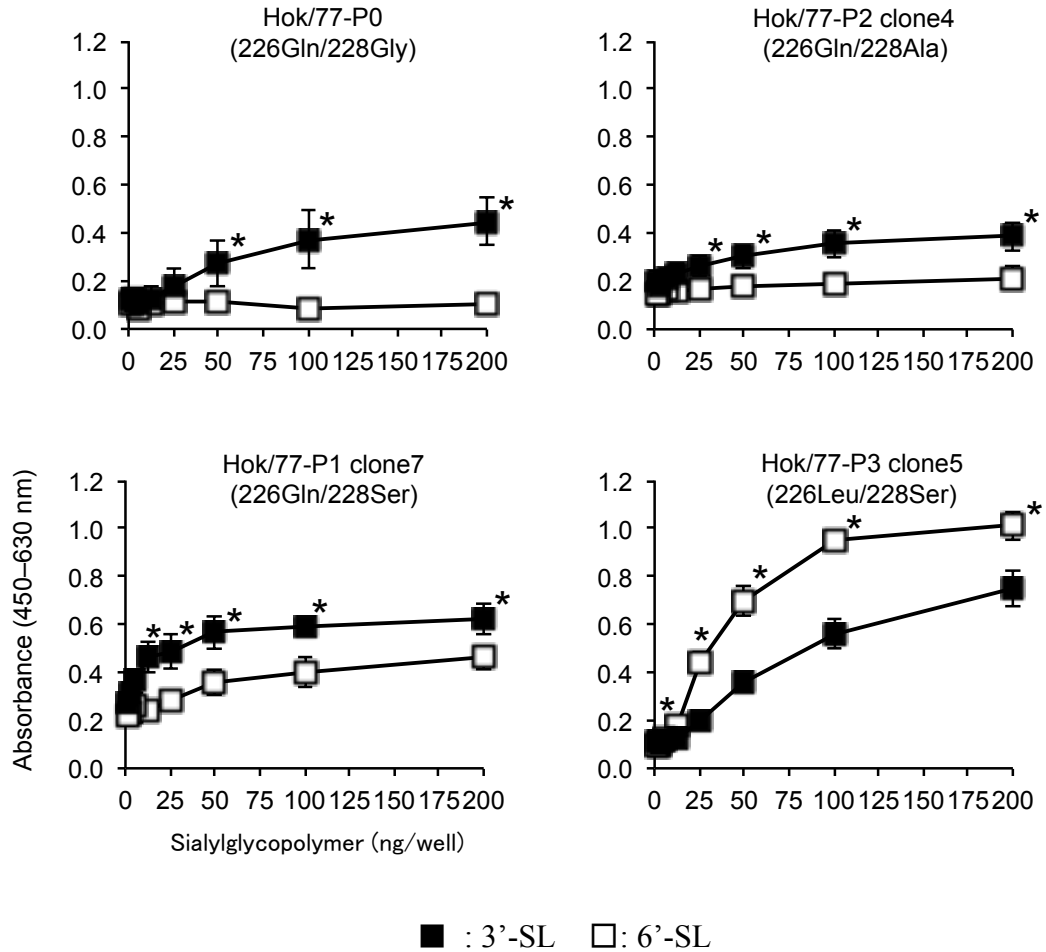


Fig. 2. Binding of Hok/77-P0 and variants to either 3'-SL or 6'-SL. Binding to 3'-SL (black square) and 6'-SL (white square) of Hok/77-P0 (226Gln/228Gly) and variants, Hok/77-P2 clone 4 (226Gln/228Ala), Hok/77-P1 clone 7 (226Gln/228Ser), and Hok/77-P3 clone 5 (226Leu/228Ser), were compared using solid-phase binding assays. Data are presented as the mean $\pm$ s.e. of triplicate experiments. Significant differences between the absorbance of 3'-SL and 6'-SL were calculated using the *t* test; * $P < 0.05$.

Discussion

HAs of avian influenza viruses preferentially bind to SA α 2,3Gal receptor, whereas those of human influenza viruses bind to SA α 2,6Gal receptor. Pigs are susceptible to both avian and human influenza viruses. Experimental infection studies revealed that genetic reassortants were generated on concurrent infections with avian and human viruses in cells of the upper respiratory in pigs, which have both SA α 2,3Gal and SA α 2,6Gal [9, 14]. Pandemic strains bind to SA α 2,6Gal receptors, although HAs originated from avian influenza viruses. It is assumed that preferential recognition of SA α 2,6Gal by influenza virus HAs is required for efficient human-to-human transmission. In the present study, it is demonstrated that variants that preferentially bind to SA α 2,6Gal were selected during consecutive passages in pigs, indicating that avian influenza viruses acquire the potential to infect humans after multiple passages in pig.

Plaque-picked virus clones with amino acid substitutions at positions 226Leu/228Ser, binding to SA α 2,6Gal, were recovered from Hok/77-P2 and Hok/77-P3. Previous studies indicate that SA α 2,6Gal is more abundant than SA α 2,3Gal in the cells of the respiratory tract of pigs [21, 39]. The A/Hong Kong/68 (H3N2), which has amino acid residues 226Leu/228Ser in HA, dominantly replicated in the respiratory

tracts of pigs compared with variants carrying 226Gln/228Gly mutations [38]. These results indicate that acquisition of preferential binding to SA α 2,6Gal is advantageous for virus replication in respiratory tracts of pigs. In addition to 226Leu/228Ser, seven other amino acid substitutions were found in Hok/77-P3. These amino acid substitutions also provided advantages for Hok/77-P0 to replicate in pigs, and SA α 2,6Gal receptor binding variants were selected in pigs.

Receptor specificity of influenza viruses is the key determinant of host range. It is assumed that binding specificity to SA α 2,6Gal receptor is required for efficient human-to-human transmission. This is the first study to demonstrate the selection of avian influenza viruses with HA that preferentially binds to SA α 2,6Gal receptor after successive passages in pigs. Influenza viruses of each HA subtype replicate in the respiratory tracts of pigs [14]. None of the HA subtypes can be ruled out as possible pandemic strain by the introduction into pig population. It is, thus, stressed that intensive surveillance of swine influenza is important to prepare for future pandemics.

Brief summary

Avian influenza viruses possess hemagglutinin (HA) which preferentially binds to SA α 2,3Gal receptor. In contrast, human influenza viruses bind to SA α 2,6Gal receptor. The A/Hong Kong/68 (H3N2) virus preferentially binds to SA α 2,6Gal receptor, although its HA gene was derived from an avian influenza virus strain. To elucidate the mechanisms behind acquisition of binding specificity for the human-type receptor, an avian influenza virus, A/duck/Hokkaido/5/77 (H3N2), which carries HA with SA α 2,3Gal receptor specificity, was consecutively passaged in pigs. Viruses that preferentially bind to the SA α 2,6Gal receptor were predominantly recovered from the nasal swabs of pigs after three passages. The present results indicate that avian influenza viruses can acquire the potential to infect humans after multiple passages in a pig population. Intensive surveillance of swine influenza is, thus, important for the preparedness for the future pandemics.

Chapter II

Potency of an inactivated influenza vaccine prepared from a non-pathogenic H5N1 virus against a challenge with antigenically drifted highly pathogenic avian influenza viruses in chickens

Introduction

Highly pathogenic avian influenza caused by H5N1 HPAI virus has spread in poultry in more than 60 countries in Eurasia and Africa since 1996 since the first outbreak occurred at a goose farm in Guangdong province in China [32, 42]. In recent intensive surveillance studies in Asia, especially in China, genetically different viruses of clades 2.3.2, 2.3.4, and 7 were characterized as dominant isolates from poultry and wild birds [11, 17, 33]. In the updated grouping of H5 HPAI viruses, it was reported that the clades of H5N1 viruses were divided into one or more newly defined second-, third-, and/or fourth-order clades, e.g. recent H5N1 isolates that had been categorized into clade 2.3.2 were defined as clade 2.3.2.1 [41]. In Japan, H5N1 HPAI viruses belonging to clade 2.3.2.1 were isolated from dead whooper swans in 2008 [24, 37],

fecal samples of ducks that flew from Siberia in October 2010 [12], and from wild birds and domestic poultry in 2011 [29]. Antigenicity of H5N1 HPAI viruses belonging to clades 2.3.2.1 and 2.3.4 was distinct from that of other HPAI viruses and non-pathogenic avian influenza viruses [24, 33], suggesting that antigenic variants of H5N1 HPAI viruses have been selected during circulation in poultry populations under immunological selection pressure.

A reassortant influenza virus, Dk/Vac-1/04, was generated using two non-pathogenic avian influenza viruses, A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1). Both viruses were isolated from fecal samples of migratory ducks [35]. Vac-1 vaccine prepared from Dk/Vac-1/04 conferred immunity to suppress the manifestation of clinical signs and the amount of virus shed in chickens after the challenge with H5N1 HPAI viruses belonging to clades 2.2 and 2.5 [8]. Vac-1 vaccine induced a high level of HI antibody response in chickens, lasting as long as 138 weeks after vaccination [31]. Vac-1 vaccine conferred protective immunity against antigenically drifted H5N1 HPAI virus, A/whooper swan/Hokkaido/1/08 (H5N1) (Ws/Hok/08), belonging to clade 2.3.2.1 in chickens [24].

In the present study, a vaccine from Dk/Vac-3/07, which is antigenically closely related with Dk/Vac-1/04, was prepared. Growth potential of Dk/Vac-3/07 in embryonated chicken eggs was higher than that of Dk/Vac-1/04 [35]. The potency of

the Vac-3 vaccine was assessed by the challenge with antigenically drifted HPAI viruses prevailing recently in Asia.

Materials and Methods

Viruses

Dk/Vac-3/07 was generated in our laboratory as a reassortant influenza virus between A/duck/Hokkaido/101/04 (H5N3) and A/duck/Hokkaido/262/04 (H6N1), isolated from fecal samples of migratory ducks [35]. The following HPAI virus isolates were used: A/muscovy duck/Vietnam/OIE-559/11 (H5N1) (Mdk/VN/11) [23], isolated from an apparently healthy muscovy duck in Vietnam in 2011, A/whooper swan/Hokkaido/4/11 (H5N1) (Ws/Hok/11), isolated from a dead whooper swan found in the Kushiro area in Hokkaido, Japan [29], and A/peregrine falcon/Hong Kong/810/09 (H5N1) (Pf/HK/09), kindly provided by Dr Luk S. M. Geraldine, Tai Lung Veterinary Laboratory (Hong Kong SAR, China). All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 h and infectious allantoic fluids were stored at –80°C until use.

The complete nucleotide sequence of Dk/Vac-1/04 and Dk/Vac-3/07 have been registered in GenBank/EMBL/DDBJ (Accession numbers: AB259709-AB259716 and AB355926-AB355933, respectively) [35]. It is also revealed that the deduced amino acids of Dk/Vac-1/04 were closely related to those of Dk/Vac-3/07 (98% similarity in HA gene, 97% similarity in NA gene, and more than 99% similarity in the other genes).

Generation of recombinant viruses

In addition to Dk/Vac-3/07, another test vaccine was prepared from attenuated Pf/HK/09. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with Pf/HK/09 using a commercial kit (TRIzol LS Reagent, Sigma-Aldrich, St Louis, MO, USA) and reverse transcribed with the Uni12 primer [3] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR-based amplification of the full-length HA and NA gene segments was performed using universal primer sets [6]. The PCR products were inserted into the vector pHW2000 [5] using an In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan). To generate a mutant virus with deletions of polybasic amino acid residue RRRK at the HA cleavage site, nucleotide mutation residue T (codon ACA) were substituted into the HA cleavage site of the Pf/HK/09 strains using a Quick Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Attenuated Pf/HK/09, Pf/mut (H5N1), was generated by reverse genetics methods according to Hoffmann *et al.* [5]. Pf/mut (H5N1) possesses the gene encoding the mutant HA of Pf/HK/09, in which polybasic amino acids at the cleavage site was deleted, NA of Pf/HK/09, and the backbone of Dk/Vac-1/04 internal genes. To confirm attenuation of Pf/mut, the IVPI test was carried out according to the OIE (World Organization for Animal Health) manual [22].

Vaccine preparation

Vac-3 and Pf/mut vaccines were prepared from Dk/Vac-3/07 or Pf/mut (H5N1), respectively. Dk/Vac-3/07 or Pf/mut (H5N1) was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs and the eggs were then incubated at 35°C for 48 h. After the allantoic fluid was harvested, formalin was added to a final concentration of 0.2%, and the mixture was incubated at 4°C for 7 days to inactivate the viruses. Virus inactivation was confirmed by the inoculation of the formalin-treated samples into embryonated chicken eggs. The inactivated Dk/Vac-3/07 and Pf/mut virus suspensions were concentrated by ultrafiltration using the Vivaflow 200 (Sartorius AG, Goettingen, Germany), and then diluted with PBS to give the required HA titer. The inactivated viruses, light liquid paraffin, sorbitan monooleate, and polysorbate 80 were mixed in a volume ratio of 9:36:4:1 and then agitated to obtain emulsion. The Vac-3 vaccine of 2.4-times antigen concentration was also prepared and designated as Vac-3 conc. vaccine. Vac-3 vaccine contains inactivated virus of 756 HA per dose and was prepared from the dilution of infectious allantoic fluid 1:1 with PBS, 378 HA per dose of Pf/mut vaccine at 1:1, and 1843 HA per dose of Vac-3 conc. vaccine at 2.4:1, respectively.

Antigenic analysis of the viruses

Polyclonal antisera were prepared from chickens immunized with inactivated Dk/Vac-3/07, A/chicken/Yamaguchi/7/04 (H5N1) (Ck/Yamaguchi/04), A/whooper swan/Mongolia/3/05 (H5N1) (Ws/Mon/05), Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. Each virus inactivated with formalin was inoculated once or twice into the lower thigh muscle of chickens. Two weeks after the final immunization, sera were obtained from each vaccinated chicken and used for HI test to assess antigenic relationships among H5 influenza viruses. HI test was performed according to Isoda *et al.* [8]. The differences within 4-times HI titers were determined as antigenically related, whereas over 4-times determined as antigenically different.

The antigenic specificities of H5 viruses, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09, were assessed by the fluorescent antibody method using monoclonal antibodies (MAb) to HA according to the method of Soda *et al.* [34]. MDCK cells infected with each of the H5 influenza viruses were fixed with 100% acetone at 8 h post-inoculation. Reactivity patterns of the H5 viruses with MAbs were investigated with FITC-conjugated goat anti-mouse IgG (ICN Biomedicals Inc., Irvine, CA, USA) using a fluorescent microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany).

Potency of test vaccines in chickens

One hundred and ten chickens (White leghorn) were hatched and raised in our laboratory. Half a milliliter of Vac-3 vaccine was injected into the lower thigh muscle of 54 four-week-old chickens. Three weeks later, 18 vaccinated and 4 non-vaccinated seven-week-old chickens in 3 groups were challenged intranasally with 100 50% chicken lethal dose (CLD₅₀) of Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. One hundred times CLD₅₀ of Mdk/VN/11, Ws/Hok/11, and Pf/HK/09 was 10^{6.3}, 10^{5.7}, and 10^{5.5} 50% egg infectious dose (EID₅₀), respectively. Twelve out of 18 vaccinated chickens in each group were monitored for their clinical signs for 2 weeks, and 6 chickens in each group were sacrificed at 3 days post challenge (dpc).

Pf/mut and Vac-3 conc. vaccines were injected into the lower thigh muscle of 2 groups of 18 four-week-old chickens. Three weeks later, 18 vaccinated and 4 non-vaccinated chickens in the 2 groups were challenged intranasally with 100 CLD₅₀ of Pf/HK/09. Twelve out of 18 vaccinated chickens in each group were monitored for their clinical signs for 2 weeks, and 6 chickens in each group were sacrificed at 3 dpc.

When chickens died or were sacrificed, tracheal and cloacal swabs were collected and soaked in MEM containing antibiotics for virus isolation, and their tissues (trachea, lungs, kidneys, and colon) were collected aseptically. To make 10% suspensions with MEM, the collected tissue samples were homogenized using a Multi-Beads Shocker

(Yasui Kikai, Osaka, Japan). Serial 10-time dilutions of the suspensions with PBS were inoculated into 10-day-old embryonated chicken eggs and incubated at 35°C for 48 h. EID₅₀ of viruses was determined by the method of Reed and Muench [26] and expressed as EID₅₀ per milliliter of swab or gram of tissue, respectively. Sera were collected from all of the 90 vaccinated and 20 non-vaccinated chickens just before challenge and examined for the presence of antibodies against the vaccine strains and challenge virus stains by the HI tests. Challenge studies were carried out in self-contained isolator units (Tokiwa kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Animal experiments were authorized by the Institutional Animal Care and Use Committee of Hokkaido University (approval numbers: 09-0119 and 10-0007) and all experiments were performed according to the guidelines of this committee.

Results

Antigenic analysis

H5N1 HPAI viruses used as the vaccine strain and challenge viruses in the present study were antigenically analyzed by the HI tests (Table 3). Dk/Vac-3/07 is antigenically closely related to Ck/Yamaguchi/04 and Ws/Mon/05, but is different from Ws/Hok/11 and Pf/HK/09. The recent H5N1 HPAI virus isolates belonging to clades 2.3.2.1, and 2.3.4 were antigenically different.

H5N1 HPAI viruses used as the vaccine strain and challenge viruses in the present study were antigenically analyzed using a panel of MAbs recognizing six different epitopes on HA of A/duck/Pennsylvania/10218/84 (H5N2). Most of the MAbs tested bound to the LPAI viruses and HPAI viruses isolated before 2005, but not to the antigens of Mdk/VN/11, Ws/Hok/11, and Pf/HK/09. It was demonstrated that the epitopes recognized by these MAbs were conserved in LPAI viruses and HPAI viruses isolated before 2005, but not in recent HPAI viruses prevailing recently in Asia (Table 4).

Efficacy of the Vac-3 vaccine in chickens

Table 3. Antigenic analysis of H5N1 subtype avian influenza viruses using polyclonal antibodies^a

Virus	Clade ^b	Antiserum to					
		Vac-3	Yama/04	Mon/05	VN/11	Hok/11	HK/09
A/duck/Hokkaido/Vac-3/07	-	<u>128</u>	16	32	4	8	<4
A/chicken/Yamaguchi/7/04	2.5	128	<u>128</u>	128	32	32	<4
A/whooper swan/Mongolia/3/05	2.2	128	256	<u>512</u>	128	128	64
A/muscovy duck/Vietnam/OIE-559/11	1.1	256	64	16	<u>256</u>	16	16
A/whooper swan/Hokkaido/4/11	2.3.2.1	32	16	64	16	<u>128</u>	<4
A/peregrine falcon/Hong Kong/810/09	2.3.4	16	8	8	16	16	<u>128</u>

^a Homologous titers are underlined

^b “-” indicate lineages not belonging to clade 0-9

Abbreviations: Vac-3, A/duck/Hokkaido/Vac-3/07; Yama/04, A/chicken/Yamaguchi/7/04; Mon/05, A/whooper swan/Mongolia/3/05; VN/11, A/muscovy duck/OIE-559/11; Hok/11, A/whooper swan/Hokkaido/4/11; HK/09, A/peregrine falcon/Hong Kong/810/09

Table 4. Antigenic analysis of H5 influenza viruses using monoclonal antibodies

Virus ^a	Clade ^b	Monoclonal antibodies ^c							
		I (88)	II (145)	III (157)	IV (168)		V (169)		VI (205)
		D101/1	A310/39	64/1	B9/5	B220/1	B59/5	B25/2	
LPAIV									
A/duck/Pennsylvania10218/84 (H5N2)	-	+	+	+	+	+	+	+	+
A/duck/Hokkaido/Vac-3/07 (H5N1)	-	+	+	+	+	+	+	+	+
HPAIV									
A/Vietnam/1194/04 (H5N1)	1	+	+	+	+	+	-	+	+
A/chicken/Yamaguchi/3/05 (H5N1)	2.5	-	+	+	+	+	-	+	+
A/whooper swan/Mongolia/3/05 (H5N1)	2.2	+	+	+	+	+	-	+	+
A/muscovy duck/Vietnam/OIE-559/11 (H5N1)	1.1	+	-	-	-	-	-	+	+
A/whooper swan/Hokkaido/4/11 (H5N1)	2.3.2.1	+	-	-	-	-	-	-	-
A/peregrine falcon/Hong Kong/810/09 (H5N1)	2.3.4	-	-	-	-	-	-	-	-

^a Viruses indicated in bold were used in the challenge study

^b “-” indicate lineages not belonging to clade 0-9

^c Reactivity of monoclonal antibodies against HA of A/duck/Pennsylvania/10218/84 (H5N2) to the representative H5 viruses was compared using fluorescent antibody methods. Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies is indicated in parentheses.

Fifty-four vaccinated chickens and 12 non-vaccinated chickens were challenged intranasally with each of the HPAI viruses, Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. The serum HI titers of the vaccinated chickens varied with each of the challenge viruses. The survival rates of the chickens challenged with each of three HPAI viruses are shown in Fig. 3. All vaccinated chickens survived without showing any disease signs after the challenge either with Mdk/VN/11 or Ws/Hok/11, whereas two vaccinated chickens died after the challenge with Pf/HK/09. All non-vaccinated chickens challenged with any of HPAI viruses died within 2 to 4 dpc (Fig 3A, B, and C).

To evaluate the potential of Vac-3 vaccine to induce immunity for the prevention of virus shedding, viruses were recovered from swabs and tissues of the vaccinated and non-vaccinated chickens after the challenge with each of HPAI viruses, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09 (Table 5). Infectivity titers of the recovered viruses from vaccinated chickens were lower than those of non-vaccinated chickens after the challenge with Mdk/VN/11 or Pf/HK/09 at 3 dpc. Infectious viruses were recovered from tracheal swabs and the organs of vaccinated chickens at 3 dpc with Ws/Hok/11, although the titers of viruses recovered from these birds were lower than those from non-vaccinated chickens.

Efficacy of the Vac-3 conc. vaccine against Pf/HK/09 in chickens

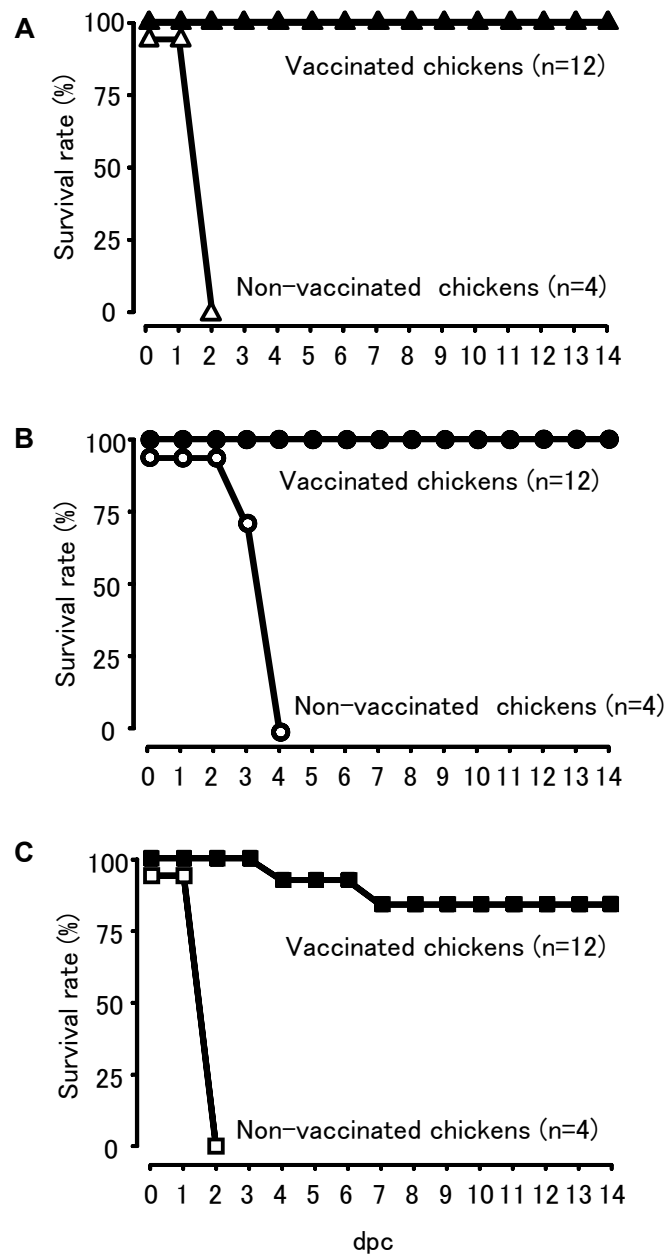


Fig. 3. Survival rates of chickens vaccinated with Vac-3 vaccine after the challenge with H5N1 HPAI viruses. Twelve four-week-old chickens from each group were immunized intramuscularly with 0.5 ml of Vac-3 vaccine. Three weeks after vaccination, the vaccinated chickens were challenged with 100 CLD₅₀ of Mdk/VN/11 (A), Ws/Hok/11 (B), and Pf/HK/09 (C), respectively.

Table 5. Virus recovery from chickens vaccinated with Vac-3 vaccine challenged with H5N1 HPAI viruses

Challenge virus	Vaccination	Sampling dpc ^a	No. of chickens	HI titer ^b		Virus recovery						
						No. of chickens from which each virus was recovered [GM value of the virus titer (log 10)]	Swab (log EID ₅₀ /ml)					
				Dk/ Vac-3/07	Challenge virus		Trachea	Cloaca	Tissue (log EID ₅₀ /g)			
									Trachea	Lungs	Colon	Kidneys
Mdk/VN/11	Vaccinated	3	6	256	8-64	1 (≤0.7)	0	0	0	0	0	0
	Non-vaccinated	2 [†]	4	<4	<4	4 (5.5)	4 (5.3)	4 (8.6)	4 (9.3)	4 (8.5)	4 (9.4)	4 (9.4)
Ws/Hok/11	Vaccinated	3	6	256-512	16-64	2 (≤1.0, ≤1.3)	0	3 (4.3)	4 (5.1)	5 (4.4)	4 (4.0)	4 (4.0)
	Non-vaccinated	3-4 [†]	4	<4	<4	4 (4.2)	4 (3.2)	4 (6.4)	4 (8.2)	4 (6.9)	4 (7.9)	4 (7.9)
Pf/HK/09	Vaccinated	3	6	64-512	<4-16	2 (≤0.7, ≤1.3)	0	0	1 (3.5)	0	0	0
	Non-vaccinated	2 [†]	4	<4	<4	4 (4.9)	4 (5.4)	4 (7.6)	4 (9.1)	4 (8.3)	4 (8.8)	4 (8.8)

^a Swab and tissue samples were collected on the following days from sacrificed (no mark) or dead (†) chickens.

^b The range of HI titers using either Dk/Vac-3/07 or each challenge virus before challenge is indicated.

In order to enhance the efficacy of Vac-3 vaccine, the antigen concentration of Vac-3 vaccine was increased 2.4 times and designated as Vac-3 conc. vaccine. The Vac-3 conc. was assessed for its efficacy against the challenge with Pf/HK/09. Pf/mut vaccine prepared from Pf/mut (IVPI=0.00) was also assessed for its potency as the homologous control. Thirty-six chickens immunized either with Vac-3 conc. or Pf/mut vaccine and 8 non-vaccinated chickens were challenged intranasally with Pf/HK/09. HI titer to Pf/HK/09 of the sera of the chickens immunized with Vac-3 conc. vaccine was 4-16 HI, which is similar to those of the chickens immunized with Vac-3 vaccine. The survival rates of the chickens challenged with Pf/HK/09 are shown in Fig. 4. All vaccinated chickens survived without showing any disease signs after the challenge with Pf/HK/09 (Fig 4A, B). All non-vaccinated chickens challenged with Pf/HK/09 died within 3 dpc. Viruses were not recovered from swabs or the organs of any of the chickens immunized with Pf/mut vaccine after the challenge with Pf/HK/09 (Table 6). Viruses were scarcely recovered from the trachea swabs of chickens immunized with Vac-3 conc. vaccine and the viral titer was lower than in non-vaccinated chickens after challenge with Pf/HK/09.

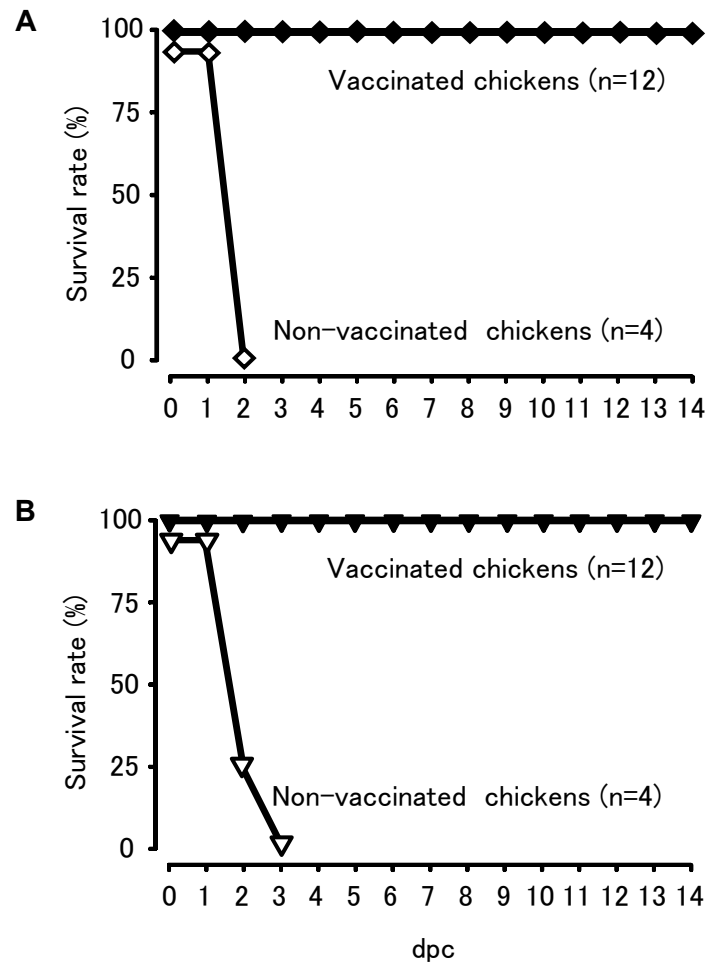


Fig. 4. Survival rates of chickens vaccinated with Pf/mut vaccine (A) and Vac-3 conc. vaccine (B) after the challenge with Pf/HK/09. Twelve four-week-old chickens from each group were immunized intramuscularly with 0.5 ml of Pf/mut vaccine or Vac-3 conc. vaccine. Three weeks after vaccination, these vaccinated chickens were challenged with 100 CLD₅₀ of Pf/HK/09.

Table 6. Virus recovery from chickens challenged with Pf/HK/09

Vaccine	Sampling dpc ^a	No. of chickens	HI titer ^b		Virus recovery						
					No. of chickens from which each virus was recovered [GM value of the virus titer (log 10)]						
			Dk/Vac-3/07		Pf/HK/09		Swab (log EID ₅₀ /ml)				
							Tissue (log EID ₅₀ /g)				
					Trachea	Cloaca	Trachea	Lungs	Colon	Kidneys	
Pf/mut	3	6	16-32	128-512	0	0	0	0	0	0	
Vac-3 conc.	3	6	256-1024	4-16	1 (1.7)	0	0	0	0	0	
Control	2-3 [†]	8	<4	<4	8 (5.1)	8 (5.3)	8 (7.8)	8 (9.0)	8 (8.3)	8 (8.7)	

^aSwab and tissue samples were collected on the following days from sacrificed (no mark) or dead (†) chickens

^bThe range of HI titers using either Dk/Vac-3/07 or Pf/HK/09 before challenge is indicated.

Discussion

Antigenic variants of H5N1 HPAI viruses have been selected in poultry under immunological selection pressure [1, 4]. In the present study, it was demonstrated that H5N1 HPAI viruses prevailing recently in Asia were antigenically different from non-pathogenic avian influenza viruses and H5N1 HPAI viruses isolated before 2005 (Table 3). It is previously demonstrated that an inactivated avian influenza vaccine prepared from Dk/Vac-1/04 conferred protective immunity and reduced the amount of virus shedding when challenge with Ck/Yamaguchi/04, Ws/Mon/05, and Ws/Hok/08 [8, 24]. In the present study, an inactivated influenza vaccine was prepared from Dk/Vac-3/07, which is a reassortant generated between non-pathogenic avian influenza viruses isolated from wild water birds [35]. It is assumed that Vac-3 vaccine has similar potency with Vac-1 vaccine against recent H5N1 HPAI viruses since Dk/Vac-3/07 is antigenically similar to Dk/Vac-1/04. However, since the growth potential of Dk/Vac-3/07 was better than that of Dk/Vac-1/04, it was possible to generate concentrated Vac-3 vaccine using Dk/Vac-3/07 in this study. The potency of Vac-3 vaccine was assessed by the challenge with antigenically drifted H5N1 HPAI viruses isolated in 2009 and 2011. Vac-3 vaccine conferred protective immunity to suppress the manifestation of clinical signs and virus shedding in chickens challenged

with antigenically drifted H5N1 HPAI viruses belonging to clades 1.1, 2.3.2.1, and 2.3.4. In order to clarify why the efficacy of Vac-3 vaccine was not sufficient to protect all vaccinated chickens from the challenge with Pf/HK/09, Pf/mut vaccine which was antigenically homologous with Pf/HK/09 was prepared (data not shown). All chickens immunized with Pf/mut vaccine survived for 14 days without showing any clinical signs and viruses were not recovered from the swabs and tissues of the chickens. These results correspond to the findings that Pf/HK/09 is antigenically different from Dk/Vac-3/07 compared with Mdk/VN/11 and Ws/Hok/11. To improve the efficacy of Vac-3 vaccine, antigen concentration was increased for Vac-3 conc. vaccine preparation. HI antibody responses of vaccinated chickens correlated with the antigen concentration in H5N1 [30] or H7N7 [19] influenza virus vaccine. Inactivated whole particle vaccine confers protective immunity against the challenge with viruses antigenically different from the vaccine strain to chickens by increasing the antigen concentration [7]. Vac-3 conc. vaccine conferred protective immunity to all vaccinated chickens after the challenge with Pf/HK/09. The vaccine with increased antigen concentration induced sufficient immunity to protect from infection with H5N1 HPAI variants in chickens.

In the present study, it was demonstrated that the vaccine prepared from non-pathogenic avian influenza virus conferred protective immunity against the

challenge with antigenically drifted H5N1 HPAI viruses, indicating that Vac-3 vaccine induces sufficient immunity in chickens. The results of the antigenic analysis indicate broad antigenic diversity among H5N1 HPAI viruses prevailing recently in Asia (Table 3). Thus, the vaccine prepared from recent H5N1 HPAI viruses may not be completely effective against HPAI viruses belonging to different clades. Since the misuse of vaccines lead to the silent spread of antigenically drifted viruses, it is recommended that avian influenza vaccine should be applied very carefully in addition to the stamping-out policy. There is an urgent need to eradicate H5N1 HPAI viruses from Asia by stamping-out without misuse of vaccines.

Brief Summary

Antigenic variants of H5N1 highly pathogenic avian influenza (HPAI) virus have selected and are prevailing in poultry populations in Asia. In the present study, the potency of inactivated influenza vaccine prepared from a non-pathogenic H5N1 avian influenza virus, A/duck/Hokkaido/Vac-3/07 (H5N1), was assessed by the challenge with H5N1 antigenic variants, A/muscovy duck/Vietnam/OIE-559/11 (H5N1), A/whooper swan/Hokkaido/4/11 (H5N1), and A/peregrine falcon/Hong Kong/810/09 (H5N1) belonging to clades 1.1, 2.3.2.1, and 2.3.4, respectively. All chickens immunized with the Vac-3 vaccine survived without showing any clinical signs after intranasal challenge either with A/muscovy duck/Vietnam/OIE-559/11 (H5N1) or A/whooper swan/Hokkaido/4/11 (H5N1). After challenge with A/peregrine falcon/Hong Kong/810/09 (H5N1), 10 out of 12 vaccinated chickens survived and the other 2 died on 4 or 7 dpc. The Vac-3 vaccine of 2.4-times antigen concentration conferred complete protective immunity in chickens against the challenge with A/peregrine falcon/Hong Kong/810/09 (H5N1).

Conclusion

There are four instances of naturally occurring emergence of new pandemic strains in humans in the last one hundred years. Each of the earliest isolates in the pandemics preferentially recognize SA α 2,6Gal receptor, although the HA genes were derived from avian influenza viruses. It is assumed that binding specificity to SA α 2,6Gal receptor is required for efficient human-to-human transmission. However, the mechanisms by which avian influenza viruses acquire binding specificity to SA α 2,6Gal and cause pandemic influenza are not fully understood. In the present study, it is revealed that H3 avian influenza virus with SA α 2,6Gal receptor binding specificity was selected after three-time passages in pigs. These results show that avian influenza viruses can acquire the potential to infect humans after multiple passages in pigs.

H5N1 HPAI viruses are prevailing in poultry populations in Asia. It is important to assess the potency of stockpile avian influenza vaccine against the viruses prevailing recently in Asia. In the present study, inactivated whole particle vaccine, Vac-3 vaccine, was prepared from non-pathogenic avian influenza virus. All chickens immunized with the Vac-3 vaccine survived without showing any clinical signs after intranasal the challenge either with A/whooper swan/Hokkaido/4/11

(H5N1) or A/muscovy duck/Vietnam/OIE-559/11 (H5N1). The Vac-3 conc. vaccine of high antigen concentration conferred complete protective immunity in chickens against challenge with A/peregrine falcon/Hong Kong/810/09 (H5N1). It was demonstrated that the test vaccine prepared from non-pathogenic avian influenza virus induced sufficient immunity in chickens against the challenge with antigenically drifted H5N1 HPAI viruses. Antigenic variants of H5N1 HPAI viruses have been selected in poultry under immunological selection pressure. Avian influenza vaccine should be applied as an optional measure in cases where the disease spreads widely, in addition to the stamping-out policy.

Acknowledgements

The author would like to acknowledge Prof. H. Kida, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University (Sapporo, Hokkaido, Japan), for his guidance and encouragement during the course of this work.

Great appreciation is extended to Prof. K. Ohashi, Laboratory of Infectious Disease, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, and Prof. A. Takada, Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University for their critical review of this manuscript.

The author is grateful to Dr. Y. Sakoda, Dr. M. Okamatsu, and Dr Naoki Yamamoto, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University for their valuable advices, supports and encouragements throughout the present studies.

The author thanks to Dr Luk S. M. Geraldine for providing A/peregrine falcon/Hong Kong/810/09 (H5N1), and its coordinator Dr. Kenji Sakurai, OIE Regional Representation for Asia and the Pacific, Tokyo, Japan for their help to carry out the present study.

The author heartily thanks to Dr. Y. Noda and Dr. T. Honda, The Chemo-Sero-Therapeutic Research Institute (Kikuchi, Kumamoto, Japan), Dr. Y. Nomoto and Dr. N. Takikawa, Kitasato Daiichi Sankyo Vaccine Co., Ltd., for producing the inactivated avian influenza vaccine with high efficacy.

The author is deeply thanks to A. Ishii and A. Ohnuma (Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan) for their technical support and help for deep sequencing, and Dr. N. Isoda for his useful advice on the preparation of Chapter II. We also thank N. Nagashima, T. Nishi, T. Tamura, T. Hiono, K. Mitsuhashi, Y. Motohashi, and R. Sawayama for their excellent technical support. The present work was supported in part by the Global Centers of Excellence (GCOE) Program from the Japan Society for Promotion of Science, the Japan Science and Technology Agency Basic Research Programs, and the Japan Initiative for Global Research Network on Infectious Disease (J-GRID).

The author earnestly appreciates all colleagues of Laboratory of Microbiology, school of Veterinaty Medicine, Hokkaido University for their invaluable help and friendships.

Finally, the author express his mourning for all of the animals sacrificed their precious lives for these studies.

References

1. **Cattoli, G., Milani, A., Temperton, N., Zecchin, B., Buratin, A., Molesti, E., Aly, M.M., Arafa, A., Capua, I.** 2011. Antigenic drift in H5N1 avian influenza virus in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to those for human influenza virus. *J Virol* 85: 8718-8724.
2. **Connor, R.J., Kawaoka, Y., Webster, R.G., Paulson, J.C.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 205: 17-23.
3. **Desselberger, U., Racaniello, V.R., Zazra, J.J., Palese, P.** 1980. The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene* 8: 315-328.
4. **Grund, C., Abdelwhab, S.M., Arafa, A.S., Ziller, M., Hassan, M.K., Aly, M.M., Hafez, H.M., Harder, T.C., Beer, M.** 2011. Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. *Vaccine* 29: 5567-5573.

5. **Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G.**
2000. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci U S A* 97: 6108-6113.
6. **Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R.** 2001.
Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 146: 2275-2289.
7. **Hwang, S.D., Kim, H.S., Cho, S.W., Seo, S.H.** 2011. Single dose of oil-adjuvanted inactivated vaccine protects chickens from lethal infections of highly pathogenic H5N1 influenza virus. *Vaccine* 29: 2178-2186.
8. **Isoda, N., Sakoda, Y., Kishida, N., Soda, K., Sakabe, S., Sakamoto, R., Imamura, T., Sakaguchi, M., Sasaki, T., Kokumai, N., Ohgitani, T., Saijo, K., Sawata, A., Hagiwara, J., Lin, Z., Kida, H.** 2008. Potency of an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 reassortant virus generated between isolates from migratory ducks in Asia. *Arch Virol* 153: 1685-1692.
9. **Ito, T., Couceiro, J.N., Kelm, S., Baum, L.G., Krauss, S., Castrucci, M.R., Donatelli, I., Kida, H., Paulson, J.C., Webster, R.G., Kawaoka, Y.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol* 72: 7367-7373.

10. **Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R.G., Kida, H.** 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. *Arch Virol* 140: 1163-1172.
11. **Jiang, W.M., Liu, S., Chen, J., Hou, G.Y., Li, J.P., Cao, Y.F., Zhuang, Q.Y., Li, Y., Huang, B.X., Chen, J.M.** 2010. Molecular epidemiological surveys of H5 subtype highly pathogenic avian influenza viruses in poultry in China during 2007-2009. *J Gen Virol* 91: 2491-2496.
12. **Kajihara, M., Matsuno, K., Simulundu, E., Muramatsu, M., Noyori, O., Manzoor, R., Nakayama, E., Igarashi, M., Tomabechi, D., Yoshida, R., Okamatsu, M., Sakoda, Y., Ito, K., Kida, H., Takada, A.** 2011. An H5N1 highly pathogenic avian influenza virus that invaded Japan through waterfowl migration. *Jpn J Vet Res* 59: 89-100.
13. **Kida, H.** 2008. Ecology of influenza viruses in nature, birds, and humans. *Global Environ Res* 12: 9-14.
14. **Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K.F., Kawaoka, Y., Webster, R.G.** 1994. Potential for transmission of avian influenza viruses to pigs. *J Gen Virol* 75: 2183-2188.

15. **Kida, H., Kawaoka, Y., Naeve, C.W., Webster, R.G.** 1987. Antigenic and genetic conservation of H3 influenza virus in wild ducks. *Virology* 159: 109-119.
16. **Kida, H., Shortridge, K.F., Webster, R.G.** 1988. Origin of the hemagglutinin gene of H3N2 influenza viruses from pigs in China. *Virology* 162: 160-166.
17. **Kou, Z., Li, Y., Yin, Z., Guo, S., Wang, M., Gao, X., Li, P., Tang, L., Jiang, P., Luo, Z., Xin, Z., Ding, C., He, Y., Ren, Z., Cui, P., Zhao, H., Zhang, Z., Tang, S., Yan, B., Lei, F., Li, T.** 2009. The survey of H5N1 flu virus in wild birds in 14 Provinces of China from 2004 to 2007. *PLoS One* 4: e6926.
18. **Kida, H., Yanagawa, R., and Matsuoka, Y.** 1980. Duck influenza lacking evidence of disease signs and immune response. *Infect immun* 30:547-553.
19. **Maas, R., Tacken, M., van Zoelen, D., Oei, H.** 2009. Dose response effects of avian influenza (H7N7) vaccination of chickens: serology, clinical protection and reduction of virus excretion. *Vaccine* 27: 3592-3597.
20. **McCauley, J. W., Hongo, S., Kaverin, N. V., Kochs, G., Lamb, R. A., Matrosovich, M. N., Perez, D. R., Palese, P., Presti, R. M., Rimstad, E., and Smith, G. J. D.** 2012. *Orthomyxoviridae*, p.749-761 In Andrew, M. Q. King, Michael, J. Adams, Eric, B. Carstens, Elliot, J. Lefkowitz (ed.), *Virus Taxonomy*

(Classification and Nomenclature of Viruses) 9th Report of the International Committee on the Taxonomy of Viruses, Elsevier Inc.

21. **Nelli, R.K., Kuchipudi, S.V., White, G.A., Perez, B.B., Dunham, S.P., Chang, K.C.** 2010. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. *BMC Vet Res* 6, 4.
22. **OIE.** 2008. Avian influenza, p.465-481. Manual of Diagnostic Tests and Vaccines For Terrestrial Animals (mammals, birds, and bees), sixth edition, Volume 1
23. **Okamatsu, M., Nishi, T., Nomura, N., Yamamoto, N., Sakoda, Y., Sakurai, K., Chu, H. D., Thanh, L. P., Nguyen, L. V., Hoang, N. V., Tien, T. N., Yoshida, R., Takada, A., Kida, H.** 2013. The genetic and antigenic diversity of avian influenza viruses isolated from domestic ducks, muscovy ducks, and chickens in northern and southern Vietnam, 2010-2012. *Virus Genes* 47: 317-329.
24. **Okamatsu, M., Tanaka, T., Yamamoto, N., Sakoda, Y., Sasaki, T., Tsuda, Y., Isoda, N., Kokumai, N., Takada, A., Umemura, T., Kida, H.** 2010. Antigenic, genetic, and pathogenic characterization of H5N1 highly pathogenic avian influenza viruses isolated from dead whooper swans (*Cygnus cygnus*) found in northern Japan in 2008. *Virus Genes* 41: 351-357.

25. **Palese, P., Shaw, M. L.** 2007. *Orthomyxoviridae*, p.1647-1689. The viruses and their replication. Fields Virology, Fifth edition, Lippincott-Raven Publishers.
26. **Reed, L.J., Muench, H.** 1938. A simple method for estimating fifty percent endpoints. *Am J Hyg* 37: 493-497.
27. **Reid, A.H., Fanning, T.G., Hultin, J.V., Taubenberger, J.K.** 1999. Origin and evolution of the 1918 “Spanish” influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A* 96: 1651-1656.
28. **Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A., Wiley, D.C.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 304: 76-78.
29. **Sakoda, Y., Ito, H., Uchida, Y., Okamatsu, M., Yamamoto, N., Soda, K., Nomura, N., Kuribayashi, S., Shichinohe, S., Sunden, Y., Umemura, T., Usui, T., Ozaki, H., Yamaguchi, T., Murase, T., Ito, T., Saito, T., Takada, A., Kida, H.** 2012. Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in the 2010-2011 winter season in Japan. *J Gen Virol* 93: 541-550.
30. **Sasaki, T., Isoda, N., Soda, K., Sakamoto, R., Saijo, K., Hagiwara, J., Kokumai, N., Ohgitani, T., Imamura, T., Sawata, A., Lin, Z., Sakoda, Y., Kida, H.** 2009a. Evaluation of the potency, optimal antigen level and lasting

immunity of inactivated avian influenza vaccine prepared from H5N1 virus. *Jpn J Vet Res* 56: 189-198.

31. **Sasaki, T., Kokumai, N., Ohgitani, T., Sakamoto, R., Takikawa, N., Lin, Z., Okamatsu, M., Sakoda, Y., Kida, H.** 2009b. Long lasting immunity in chickens induced by a single shot of influenza vaccine prepared from inactivated non-pathogenic H5N1 virus particles against challenge with a highly pathogenic avian influenza virus. *Vaccine* 27: 5174-5177.
32. **Smith, G.J., Fan, X.H., Wang, J., Li, K.S., Qin, K., Zhang, J.X., Vijaykrishna, D., Cheung, C.L., Huang, K., Rayner, J.M., Peiris, J.S., Chen, H., Webster, R.G., Guan, Y.** 2006. Emergence and predominance of an H5N1 influenza variant in China. *Proc Natl Acad Sci U S A* 103: 16936-16941.
33. **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.** 2009. Characterization of avian influenza viruses A (H5N1) from wild birds, Hong Kong, 2004-2008. *Emerg Infect Dis* 15: 402-407.
34. **Soda, K., Ozaki, H., Sakoda, Y., Isoda, N., Haraguchi, Y., Sakabe, S., Kuboki, N., Kishida, N., Takada, A., Kida, H.** 2008a. Antigenic and genetic

analysis of H5 influenza viruses isolated from water birds for the purpose of vaccine use. *Arch Virol* 153: 2041-2048.

35. **Soda, K., Sakoda, Y., Isoda, N., Kajihara, M., Haraguchi, Y., Shibuya, H., Yoshida, H., Sasaki, T., Sakamoto, R., Saijo, K., Hagiwara, J., Kida, H.** 2008b. Development of vaccine strains of H5 and H7 influenza viruses. *Jpn J Vet Res* 55: 93-98.
36. **Totani, K., Kubota, T., Kuroda, T., Murata, T., Hidari, K.I., Suzuki, T., Suzuki, Y., Kobayashi, K., Ashida, H., Yamamoto, K., Usui, T.** 2003. Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses. *Glycobiology* 13: 315-326.
37. **Uchida, Y., Mase, M., Yoneda, K., Kimura, A., Obara, T., Kumagai, S., Saito, T., Yamamoto, Y., Nakamura, K., Tsukamoto, K., Yamaguchi, S.** 2008. Highly pathogenic avian influenza virus (H5N1) isolated from whooper swans, Japan. *Emerg Infect Dis* 14: 1427-1429.
38. **Van Poucke, S., Uhlenborff, J., Wang, Z., Billiau, V., Nicholls, J., Matrosovich, M., Van Reeth, K.** 2013. Effect of receptor specificity of A/Hong Kong/1/68 (H3N2) influenza virus variants on replication and transmission in pigs. *Influenza Other Respi Viruses* 7: 151-159.

39. **Van Poucke, S.G., Nicholls, J.M., Nauwynck, H.J., Van Reeth, K.** 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. *Virol J* 7, 38.
40. **Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y.** 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev* 56: 152-179.
41. **WHO/OIE/FAO H5N1 Evolution Working Group.** 2012. Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. *Influenza Other Respi Viruses* 6: 1-5.
42. **Xu, X., Subbarao, Cox, N.J., Guo, Y.** 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 261: 15-19.

和文要旨

インフルエンザウイルスのヘマグルチニン糖蛋白 (HA) の HA1 サブユニット上の複数のアミノ酸配列がレセプター特異性を決定している。鳥のウイルスはシアル酸 (SA) $\alpha 2,3$ ガラクトース (Gal) を、ヒトのウイルスは SA $\alpha 2,6$ Gal をレセプターとして認識し、結合する。ブタの呼吸器上皮細胞には SA $\alpha 2,3$ Gal と SA $\alpha 2,6$ Gal 両レセプターが存在する。1968 年に出現したパンデミックインフルエンザウイルス A/Hong Kong/68 (H3N2)株は、ヒトの H2N2 流行株と鳥の H3 ウイルスがブタに同時感染して生じた遺伝子再集合体である。その HA 遺伝子は鳥のウイルスに由来する。したがって、ブタに感染した当初の H3HA は SA $\alpha 2,3$ Gal をレセプターとする。A/Hong Kong/68 (H3N2)ウイルスの HA は SA $\alpha 2,6$ Gal を認識する。これまで SA $\alpha 2,6$ Gal を認識する鳥インフルエンザウイルスが出現するメカニズムは明らかになっていない。本研究では、鳥のウイルスがブタに感染し、受け継がれる間に SA $\alpha 2,6$ Gal レセプターを認識するウイルスが選択されるか否かを調べた。A/duck/Hokkaido/5/77 (H3N2)を 3 週齢のブタに経鼻接種した。7 日間毎日スワブを採取し、MDCK 細胞で回収したウイルスを分離培養した後、これを新たなブタに経鼻接種して、継代を重ねた。継代毎に回収したウイルスをブラッククロニングし、遺伝子の塩基配列を決定し、推定されるアミノ酸配列を決定した。さらに、配列の異なるウイルスクローンの糖

鎖結合特異性を調べた。継代前のウイルスは HA のレセプター特異性を決定する 226-227-228 番目のアミノ酸が Gln-Ser-Gly で SA α 2,3Gal 糖鎖に特異的に結合した。ブタ継代 2 代目の回収ウイルスに、Leu-Ser-Ser の配列を持つウイルスが含まれており、3 代目でこれが優勢になった。このウイルスは SA α 2,6Gal 糖鎖に特異的に結合した。すなわち、鳥インフルエンザウイルスがブタに感染を繰り返すことによってヒトにパンデミックを起こし得るウイルスが選択された。本成績は、これまでのパンデミックウイルスはブタで産生された遺伝子再集合体であるとの提案が正しいことを支持するものである。加えて、ブタインフルエンザのサーベイランスによりパンデミックウイルスの出現を予測し得ることを示している。

H5N1 高病原性鳥インフルエンザウイルスの感染がアジア、アフリカの家禽に広がっている。本研究は、当研究室のインフルエンザウイルスライブラリーから選抜した H5N1 非病原性鳥インフルエンザウイルス A/duck/Hokkaido/Vac-3/07 (H5N1) [Dk/Vac-3/07]株で試製したワクチンが近年アジアで流行している 3 つの異なる系統の抗原変異株の攻撃に対する防御免疫を誘導するか否かをニワトリで評価した。不活化した Dk/Vac-3/07 とオイルアジュバントを混合し試製した Vac-3 ワクチンを 4 週齢のニワトリに接種し、3 週後に A/muscovy duck/Vietnam/OIE-559/11 (H5N1) [Mdk/VN/11]、A/whooper swan/Hokkaido/4/11 (H5N1) [Ws/Hok/11]、または A/peregrine falcon/Hong

Kong/810/09 (H5N1) [Pf/HK/09]を鼻腔内に接種して攻撃した。無免疫のニワトリは攻撃後 2 から 4 日の間に全羽死亡した。Mdk/VN/11 または Ws/Hok/11 で攻撃したニワトリは臨床症状を示すことなく全羽生残した。Pf/HK/09 の攻撃後、臨床観察群のニワトリ 12 羽中、攻撃後 4 日目と 7 日目に 1 羽ずつ死亡し、10 羽が生残した。Vac-3 ワクチンより高い抗原量の Vac-3 conc. ワクチンを接種したニワトリを Pf/HK/09 で攻撃したところ、全羽が臨床症状を示すことなく生残した。

本研究の結果から、Dk/Vac-3/07 を用いて試製したワクチンは近年アジアで流行している抗原変異株に対する防御免疫をニワトリに誘導し、ウイルスの排出量を低減することが判った。試製ワクチンは緊急用備蓄ワクチンとして有効である。ワクチンの濫用によって、H5N1 高病原性鳥インフルエンザウイルスが免疫圧下で選択されたことにより、抗原性および遺伝子が多様になった。鳥インフルエンザ対策は摘発淘汰を基本とし、ワクチンを使用するとしても緊急時の補助的な手段としてのみ用いるべきである。