Original article

Hemagglutinin and neuraminidase of an H7N7 non-pathogenic avian influenza virus co-evolved during the acquisition of intranasal pathogenicity in chickens

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Abstract

Polybasic amino acid residues at the hemagglutinin (HA) cleavage site are insufficient to induce the highly pathogenic phenotype of avian influenza viruses in chickens. In our previous study, an H7N7 avian influenza virus named Vac2sub-P0, which is nonpathogenic despite carrying polybasic amino acids at the HA cleavage site, was passaged in chick air sacs, and a virus with high intravenous pathogenicity, Vac2sub-P3, was obtained. Intranasal infection with Vac2sub-P3 is only partially lethal in chickens; therefore, in this study, this virus was further passaged in chicken lungs, and the resultant virus, Vac2sub-P3L4, acquired high intranasal pathogenicity. Experimental infection of chickens with recombinant viruses demonstrated that mutations in HA and neuraminidase (NA) found in consecutive passages were responsible for increased pathogenicity. The HA and NA functions of Vac2sub-P3L4 were compared with the parental virus *in vitro*; the virus growth at 40 °C was higher, the binding affinity to a sialic acid receptor was lower, and the release activity by NA from the cell surface was lower, suggesting that these changes enabled the virus to replicate efficiently in chickens with high intranasal pathogenicity. This study demonstrates that intranasally highly pathogenic viruses require additional adaptations for increased pathogenicity to be highly lethal to intranasally infected chickens.

KEYWORDS: High pathogenicity avian influenza virus, Intranasal pathogenicity, Chicken, Hemagglutinin, Neuraminidase

Introduction

The natural hosts of influenza A viruses are wild waterbirds, particularly migratory ducks [10, 17, 39]. Previous studies revealed that duck influenza viruses are transmitted to chickens via domestic waterfowl or terrestrial birds [19, 24]. With multiple repeated infections in a chicken population, high pathogenicity avian influenza viruses (HPAIVs) can only be selected from H5 or H7 viruses [1, 16, 27]. The hemagglutinin (HA) of low pathogenicity avian influenza viruses (LPAIVs) is cleaved only by trypsin-like serine proteases expressed in the respiratory or intestinal epithelia, leading to mild or asymptomatic local infection. In contrast, the HA of HPAIV has polybasic amino acid residues at its cleavage site, which permit ubiquitous proteases such as furin and PC6 to cleave the precursor HA0 into HA1 and HA2 subunits, causing severe systemic infection in chickens [29]. Polybasic amino acids are a prerequisite for fatal pathogenicity in chickens. In contrast, previous investigations have demonstrated that LPAIVs did not show high pathogenicity after the artificial introduction of a polybasic amino acid motif at the HA cleavage site [3, 20, 32, 35, 38]. These results strongly suggested that additional viral factors are required for LPAIVs to acquire high pathogenicity in chickens. For example, a virus with three characteristics–filamentous form, high polymerase activity, and high stability of the matrix (M1) protein–was selected during the passage of a genetically modified H5N1 virus in chickens [42].

In our previous study, a genetically modified H7N7 duck influenza virus was designed by artificially introducing basic amino acid residues KRRRRR at the HA cleavage site of an H7N7 LPAIV, A/duck/Hokkaido/Vac-2/2004 (Vac2), namely, the Vac2sub-P0 strain [20]. After consecutive passages of Vac2sub-P0 in chicks, the resultant virus Vac2sub-P3 acquired high intravenous pathogenicity (Intravenous Pathogenicity Index: IVPI=2.54). Based on experimental infections of chickens with mutant viruses generated by site-directed mutagenesis and reverse genetics, amino acid substitutions at two positions, E227G and I388T, in the HA of Vac2sub-P3 are essential for its high pathogenicity in chickens. In contrast to intravenous infection, intranasal inoculation with Vac2sub-P3 resulted in limited viral replication in the organs and mortality in chickens. Discrepancies may arise in the pathogenicity of the intranasal and intravenous inoculation routes. In

the present study, Vac2sub-P3, which showed high pathogenicity via intravenous inoculation, but partial pathogenicity via intranasal inoculation, was serially passaged in the lungs of chickens to acquire higher intranasal pathogenicity. We obtained a virus with pathogenicity comparable to field HPAIV isolates. We characterized the virus to demonstrate the functional changes in viral proteins during the acquisition of intravenous and intranasal pathogenicity in chickens.

Materials and methods

Viruses

The viruses used in this study were obtained after three passages in chick air sacs (Vac2sub-P3) using the A/duck/Hokkaido/Vac-2/2004 (H7N7) strain, which introduced the amino acid residue KRRRRR at the HA cleavage site (rgVac2sub-P0), as described previously [20]. All viruses were propagated in 10-day-old embryonated chicken eggs for 30–48 h at 35 °C.

Cells

Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM, Nissui, Tokyo, Japan) supplemented with 0.3 mg/mL L-glutamine (FUJIFILM Wako Pure Chemical, Osaka, Japan), 5% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin G (Meiji Seika Pharma, Tokyo, Japan), 0.1 g/mL streptomycin (Meiji Seika Pharma), and 8.0 μg/mL gentamicin (MSD, Rahway, NJ, USA). Chicken embryonic fibroblasts (CEFs) were prepared from 10-day-old chicken embryos and cultured in MEM supplemented with L-glutamine, fetal bovine serum, and antibiotics.

Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were removed by washing the cells with MEM. Cells were then overlaid with

MEM containing 0.7% Bacto-agar (Becton Dickinson, Franklin Lakes, NJ, USA) in the presence of 5 μg/mL trypsin acetylated (Sigma-Aldrich, St. Louis, MO, USA). After 48 h of incubation at 35 °C, cells were stained with 0.005% neutral red (Sigma-Aldrich).

Consecutive passages of Vac2sub-P3 in chickens

Four-week-old chickens (Boris Brown) free from antibodies against the H7 influenza virus, which was confirmed by the hemagglutination inhibition (HI) test against the Vac2 strain, were obtained from Hokkaido Chuo Shukeijou, Hokkaido, Japan. Three chickens were intranasally inoculated with 100 μL of Vac2sub-P3. Three days post-inoculation (dpi), the chickens were sacrificed, and their lungs were collected. A pooled 10% tissue suspension of infected organs was serially passaged intranasally into three 4-week-old chickens. After four passages, the isolate named Vac2sub-P3L4 ("L" indicates lungs) was obtained. Resultant viruses were propagated in the allantoic cavities of 10-day-old embryonated eggs for 48 h at 35 °C.

Experimental infection of chickens with mutant viruses

Four-week-old chickens were used to test the pathogenicity of the viruses used in this study. Six chickens were intranasally inoculated with 100 μL of allantoic fluid containing each virus at $10^{5.0}$ plaque-forming units (PFU) and observed for 14 days. To study viral replication, three additional chickens were inoculated with each virus, as described above and euthanized at 3 dpi, after which tissue and blood were collected aseptically. To prepare a 10% suspension in MEM, the tissue samples were homogenized using a multi-bead shocker (Yasui Kikai, Osaka, Japan). The viral titers of these suspensions were calculated using a plaque assay and expressed as PFU/g of tissue or mL of blood. All experiments were performed in self-containing isolator units (Tokiwa Kagaku, Tokyo, Japan) in a biosafety level 3 (BSL3) facility at the Faculty of Veterinary Medicine, Hokkaido University, Japan.

Reverse genetics

Mutations were introduced into specific regions of the plasmid-encoded PA, HA, NA, M, and NS genes of Vac2sub-P3 using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) as previously described [32]. The mutant viruses listed in Fig. 1A were rescued by reverse genetics [12], and all eight segments of the genome were sequenced to confirm that the desired mutations were induced and the undesired ones were eliminated. Co-cultures of human embryonic kidney 293T and MDCK cells were transfected with plasmids using 2 mg/mL polyethyleneimine (Polysciences, Warrington, PA, USA). At 48 h post-transfection, the culture supernatant was collected and propagated in 10-day-old embryonated chicken eggs.

Three-dimensional (3D) structural-based mapping of the HA protein

Mutations were mapped to the H7 HA protein (Protein Data Bank accession 1TI8 [26]) using UCSF Chimera X version 1.2 (University of California, San Francisco, CA, USA). Monomeric HA is depicted as a soft-surface model and ribbon diagram.

Virus growth in cell culture

Viruses at a multiplicity of infection (M.O.I.) of 0.01 were inoculated onto confluent monolayers of CEF cells and incubated at 35 °C for 1 h. Unbound viruses were removed by washing the cells with MEM, and the cells were overlaid with MEM in the presence of trypsin accetylated (5 μg/mL). The plates were incubated at 35 or 40 °C under 5% CO_2. At various times post-infection, viral titers in the cell culture supernatant were determined using a plaque assay with MDCK cells.

Solid-phase direct binding assay

The viral receptor binding affinity was assessed using a solid-phase direct binding assay with the sialylglycopolymer 3′-Sialyllactose-PAA (3′-SL) (Cosmo Bio, Tokyo, Japan), as described previously [30]. 3′-

SL was added to each well of a Universal-BIND™, 96-well polystyrene stripwell microplate (Corning, Corning, NY, USA). After blocking the wells with 2% bovine serum albumin, a solution containing influenza viruses (32 HA units) was added and incubated at 4 °C for 16 h. Mouse anti-H7 HA monoclonal antibody 253/1 [28] and goat anti-mouse IgG-HRP conjugate (Bio-Rad, Hercules, CA, USA) were used to detect the viruses. 3,3′-tetramethylbenzidine (TMB) and 0.04% H_2O_2 were added, and the absorbance was measured at 450/630 nm using a Model 680 Microplate Reader (Bio-Rad).

The affinity constant for each virus was calculated as previously described [25]. Blank-corrected data were plotted and fitted to an established binding model using R version 3.0.1 (The R Foundation for Statistical Computing, Vienna, Austria). The formula for this model is as follows:

$$\text{absorbance} = \frac{A_{max} \times C}{Kd + C}$$

Where C is the concentration of 3′-SL, Amax is the maximum binding absorbance, and Kd is the dissociation constant. The affinity constant (Ka) determines the reciprocal of Kd.

Erythrocyte elution assay

The function of NA was evaluated via ability of viruses to elute agglutinated chicken erythrocytes as previously described [5]. Briefly, viruses containing 128 HA units were serially diluted in calcium saline buffer (6.8 mM $CaCl_2$ - 154 mM NaCl in 20 mM borate buffer, pH7.2) and incubated with 50 µL of 0.5% chicken erythrocytes in microtiter plates at 4 °C for 1 h. The plates were then stored at 37 °C, and the reduction of HA titers was monitored periodically for 10 h.

NA enzymatic activity

NA enzymatic activity was measured using 2′-(4-Methylumbelliferyl)–α–D–*N*-acetylneuraminic acid (MUNANA; Sigma-Aldrich) as a soluble substrate, as described previously [7]. Briefly, viruses were mixed with serially diluted MUNANA in 96-well black-bottomed plates. The mixture was subjected to exertion at 360 nm

at 1-min intervals, and the emission signal was periodically monitored for 30 min at 37 °C using POWER SCAN 4 (Agilent Technologies). The initial reaction rate under each condition was calculated and plotted using R software to obtain the Michaelis constant (Km).

Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers: 13-0108, 13-0162, 18-0037, 18-0040). All experiments involving genetically modified organisms, including mutant viruses generated by reverse genetics, were authorized by the Safety Committee on Genetic Recombination Experiments of Hokkaido University (approval numbers: 2015-019, 2020-009) and the Ministry of Education, Culture, Sports, Science, and Technology, Japan (30-963, 2-654). This study was approved by the Biosafety Management Committee on Pathogens and Other Hazardous Agents of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers: 2017-1-61, 2018-1-59, and 2019-1-62).

Results

Amino acid changes of the viruses during consecutive passages of Vac2sub-P3 in chickens

The Vac2sub-P3 virus was obtained from a previous study and was used for additional consecutive passages. Each isolate obtained from the lungs was tested for pathogenicity. After four passages, the resultant virus Vac2sub-P3L4 showed high intranasal pathogenicity in chickens; all six chickens intranasally inoculated with the virus died within 5 days. The sequences of the resultant viruses were compared to identify amino acid substitutions (Table 1). Seven amino acid substitutions (S409I in PA, T32I and R65K in HA, L10S in NA, R101K in M1, D44G in M2, and E229K in NS1) and one deletion (at positions 40–73 in NA) were observed between Vac2sub-P3 and Vac2sub-P3L4. Among the six substitutions (K123E in PB2, N16D in PB1, E227G, and I388T in HA, G228R in M1, and L46P in M2) between Vac2sub-P0 and Vac2sub-P3, G228R in M1 and L46P in M2

were reverted during additional passages to Vac2sub-P3L4.

Intranasal pathogenicity of rgVac2sub-P0, rgVac2sub-P3, rgVac2sub-P3L4, and mutant viruses in chickens

To investigate the contribution of each mutation to the high intranasal pathogenicity of the resultant virus, we generated recombinant viruses using reverse genetics and compared their pathogenicity with those of Vac2sub-P0, Vac2sub-P3, and Vac2sub-P3L4 (Fig. 1B). Consistent with a previous report [17], all chickens that were intranasally inoculated with rgVac2sub-P0 survived. Two of the six chickens inoculated with rgVac2sub-P3 died during the observation period. All chickens inoculated with rgVac2sub-P3L4 died within four days. A previous study demonstrated that two mutations in HA, E227G, and I388T, are critical for the acquisition of intravenous pathogenicity via Vac2sub-P3 [20]. Accordingly, we first focused on the contribution of these mutations to HA segments. The virus rgP3L4/P0-HA, which has a Vac2sub-P0 HA segment with a Vac2sub-P3L4 backbone, did not kill any inoculated chickens. These results highlighted the importance of these four HA mutations in acquiring pathogenicity throughout consecutive passages. Moreover, infection with rgP3L4/P3-HA resulted in significantly delayed mortality compared with infection with rgVac2sub-P3L4, suggesting that two additional mutations in HA, T32I and R65K, also contribute to the acquisition of intranasal pathogenicity.

The functional balance between HA and NA is important for viral replication in cultured cells and pathogenesis in animals [2, 22, 31, 40]. Therefore, we focused on mutations observed in the NA. For this purpose, rgP3L4/P0-NA, harboring the NA of Vac2sub-P0 with a Vac2sub-P3L4 backbone, was generated. Similar to rgP3L4/P3-HA, infection with rgP3L4/P0-NA resulted in significantly delayed mortality compared to infection with rgVac2sub-P3L4. However, infection with rgP0/P3L4-HA,NA, which possesses HA and NA derived from Vac2sub-P3L4 with the Vac2sub-P0 backbone, resulted in a 2/3 mortality rate in infected chickens, indicating a significant contribution of mutations in other gene segments.

We euthanized the infected chickens at 3 dpi to evaluate viral growth in chickens and collected organ

and blood samples to determine viral titers (Table 2). No viruses were detected in chickens inoculated with rgVac2sub-P0 or rgP3L4/P0-HA, whereas rgVac2sub-P3L4 was systemically recovered from chickens at high titers. Viral titers recovered from chickens inoculated with rgP3L4/P3-HA or rgP3L4/P0-NA were lower than those recovered from chickens inoculated with rgVac2sub-P3L4. No virus was recovered from one of the three chickens inoculated with rgP0/P3L4-HA or NA, consistent with their mortality rates and delayed mortality (Fig. 1B).

Phenotypical analyses of the HA of resultant viruses

In vivo analyses suggested that functional changes in HA and NA are crucial for acquiring intranasal pathogenicity of the resultant viruses in chickens. We visualized the mutations observed in the 3D structure of HA (Fig. 2A). Amino acid position 227 is located in the peripheral region of the receptor binding site. Position 388 is located in the alpha helix of the HA2 subunit. Position 32 is located in the N-terminal region of the HA1 subunit and near the HA1/2 cleavage site in the 3D structure. Position 65 is located in the hinge domain between the globular head and the stem of the HA1 subunit.

Notably, mutations at positions 227 and 388 were observed during the acquisition of intravenous pathogenicity in chickens in our previous study [20]. Position 227 faces opposite to the receptor-binding pocket. Accordingly, we hypothesized that this mutation would affect receptor-binding avidity. Then, binding analyses of rgVac2sub-P0, rgVac2sub-P3L4, and rgP0/HA-227G with 3′-SL were conducted to calculate the affinity constant (Ka) of each virus with the receptor analog. 3′-SL is a sialylglycopolymer terminating with sialic acid α2,3 galactose and a model of the receptor for avian influenza viruses (Fig. 2B). The Ka values of rgVac2sub-P3L4 and rgP0/HA-227G were significantly lower than that of rgVac2sub-P0 (Fig. 2C). This result indicated that the HA-E227G mutation attenuated receptor-binding avidity.

Mutations in the helix of the HA2 stem affect the thermostability of the HA molecule [15]. The growth of these viruses at different temperatures was assessed using CEFs. Although a few differences were observed

between the viruses at 35 °C (Fig. 2D), the virus titer of rgVac2sub-P3L4 and rgP0/HA-388T was significantly higher than that of rgVac2sub-P0 and rgP0/HA-227G at 40 °C (Fig. 2E). These results indicate that viruses carrying HA-388T have a higher capacity to grow at high temperatures, such as in chicken bodies.

Phenotypical analyses of the NA of resultant viruses

HA and NA functionally co-evolve during viral adaptation to novel host species. Deletion of the NA stalk domain is one of the most well-characterized changes in molecular coevolution. To examine the effects of the NA stalk deletion on its function, the elution abilities from chicken erythrocytes of rgVac2sub-P0, rgVac2sub-P3L4, and rgP0/NAΔ40-73 (the mutant virus with the 34 amino acid truncation at the NA stalk with the Vac2sub-P0 backbone) were assessed using an erythrocyte elution assay (Fig. 3A). The rgVac2sub-P0 virus was eluted over time, whereas rgVac2sub-P3L4 and rgP0/NAΔ40-73 with shortened NA stalks, showed little or no elution. The sialidase activities of rgVac2sub-P0 and rgVac2sub-P3L4 were tested in a standard assay using MUNANA as a soluble substrate. As expected, no significant difference was observed in the sialidase activity between these viruses (Fig. 3B), indicating that NA stalk deletion does not affect the removal of sialic acid from a soluble substrate [8].

Discussion

The sequence of polybasic amino acids at the HA cleavage site permits ubiquitous proteases to cleave HA, which is necessary to induce severe systemic infections [9, 16, 32]. Some viral factors are also required for pathogenicity in chickens [11, 36, 38, 42]. In addition, this study demonstrates that intranasally highly pathogenic viruses require additional adaptations for increased pathogenicity to be highly lethal to intranasally infected chickens. In this study, we investigated the characteristics of passaged H7N7 avian influenza viruses in a laboratory setting to determine the key factors responsible for their high intranasal pathogenicity.

The receptor-binding analysis demonstrated that viruses with HA-E227G showed lower glycan-

binding avidity. Our previous study showed that this mutation, located near the receptor-binding domain, critically contributes to the acquisition of intravenous pathogenicity in chickens [20]. Soon after the additional passage of rgVac2sub-P3 into the respiratory tract of chickens, a 34 amino acid deletion was observed in the NA stalk between Vac2sub-P3 and Vac2sub-P3L1. Such deletions are often detected in avian influenza viruses isolated from terrestrial poultry, such as chickens and quail, but not in aquatic birds [1, 18, 21, 34]. The NA stalk deletion facilitates the adaptation of avian influenza viruses to terrestrial poultry [4, 11, 41] and increases their pathogenicity in the hosts [23, 43]. Given that the virus used in this study was derived from the duck influenza virus [a reassortant strain between A/duck/Mongolia/736/2002 (H7N7) and A/duck/Hokkaido/49/1998 (H9N2)] [33], NA stalk deletion following the present passages is consistent with previous findings. Viruses with shortened NA stalks, such as rgVac2sub-P3L4 and rgP0/NAΔ40-73, showed little or no elution from chicken erythrocytes, in contrast to rgVac2sub-P0, indicating that the release activity of the resultant virus from the infected cells was lower than that of the parent virus. Several studies have suggested that balancing HA and NA functions is important for viral replication in cultured cells and experimental animals [2, 22, 31, 40].

Accordingly, this selection may occur to adjust its function to optimize the balance with the HA function, which decreases the receptor-binding affinity during previous passages by Vac2sub-P3. However, the influence of a shortened NA stalk region on the adaptation of aquatic birds to terrestrial poultry remains unclear. We hypothesized that the difference in tissue tropism between water birds and terrestrial poultry could be important; viruses infect and replicate in the colon of water birds and in the respiratory tract of terrestrial poultry, respectively. The basis of this hypothesis is that no NA stalk deletion was observed during other consecutive passages of Vac2sub-P3, in which the brain was used as a pooled sample in parallel (data not shown), and viruses with shortened NA stalks replicated efficiently in the respiratory tract of chickens [23, 34]. Viruses with low binding affinities and release activities may have advantages in replicating ciliated epithelial cells in the respiratory tract.

The ancestral strain of H5 HPAIVs, A/goose/Guangdong/1/1996 (H5N1) (Gs/GD), had no deletion in

its NA stalk domain, whereas the N1 NA of subsequent Gs/GD-like strains had 19 or 20 amino acid deletions in the stalk domain after the outbreak in Hong Kong in 1997. Later, descendants of the Gs/GD strain belonging to the HA genetic clade 2.3.4.4 underwent reassortment with viruses of various NA subtypes, resulting in the generation of H5Nx viruses. Among these, the N6 NA of H5N6 viruses contained an 11 amino acid deletion in the stalk region, whereas no deletion was observed in the N8 NA of H5N8 viruses. Furthermore, contemporary H5N1 viruses belonging to the HA genetic clade 2.3.4.4b obtained an N1 NA without deletion in the NA-stalk domain by reassortment with non-pathogenic avian influenza viruses circulating among wild birds. Interestingly, these viruses show lethal pathogenicity in chickens in the field, although strain-by-strain differences exist in their pathogenicity [37]. This suggests that HPAIVs without deletions in the NA stalk are highly pathogenic in field settings. In contrast, H5Nx viruses show altered receptor-binding specificity; these viruses recognize fucosylated receptors in addition to non-modified receptors [12]. Changes in the receptor-binding specificity of these viruses may be related to their potential to accept various NAs with or without stalk deletion.

Viruses harboring HA-I388T (rgVac2sub-P3L4 and rgP0/HA-388T) exhibited significantly higher growth and thermostability in CEFs. Mutations in the stem region of HA are often observed during experimental adaptation to influenza viruses and are associated with a prior mutation in the (vicinity of) receptor-binding domain. The most well-known mutation is the T318I substitution in HA, which was observed during the selection of airborne transmissible HPAIV in ferrets and increased the acid stability of HA [15]. It remains unclear whether these variants are selected to compensate for the instability of HA caused by the mutation in the receptor-binding domain or whether the phenotypic changes caused by the HA stem mutation are essential for adaptation. Since rgP0/HA-227G showed almost similar growth to rgVac2sub-P0 at both 35 and 40 °C, the mutation at the HA receptor-binding domain did not result in lowered HA stability. Accordingly, the higher growth at 40 °C might be one of the important biomarkers associated with the virus pathogenicity in chickens. In addition, amino acid T32 contains an *N*-glycosylation sequon, and the mutation T32I, which was observed after P3L3, abrogated glycosylation at N30. However, we did not obtain critical evidence to support glycosylation at N30 in P0-HA or

non-glycosylation in P3L4-HA (data not shown). Perhaps minor changes in glycan occupancy at this site may have contributed to the further adaptation of Vac2sub-P3L4 from Vac2sub-P3.

The results of the experimental infections also clearly demonstrated the contribution of mutations in internal genes. As previously demonstrated by Maruyama et al., mutations PB2-K123E and PB1-N16D significantly contribute to the acquisition of intravenous pathogenicity of Vac2sub-P3 in chickens, although the detailed mechanism is unclear [20]. M2-L46P is also involved in acquiring intravenous pathogenicity of Vac2sub-P3 in chickens. Interestingly, this was reverted during additional passages, and the M2-D44G mutation was detected soon after. Since amino acid positions 44 and 46 are in the vicinity of the amphipathic helix of the M2 protein [44], these mutations may have a similar effect on protein function. Position 229 of the NS1 protein is located in the PDZ domain [13]. The NS1-E229K mutation collapsed the consensus sequence of the PDZ domain ligands (ESEV and EPEV). The function of the NS1 PDZ domain is often discussed in the context of the pathogenesis of avian influenza viruses against mammals. However, its effects on viral pathogenesis in avian species have not been clarified. Unfortunately, we could not find any studies on the roles of PA-S409I and M1-R101K [6]. These mutations synergistically or additively increase the pathogenicity of the resulting viruses in chickens.

In conclusion, our study revealed that HA and NA underwent functional co-evolution while acquiring high intranasal pathogenicity in chickens. Phenotypic changes in these proteins and the polybasic amino acid residues at the HA cleavage site are critical for this process. We introduced polybasic amino acid residues at the HA cleavage sites to accelerate the adaptation process in the laboratory, and consecutive passages of the virus enhanced its growth capacity in chickens. The processes are vice versa; in the natural setting, the virus first acquires the ability to grow and be transmitted efficiently in chickens, and then the virus acquires the polybasic amino acid residues in the HA cleavage sites during the multiple transmission events in chicken flocks. Further studies are needed to elucidate the definitive factors responsible for the differences between the high pathogenicity of HPAIVs caused by intravenous and intranasal inoculations in chickens.

Acknowledgments

We thank Ms. Yuka Sato and Ms. Mayumi Endo for their technical assistance.

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Statements and Declarations

Funding

Funding: This work was supported by the Japan Initiative for World-Leading Vaccine Research and Development Centers [Grant No. JP233fa627005]. This study was partially funded by the Japan International Cooperation Agency under the Science and Technology Research Partnership for Sustainable Development [Grant No. JP23jm0110019]. This study was partially supported by the Japanese Initiative for the Progress of Research on Infectious Diseases for Global Epidemics (J-PRIDE) (grant no. JP18fm0208026), and Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) (Grant No. JP18fm0108008) of the Japan Agency for Medical Research and Development (AMED). This study was partially supported by the Japan Science and Technology Agency (Grant No. JPMJSP2119); the World-Leading Innovative and Smart Education Program (1801) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and the Japan Society for the Promotion of Science KAKENHI (Grant No. JP23KJ0059).

Competing interests

The authors declare that they have no competing interests.

Author contributions

Conceptualization: TI, MO, TH, HK, and YS; methodology: TI, MO, and TH; formal analysis: TI, MO, TH, and KM; investigation: TI, MO, TH, JM, DK, and YS; data curation: MO, TH, and KM; writing and original draft preparation: TI and MO. TH: Writing, review, and editing, all authors; supervision, MO, HK, and YS; funding acquisition, MO and HK. All the authors have read and agreed to the published version of this manuscript.

Data availability statement

All data are available within the manuscript.

Ethics approval

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers: 13-0108, 13-0162, 18-0037, 18-0040). All experiments involving genetically modified organisms, including mutant viruses generated by reverse genetics, were authorized by the Safety Committee on Genetic Recombination Experiments of Hokkaido University (approval numbers: 2015-019, 2020-009), and the Ministry of Education, Culture, Sports, Science, and Technology, Japan (30-963, 2-654). This study was approved by the Biosafety Management Committee on Pathogens and Other Hazardous Agents of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers: 2017-1-61, 2018-1-59, and 2019-1-62).

505 **Figure Legends**

506 Fig. 1. Survival of chickens intranasally inoculated with each virus.

507 (A) rgVac2sub-P0, rgVac2sub-P3, rgVac2sub-P3L4, and hemagglutinin/neuraminidase (HA/NA) reassortant
508 viruses were generated using reverse genetics. Open boxes indicate gene segments derived from rgVac2sub-P0,
509 shaded boxes indicate those from rgVac2sub-P3, and closed boxes indicate those from rgVac2sub-P3L4. (B) Six
510 4-week-old chickens were intranasally inoculated with 100 μL of each virus at $10^{5.0}$ plague forming units (PFU)
511 and observed for 14 days. ** indicates $p < 0.01$, * indicates $p < 0.05$ in the comparison of rgVac2sub-P3L4 using
512 a log-rank test.

513

514 Fig. 2. Evaluation of the NA function of each virus.

515 (A) The positions of four substitutions were mapped on the 3D structure of the H7 HA (Protein Data Bank
516 accession 1TI8). The HA was depicted in the soft surface model (left panel) and ribbon diagram (right panel).
517 HA1 was colored blue, and HA2 was colored red in the surface model, whereas the protein Cα-chain was colored
518 dim gray in the ribbon diagram. Mutations were colored lime in both models. (B), (C) The binding of rgVac2sub-
519 P0, rgVac2sub-P3L4, and rgP0/HA-227G to the receptor was investigated using a solid-phase direct binding
520 assay to a sialylglycopolymer containing 3′-Sialyllactose-PAA (3′-SL), SAcα2-3Galβ1-4Glc (B). The binding
521 affinity constant (Ka) of each virus to 3′-SL was calculated and represented as the mean ±SEM of triplicate
522 experiments. * indicates $p < 0.05$ in the comparison of rgVac2sub-P0 (C). (D), (E) Chicken embryonic fibroblasts
523 (CEFs) were inoculated with rgVac2sub-P0, rgVac2sub-P3L4, rgP0/HA-227G, and rgP0/HA-338T at M.O.I of
524 0.01 at 35 °C (D) or 40 °C (E). The data is represented as the mean ± SEM of triplicate experiments. a: $p < 0.05$
525 between rgVac2sub-P0 and rgVac2sub-P3L4, b: $p < 0.05$ between rgVac2sub-P0 and rgP0/HA-388T.

526

527 Fig. 3. Evaluation of the NA function of each virus.

528 (A) The abilities of the NAs of rgVac2sub-P0 with a long NA stalk and those of rgVac2sub-P3L4 and rgP0/NA

529 Δ40–73 with short NA stalks were assessed using the erythrocyte elution assay. Viruses containing HA titers of
530 1:128 were incubated with chicken erythrocytes at 4 °C for 1 h and then incubated at 37 °C. The HA titers were
531 monitored periodically for 10 h. (B) Sialidase activity of rgVac2sub-P0 (open circles) and rgVac2sub-P3L4
532 (closed circles) to MUNANA was measured. The initial reaction rate at each condition was calculated and plotted
533 to obtain the Michaelis constant, Km. The data are presented as the mean ± SEM of three independent
534 experiments.
535

Table 1

Amino acid changes during consecutive passages of Vac2sub-P0

Virus		PB2	PB1	PA	HA				NA		M1		M2		NS1
		123[a]	16	409	32	65	227	388	10	40-73	101	228	44	46	229
Vac2sub-	P0	K	N	S	T	R	E	I	L	∗[b]	R	G	D	L	E
	P3	E	D	・[c]	・	・	G	T	・	∗	・	R	・	P	・
	P3L1	E	D	・	・	・	G	T	S	Δ[d]	・	・	D/G[e]	・	・
	P3L2	E	D	・	・	・	G	T	S	Δ	・	・	G	・	・
	P3L3	E	D	・	T/I	・	G	T	S	Δ	・	・	G	・	・
	P3L4	E	D	I	I	K	G	T	S	Δ	K	・	G	・	K

[a] Methionine encoded by the AUG start codon is defined as position 1

[b] Asterisk indicates amino acids sequence GPKQKENLTCTTINQNNTTVVENTYVNNTTIITK

[c] Periods indicate same amino acids as the parent virus

[d] Delta (Δ) indicates the deletion of 40 to 73 amino acid residues in NA

[e] Amino acid quasispecies were observed

Amino acid diffenrence between Vac2sub-P0 and Vac2sub-P3 were already published by Maruyama et al., [18]

Table 2

Virus recovery from the chickens intranasally inoculated with each virus on 3 dpi

Virus	Chicken ID	Virus recovery (log PFU/g)						
		Brain	Trachea	Lung	Liver	Kidney	Colon	Blood[a]
rgVac2sub-P0	1	-[b]	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
rgVac2sub-P3	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-
rgVac2sub-P3L4	7	6.7	5.0	4.7	2.8	5.3	4.3	3.0
	8	7.7	6.3	6.7	4.3	7.2	5.4	4.2
	9	7.7	5.6	5.2	3.2	6.6	4.3	3.5
rgP3L4/P0-HA	10	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-
rgP3L4/P3-HA	13	4.6	4.3	4.1	2.9	4.1	3.5	3.1
	14	6.3	3.4	3,0	2.3	4.4	-	3.0
	15	4.6	3.8	3.2	2.3	3.7	2.3	-
rgP3L4/P0-NA	16	3.4	3.8	3.0	-	3.9	2.5	-
	17	6.0	4.3	4.3	-	5.6	2.3	-
	18	6.8	4.2	4.6	2.3	6.0	3.0	-
rgP0/P3L4-HA,NA	19	6.6	5.3	4.5	3.3	5.1	3.7	3.0
	20	-	-	-	-	-	-	-
	21	5.3	4.4	4.1	3.9	5.5	4.5	3.0

[a] log PFU/ml

[b] < 2.0 (< 1.3 for blood samples)

A

Virus	Viral proteins									
	PB2	PB1	PA	HA	NP	NA	M1	M2	NS1	NS2
rgVac2sub-P0	123 K	16 N	409 S	32 65 227 388 TR E I		10 L	101 228 R G	44 46 D L	229 E	
rgVac2sub-P3	123 E	16 D		227 388 G T			228 R	46 P		
rgVac2sub-P3L4	123 E	16 D	409 I	32 65 227 388 I K G T		10 Δ40-73 S	101 K	44 G	229 K	
rgP3L4/P0-HA										
rgP3L4/P3-HA										
rgP3L4/P0-NA										
rgP0/P3L4-HA,NA										

B

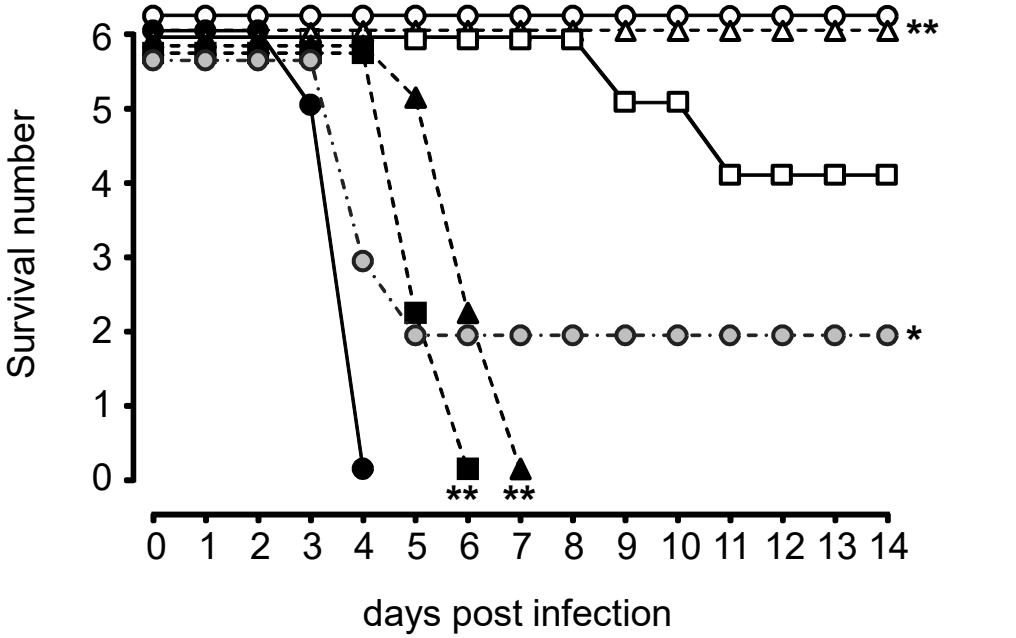


Fig. 1

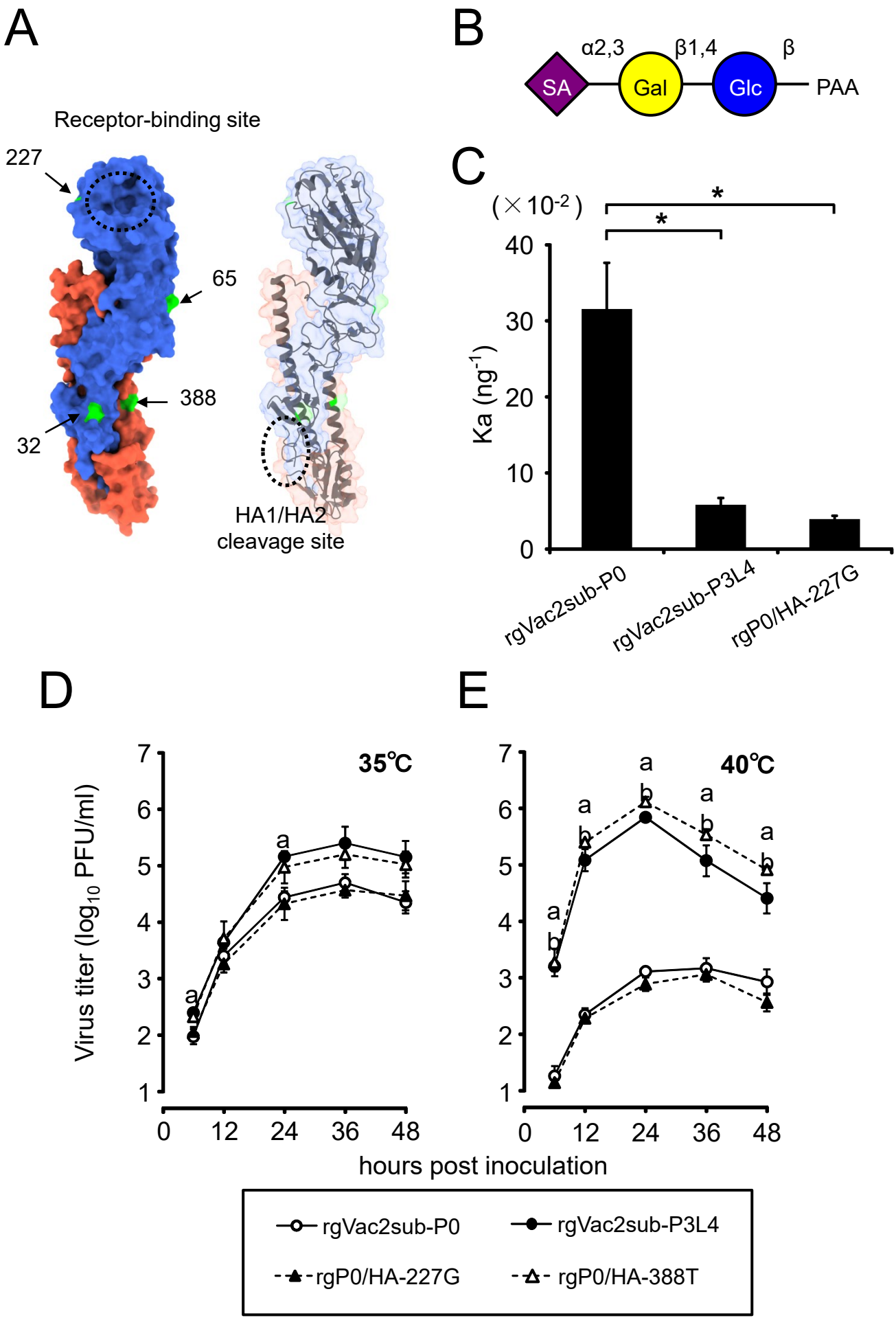
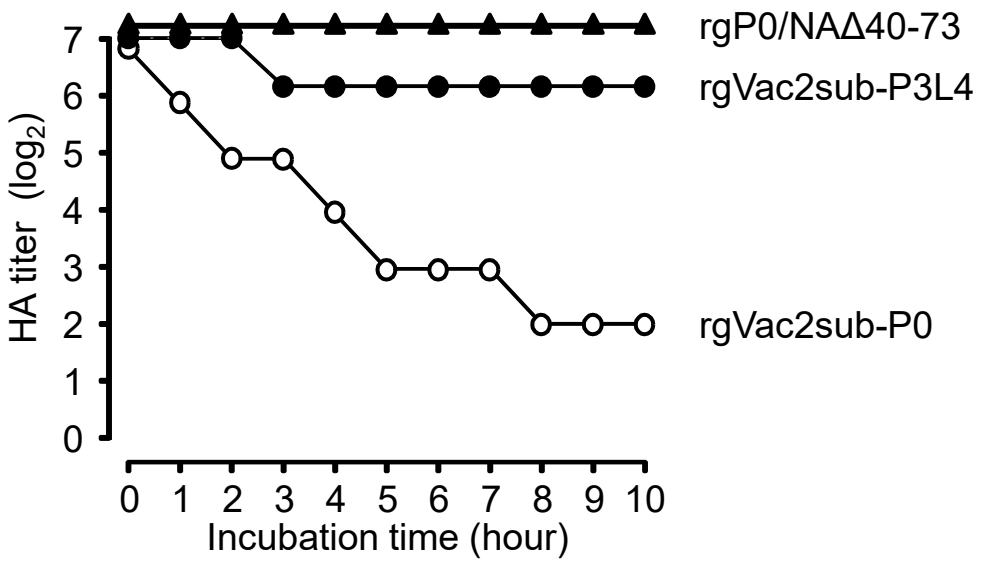
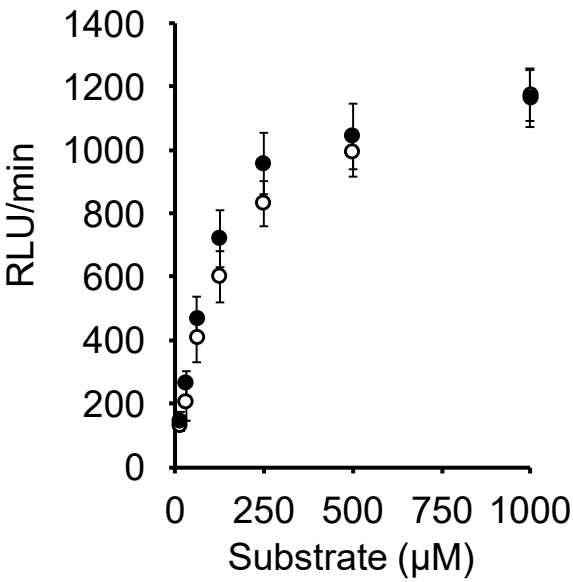

A
Receptor-binding site
227
65
388
32
HA1/HA2
cleavage site
B
α2,3
β1,4
β
SA
Gal
Glc
PAA
C
(×10-2)
*
*
40
30
20
10
0
Ka (ng-1)
rgVac2sub-P0
rgVac2sub-P3L4
rgP0/HA-227G
D
35℃
Virus titer (log10 PFU/ml)
7
6
5
4
3
2
1
0
12
24
36
48
hours post inoculation
E
40℃
rgVac2sub-P0
rgVac2sub-P3L4
rgP0/HA-227G
rgP0/HA-388T

Fig. 2

A



B



Virus	Km (μM)
○ rgVac2sub-P0	111±16.0
● rgVac2sub-P3L4	113±19.5

Fig. 3