Original article

E190V substitution of H6 hemagglutinin is one of key factors for binding to sulfated sialylated glycan receptor and infection to chickens

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Abstract

Avian influenza viruses (AIVs) recognize sialic acid linked α2,3 to galactose (SAα2,3Gal) glycans as receptors. In this study, the interactions between hemagglutinins (HAs) of AIVs and sulfated SAα2,3Gal glycans were analyzed in order to clarify the molecular basis of interspecies transmission of AIVs from ducks to chickens. It was revealed that E190V and N192D substitutions of the HA increased the recovery of viruses derived from an H6 duck virus isolate, A/duck/Hong Kong/960/1980 (H6N2), in chickens. Recombinant HAs from an H6 chicken virus, A/chicken/Tainan/V156/1999 (H6N1), bound to sulfated SAα2,3Gal glycans, whereas the HAs from an H6 duck virus did not. Binding preference of mutant HAs revealed that an E190V substitution is critical for the recognition of sulfated SAα2,3Gal glycans. These results suggest that the binding of the HA from H6 AIVs to sulfated SAα2,3Gal glycans explains a part of mechanisms of interspecies transmission of AIVs from ducks to chickens.

Keywords

Avian influenza virus, Interspecies transmission; Sialic acid receptor; Hemagglutinin; Sulfated glycans

Introduction

Migratory ducks are the natural reservoir of avian influenza viruses (AIVs) (1). AIVs can infect most avian species and be transmitted to mammalian species, including humans. Chickens, however, are rarely infected directly with AIVs circulating in ducks and are thought to be infected by them following their adaptation to other galliform species, including turkeys and quails (2). Therefore, there must be some interspecies barrier between ducks and chickens for AIVs. H6 low pathogenic AIVs have circulated in domestic poultry in Asian countries (3, 4) and infected humans (5). Generally, a single infection of H6 AIVs does not induce clinical signs in poultry; however, the co-infection of bacteria aggravates the clinical signs (6). The adaptation of H6 duck influenza viruses to chickens increases the risk of economic damages.

Influenza viruses recognize sialylated glycans on host epithelial cells as their receptor. The binding specificity of hemagglutinin (HA) of influenza viruses to sialylated glycans depends on the host species from which the virus was isolated: HAs of human influenza viruses preferentially bind to glycans terminating with sialic acid linked α2,6 to galactose (SAα2,6Gal), whereas the HAs of AIVs preferentially bind to glycans with sialic acid linked α2,3 to galactose (SAα2,3Gal) (7, 8). It has been reported that AIVs isolated from terrestrial birds, including chickens, prefer 6-sulfo sialyl Lewis X (Le^x) (Siaα2-3Galβ1-4(Fucα1-3)(6-Sulfo)GlcNAc) as receptors, to which AIVs

73 isolated from ducks do not bind (9). The result suggests that the binding of AIVs to
74 fucosylated and/or sulfated SAα2,3Gal glycans is one of the determinants for a
75 successful infection in chickens. Previous studies have revealed that H5Nx chicken
76 influenza viruses preferentially bind to fucosylated SAα2,3Gal glycans (10, 11).
77 However, the role of sulfation in SAα2,3Gal glycans which are not fucosylated, in
78 infections by chicken influenza viruses is still unknown.

79 Several amino acids in the 190-helix and 220-loop in HA are known to alter the
80 receptor-binding specificity of influenza viruses; amino acid residues at the 226 and 228
81 positions of H3 HA (H3 numberings is used throughout) determine the binding
82 specificity of influenza viruses to SAα2,6Gal glycans or SAα2,3Gal glycans (12, 13).
83 In several studies, a relationship has been reported between fucosylated and/or sulfated
84 SAα2,3Gal glycans and the 190-helix and 220-loop on the HA of influenza viruses; the
85 fucose moiety of the host fucosylated SAα2,3Gal glycans is positioned close to the 220-
86 loop on the HA of the influenza virus (14). In particular, amino acid residues at positions
87 222 and 227 on the 220-loop of HA are involved in binding H5 chicken influenza viruses
88 to sialyl Le^x (11, 15). The sulfo moiety of 6-sulfo sialyl Le^x is thought to be positioned
89 close to the 190-helix on the HA of chicken influenza viruses (9); however, reports on
90 the mechanisms of the recognition of the sulfo moiety are limited. Previously, the
91 cocrystallization of HA from an H7N9 human isolate and sulfated SAα2,3Gal glycan

92 revealed the mechanism of the recognition of the sulfo moiety: amino acid residues at
93 positions 190 and 227 of H7 HA determine the binding of HA to sulfated SAα2,3Gal
94 glycans (16), whereas the situation for other subtypes remains unreported.

95 The distribution of sialylated glycans is highly variable in each species; SAα2,6Gal
96 glycans are predominantly detected in human respiratory epithelial cells (17), whereas
97 SAα2,3Gal glycans are dominant on duck cloacal epithelial cells (18). The tissue
98 tropism of influenza viruses, which circulate in each host, is consistent with the
99 distribution of their preferred glycans in hosts. In a previous study, we detected
100 fucosylated SAα2,3Gal glycans in chicken tracheal epithelial cells, where chicken
101 influenza viruses primarily replicate, whereas this glycan structure was not detected in
102 duck colon (10). On the other hand, there are no reports on the detection of sulfated
103 SAα2,3Gal glycans on chicken tracheal epithelial cells to date.

104 In the present study, we aimed to clarify the mechanism of interspecies transmission
105 between ducks and chickens. The growth capacity in chickens and the binding
106 specificity to sulfated glycans of AIVs isolated from chicken and duck were
107 comparatively assessed. The results indicated that E190V and N192D substitutions of
108 the HA increased the virus recovery in chickens and the binding to sulfated SAα2,3Gal
109 glycans is one of the determinants for the increasing infectivity of duck derived AIVs in
110 chickens.

Materials and methods

Viruses and cells

Influenza virus A/chicken/Tainan/V156/1999 (H6N1) (Ck/Tainan) was provided by the Animal Health Research Institute, Council of Agriculture, Taiwan. Influenza virus A/duck/Hong Kong/960/1980 (H6N2) (Dk/HK) was kindly provided by Professor Ken F. Shortridge, The University of Hong Kong, Hong Kong SAR. These viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and the infectious allantoic fluids were used as virus stocks. Madin-Darby canine kidney (MDCK) cells were maintained in Minimum Essential Medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mg/mL L-glutamine, 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 8 μg/mL gentamicin, and 10% fetal calf serum (FCS; Sigma Aldrich, St. Louis, MO, USA). Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with the same concentration of L-glutamine and antibiotics with MEM and 10% FCS (Cambrex, East Rutherford, NJ, USA). HEK 293S GnT (–) cells, which lack N-acetyl-glucosaminyltransferase I (GnTI) activity, were maintained in pyruvate-free DMEM (Thermo Fisher Scientific) with the same supplements as DMEM.

130 **Reverse genetics**

131 Eight gene segments from Dk/HK were cloned into pHW2000 vector according to
132 previously described methods (19-21). Amino acid substitutions of E190V and
133 N192D in the HA of Dk/HK were generated by site-directed mutagenesis using KOD
134 -Plus- Neo (TOYOBO, Tokyo, Japan) with specific primers (Supplemental Table 1).
135 All eight plasmids were transfected into a mixed culture of MDCK and HEK 293T
136 cells and mutant viruses were rescued (Supplemental Figure). These viruses were
137 propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and the
138 infectious allantoic fluids were used as virus stocks. Virus stocks were kept at –80°C
139 until use. All eight gene segments of rescued viruses were sequenced to confirm the
140 existence of the introduced mutations and the absence of undesired mutations.
141

142 **Virus titration**

143 Plaque assays were performed to titrate the viruses. 10-fold dilutions of viruses
144 were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for
145 1 h. Unbound viruses were removed, and the cells were washed with PBS. The cells
146 were then overlaid with FCS-free MEM containing 0.7% Bacto Agar (Thermo Fisher
147 Scientific) and 5 μg/mL Trypsin Acetylated (Sigma Aldrich). After incubation for 48
148 h at 35°C, cells were overlaid with FCS-free MEM containing 0.7% Bacto Agar and

149 0.005% neutral red. After 24 h, visible plaques were counted and expressed as plaque
150 forming unit (PFU)/mL.
151
152 **Experimental infection of chicken with viruses**
153 Four-week-old conventional chickens (*Gallus gallus*, Julia), which were free of
154 anti-influenza virus antibodies, were obtained from Hokkai Starchick, Hokkaido, Japan.
155 Each of three chickens was intranasally inoculated with $10^{5.0}$ PFU of either Ck/Tainan
156 or Dk/HK viruses. On three and five days post inoculation (d.p.i.), oral and cloacal
157 swabs were collected. Also, other three chickens were inoculated with $10^{5.0}$ PFU of
158 mutant viruses derived from Dk/HK. Tracheal and cloacal swabs and tissue samples
159 were collected at three d.p.i. The tissue samples were homogenized using a Multi-
160 Beads Shocker (Yasui Kikai, Osaka, Japan) to prepare 10% suspensions with MEM.
161 The infectivity titers of the swabs were calculated by a plaque assay. All infected
162 chickens were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan).
163 Although the experiments were possibly conducted in ABSL2 condition, all the animal
164 experiments were conducted in the higher biosafety level, ABSL3 facility at the Faculty
165 of Veterinary Medicine, Hokkaido University, Japan due to the limitation of access to
166 the animal facility.
167
168 **Expression of the recombinant HAs (rHAs)**

169 The cDNAs of the HA genes of Ck/Tainan, Dk/HK and their mutants were cloned
170 into a pCD5 expression vector (22). In this vector, HA genes without transmembrane
171 regions were fused with a GCN4IL motif and Strep-tag II (WSHPQFEK; IBA,
172 Göttingen, Germany). The soluble trimeric rHA proteins were expressed in HEK293S
173 GnT (–) cells and purified from the cell culture supernatants according to a previously
174 described method (22). Briefly, rHA proteins were purified using Strep-Tactin
175 Sepharose beads (IBA). The rHA bound to Strep-Tactin beads were treated by d-
176 Desthiobiotin buffer (IBA) for 15 min at 4°C to elute the rHAs from the beads. Mutant
177 HAs were prepared by the single-site mutagenesis method used specific primers
178 (Supplemental Table 1).
179
180 **Glycan microarray**
181 Purified rHAs were analyzed according to previously described method (17). The
182 glycan microarray contains two nonsialylated glycans (glycans 1 and 2) and 56 α2,3
183 and α2,6 sialylated glycans (glycans 3–58). Of the 56 sialylated glycans, 5 were
184 sulfated α2,3 sialylated glycans (glycans 3–7), 28 were nonsulfated α2,3 sialylated
185 glycans (glycans 8–35), and 22 α2,6 sialylated glycans (glycans 36–58) were printed
186 on the array side (Supplemental Table 2). For each glycan, the mean signal intensity
187 was calculated from six replicates. The highest and lowest signals of the six were

188 removed, and the remaining four replicates were used to calculate the mean signal
189 shown as relative fluorescence unit (RFU) and standard error (SE).
190
191 **Solid-phase direct binding assay**
192 The receptor-binding specificity of rHAs was assessed using a solid-phase direct
193 binding assay with Neu5Acα2-3Galβ1-4GlcNAc-β-PEG-biotin (SAα2,3Gal-bio) and
194 Neu5Ac α2-3Galβ1-4(6-Sulfo)GlcNAc-β-PEG-biotin (Su-SAα2,3Gal-bio) (Tokyo
195 Chemical Industry Co., Ltd., Tokyo, Japan). Each sialylated glycan was serially diluted
196 from 0.039–5 μM/well and added to each well of a Nunc immobilizer streptavidin 12
197 × 8 strips microplate (Thermo Fisher Scientific). Each well was blocked with 2%
198 bovine serum albumin (BSA) at room temperature for 1 h. After washing with PBS
199 containing 0.05% Tween 20 (PBST), the rHAs (5 μg in PBST containing 1% BSA)
200 were added to each well, and plates were incubated at 4°C for 12 h. After washing,
201 anti-Strep-tag mouse antibody (IBA; 1,000-fold dilution in PBST containing 1% BSA)
202 was added to each well, and the plates were incubated at 4°C for 2 h. The wells were
203 then washed and incubated with goat anti-mouse IgG-HRP conjugate (Bio-Rad,
204 Hercules, CA, USA) at 4°C for 2 h. After washing, 100 μL of the substrate solution
205 including 0.5 mM 3,3′-tetramethylbenzidine and 0.04% H_2O_2, was added to each well.
206 After incubation at room temperature for 30 min, the reactions were stopped using 50

207 μL of 2 N H_2SO_4, and absorbance at 450/630 nm was measured using a MULTISCAN
208 JX microplate photometer (Thermo Fisher Scientific). The data were presented with
209 the mean values of three technical replicates with SE.
210
211 **Amino acid sequence comparison of the H6 HA**
212 A total of 638 amino acid sequences of H6 chicken and duck influenza viruses was
213 obtained from GenBank. Sequence data were aligned with GENETYX ver. 12.01
214 (GENETYX Co., Tokyo, Japan).
215
216 **Ethics statement**
217 All *in vivo* experiments were authorized by the Institutional Animal Care and
218 Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval
219 number: 18-0037; 18-0040) and performed according to the guidelines of this committee.
220
221 **Results**
222 **Infectivity of chicken and duck influenza viruses in chickens**
223 To confirm the growth of chicken virus, Ck/Tainan, and duck virus, Dk/HK, in
224 chickens, the viral growth was compared *in vivo*. Each virus containing $10^{5.0}$ PFU was
225 intranasally inoculated into three four-week-old chickens. All of chickens inoculated

226 with each virus did not show any clinical signs. Ck/Tainan viruses were recovered from
227 the respiratory tracts of all three infected chickens at three and five d.p.i., whereas
228 Dk/HK was not recovered from any of the inoculated chickens (Table 1).
229
230 **Preparation of E190V and N192D mutant viruses derived from Dk/HK and their**
231 **viral growth in chickens**
232 A previous study reported that 190-helix of HA is thought to be positioned close to
233 the sulfo moiety of sulfated SAα2,3Gal glycans (9). Therefore, the amino acid
234 sequences of 190-helix on HA of these two viruses were compared. As a result, the
235 amino acid properties of positions 190 and 192 are different between them. The majority
236 of H6 AIVs isolated from ducks have amino acid residues E190 and N192 based on
237 information from a public database (GenBank/DDBJ/EMBL). Almost one-third of H6
238 AIVs isolated from chickens, including Ck/Tainan, have amino acid residues V190 and
239 D192 (Table 2). To clarify the role of amino acid residues at positions 190 and 192 of
240 HA in the viral growth in chickens, Dk/HK viruses containing various amino acid
241 substitutions at these positions (rgDk/HK, rgDk/HK-HA190V, rgDk/HK-HA192D, and
242 rgDk/HK-HA190V/192D) were prepared using a reverse genetics method. To compare
243 the virus growth in chickens, $10^{5.0}$ PFU of each recombinant virus was intranasally
244 inoculated to three 4-week-old chickens. Viruses were recovered from tracheal swabs at

245 3 d.p.i from the chickens infected with rgDk/HK-190V, rgDk/HK-HA192D and
246 rgDk/HK-HA190V192D (Table 3). Viruses were also recovered from respiratory organs
247 of one of three rgDk/HK-HA190V infected chicken and of all three rgDk/HK-
248 HA190V192D infected chickens. These results suggest that both E190V and N192D
249 substitutions are involved in the replication of mutants derived from Dk/HK in chickens.
250

251 **Glycan-binding specificity of mutant viruses derived from Dk/HK**

252 To clarify the molecular basis of different viral growth in chickens, the glycan-
253 binding specificities of HAs from these two viruses were compared in a glycan
254 microarray (Fig. 1, Supplemental Table 3). The rHAs of Ck/Tainan bound to sulfated
255 SAα2,3Gal glycans (glycans 3, 4, and 7, Fig. 2). The rHAs of Dk/HK bound to linear
256 and non-sulfated SAα2,3Gal glycans (glycans 14 and 24–26, Fig. 2) with high RFU.
257 The rHAs of Dk/HK also bound to sulfated SAα2,3Gal glycans with lower RFU than
258 those of the rHAs of Ck/Tainan (glycans 3, 4, and 7), indicating that the glycan-binding
259 profile of rHA of Dk/HK is different from that of rHA of Ck/Tainan. These results
260 indicated that the rHA of the chicken isolate, Ck/Tainan, selectively bound to sulfated
261 SAα2,3Gal glycans, whereas that of the duck isolate, Dk/HK, preferentially bound to
262 linear and non-sulfated SAα2,3Gal glycans.
263

Key amino acid residues for recognition of sulfated SAα2,3Gal glycans by HA

To evaluate the contribution of amino acid residues at positions 190 and 192 of the HA to the glycan-binding specificity of these viruses, mutant rHAs of Ck/Tainan and Dk/HK, in which amino acid residues at positions 190 and/or 192 were substituted (Ck/Tainan-HA190E, Ck/Tainan-HA192N, Ck/Tainan-HA190E/192N, Dk/HK-HA190V, DK/HK-HA192D and Dk/HK-HA190V/192D), were prepared and these rHAs were subjected to solid-phase direct binding assays (Figs. 3, 4). The rHAs of Ck/Tainan preferentially bound to Su-SAα2,3Gal-bio (Fig. 3A) and Ck/Tainan-HA190E HAs preferentially bound to SAα2,3Gal-bio (Fig. 3B). The introduction of the 192N mutation to Ck/Tainan-HA and its mutant resulted in the diminished binding to both SAα2,3Gal-bio and Su-SAα2,3Gal-bio; yet the binding preference to Su-SAα2,3Gal-bio was slightly observed in 190V HA (Ck/Tainan-HA192N) and to SAα2,3Gal-bio in 190E HA (Ck/Tainan-HA190E/192N) (Figs. 3C, 3D). The rHAs of Dk/HK bound to SAα2,3Gal-bio (Fig. 4A), whereas Dk/HK-HA190V acquired binding to Su-SAα2,3Gal-bio (Fig. 4B). The introduction of the 192D in Dk/HK-HA resulted in weaker binding to glycans without changing binding preference to SAα2,3Gal-bio (Fig. 4C). The introduction of the same mutation in Dk/HK-HA190V (Dk/HK-HA190V/192D) also resulted in diminished binding to the glycans, although the binding to Su-SAα2,3Gal-bio was more affected when compared with that to SAα2,3Gal-bio

283 (Fig. 4D). These results indicate that E190V substitution of the HA determines the
284 binding of H6 AIVs to sulfated SAα2,3Gal glycans. On the other hand, the discrepancy
285 of the 192D/N and the backbone of the HA resulted in the diminished binding to the
286 glycans.
287
288 **Discussion**
289 In the present study, the virus growth in chickens and the receptor-binding
290 specificities of viruses were analyzed to understand the mechanisms of interspecies
291 transmission of AIVs from ducks to chickens. From the growth capacity of mutant
292 viruses, E190V and N192D substitutions of the HA increased the recovery of the viruses
293 derived from the H6 duck virus, Dk/HK, in experimentally infected chickens. The H6
294 AIV isolated from a chicken, Ck/Tainan, bound specifically to sulfated SAα2,3Gal
295 glycans, whereas Dk/HK preferred to non-sulfated SAα2,3Gal glycans. The difference
296 of the binding specificity was thought to be related to the host range of H6 AIVs. Solid-
297 phase direct binding assay of rHAs revealed that only an E190V substitution in the HA
298 of Dk/HK altered its glycan-binding specificity from linear and non-sulfated SAα2,3Gal
299 glycans to sulfated SAα2,3Gal glycans. These results indicate that the amino acid
300 residue at position 190 of H6 HA is a key component for the recognition of sulfated

301 SAα2,3Gal glycans. Furthermore, position 190 of HA might be the key for the
302 interspecies transmission of AIVs from ducks to chickens.

303 On the other hand, both V190 and D192 were important for the virus growth in
304 chickens (Table 3). Interestingly, viruses with V190/D192 were selected in chickens in
305 the field (32/101 strains) rather than those with V190/N192 (4/101 strains) (Table2).
306 These facts suggested that these two mutations, E190V and N192D, synergistically act
307 in the adaptation process of duck influenza viruses to chickens. Moreover, the mutation
308 at position 192 of the HA modulated receptor binding avidity (Figs. 3, 4). A previous
309 report on the structural analysis demonstrated that the amino acid residue at the 192
310 positioned close to penultimate Gal moiety of the sialylated glycan (23). Perhaps, the
311 mutation at position 192 might be related to the recognition of modified Gal moiety (e.g.,
312 6-sulfo-Gal), which was not analyzed in the present study.

313 Nevertheless, approximately 40% of chicken H6 viruses still have the E residue at
314 position 190 of the HA (Table 2). On the other hand, viruses possessing the V residue
315 at the same position were exceptional among duck H6 viruses; only two out of 537
316 strains possess V at the position. Interestingly, similar phenomena were observed in the
317 combination of H5 viruses and fucosylated SAα2,3Gal (15). These facts suggest that
318 viruses recognizing linear and non-sulfated SAα2,3Gal were selected among the duck
319 population. In addition, a previous study demonstrated that co-infection of *Mycoplasma*

320 *gallisepticum* exaggerated infection of duck influenza viruses in experimentally infected
321 chickens (24). This suggested that duck influenza viruses, which originally recognize
322 linear and non-sulfated SAα2,3Gal are able to establish infections in chickens in the
323 field without altering their receptor-binding specificity and viruses with "chicken-type
324 receptor specificity" were selected during multiple replications and transmission events
325 in chicken flocks, rather than in the adaptation among ducks or non-chicken terrestrial
326 poultry.

327 Although rgDk/HK-HA190V, rgDk/HK-HA192D, and rgDk/HK-HA190V/192D
328 were recovered from infected chickens, the virus recovery titers from swabs at 3 d.p.i
329 were slightly lower compared with those from the chickens inoculated with Ck/Tainan.
330 These viruses contain E190V and/or N192D substitution in their HA, indicating that
331 these both mutations enhance viral infection and replication in chickens; however, the
332 backbone of the HA as well as other viral proteins remains the same as wild-type Dk/HK,
333 which could not replicate well in chickens. Therefore, some other interspecies barrier
334 between ducks and chickens for AIVs may explain these lower titers of mutant viruses
335 in chickens. Influenza viruses have eight segmented genes, and each gene encodes for
336 different proteins. Of these proteins, HA is responsible for the attachment and fusion of
337 viral and cellular membranes. Other proteins have roles for each step of viral replication.
338 It has been reported that PB2 and NS proteins are important for the interspecies

339 transmission of influenza viruses (25, 26). In a previous study, a duck influenza virus
340 adapted to chickens through mutations in its PB2 and NP proteins (27), although the
341 functional details of these mutations have not been reported.

342 The mechanisms of receptor-recognition by HA of influenza viruses were revealed
343 through the cocrystallization of HA with sialylated glycans (9). However, reports on the
344 detailed molecular interactions between HAs with modified SAα2,3Gal glycan
345 receptors, such as fucosylated and/or sulfated receptors, are limited. One possible
346 explanation for the lack of receptor-binding of HAs form duck influenza viruses to
347 sulfated SAα2,3Gal glycans is the negative charge of the sulfo moiety (9). Position 190
348 in the HA of Dk/HK is glutamic acid, E, whereas in Ck/Tainan, it is valine, V. The
349 negatively charged glutamic acid is perhaps not compatible with the similarly negatively
350 charged sulfo-moiety of the glycans. Therefore, the amino acid substitution to the
351 uncharged amino acid, E190V in HA must be involved in binding to sulfated glycans
352 and allowing H6 viruses to infect and replicate in chickens.

353 In conclusion, it is speculated that sulfated SAα2,3Gal glycans are expressed on the
354 chicken trachea and binding to sulfated SAα2,3Gal glycans contributes to the
355 interspecies transmission of H6 AIVs from ducks to chickens. There is limited
356 information on the distribution of sialylated glycans, which can be detected by lectins
357 and anti-glycan antibodies on the surface of host cells. In order to clarify the role of

358 sulfated SAα2,3Gal glycans in the interspecies transmission, it is necessary to analyze
359 the receptor distribution on the host cells. Furthermore, to fully understand the molecular
360 mechanisms of interspecies transmission of AIVs, it is necessary to clarify the structural
361 and quantitative details of sialylated glycans used as receptors by these viruses.
362
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364 The authors have no conflicts of interest to declare.
365
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References

1 Webster R.G., Morita M., Pridgen C., Tumova B. (1976) Ortho- and paramyxoviruses from migrating feral ducks: characterization of a new group of influenza A viruses. *J Gen Virol,* **32**: 217-25.

2 Perez D.R., Lim W., Seiler J.P., Yi G., Peiris M., Shortridge K.F., Webster R.G. (2003) Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. *J Virol,* **77**: 3148-56.

3 Chu D.H., Okamatsu M., Matsuno K., Hiono T., Ogasawara K., Nguyen L.T., Van Nguyen L., Nguyen T.N., Nguyen T.T., Van Pham D., Nguyen D.H., Nguyen T.D., To T.L., Van Nguyen H., Kida H., Sakoda Y. (2016) Genetic and antigenic characterization of H5, H6 and H9 avian influenza viruses circulating in live bird markets with intervention in the center part of Vietnam. *Vet Microbiol,* **192**: 194-203.

4 Li J., Quan C., Xie Y., Ke C., Nie Y., Chen Q., Hu T., Chen J., Wong G., Wang Q., Feng L., Yu H., Liu Y., Liu W., Gao G.F., Liu W.J., Shi W., Bi Y. (2019) Continued reassortment of avian H6 influenza viruses from Southern China, 2014-2016. *Transbound Emerg Dis,* **66**: 592-98.

5 Yuan J., Zhang L., Kan X., Jiang L., Yang J., Guo Z., Ren Q. (2013) Origin and molecular characteristics of a novel 2013 avian influenza A(H6N1) virus causing human infection in Taiwan. *Clin Infect Dis,* **57**: 1367-8.

6 Umar S., Delverdier M., Delpont M., Belkasmi S.F.Z., Teillaud A., Bleuart C., Pardo I., El Houadfi M., Guérin J.L., Ducatez M.F. (2018) Co-infection of turkeys with Escherichia coli (O78) and H6N1 avian influenza virus. *Avian Pathol,* **47**: 314-24.

7 Rogers G.N., Paulson J.C. (1983) Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology,* **127**: 361-73.

8 Rogers G.N., D'souza B.L. (1989) Receptor binding properties of human and animal H1 influenza virus isolates. *Virology,* **173**: 317-22.

9 Gambaryan A.S., Tuzikov A.B., Pazynina G.V., Desheva J.A., Bovin N.V., Matrosovich M.N., Klimov A.I. (2008) 6-sulfo sialyl Lewis X is the common receptor determinant recognized by H5, H6, H7 and H9 influenza viruses of terrestrial poultry. *Virol J,* **5**: 85.

10 Hiono T., Okamatsu M., Nishihara S., Takase-Yoden S., Sakoda Y., Kida H. (2014) A chicken influenza virus recognizes fucosylated α2,3 sialoglycan

receptors on the epithelial cells lining upper respiratory tracts of chickens. *Virology,* **456-457**: 131-8.

11 Guo H., De Vries E., Mcbride R., Dekkers J., Peng W., Bouwman K.M., Nycholat C., Verheije M.H., Paulson J.C., Van Kuppeveld F.J., De Haan C.A. (2017) Highly Pathogenic Influenza A(H5Nx) Viruses with Altered H5 Receptor-Binding Specificity. *Emerg Infect Dis,* **23**: 220-31.

12 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**: 76-8.

13 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.

14 Xiong X., Tuzikov A., Coombs P.J., Martin S.R., Walker P.A., Gamblin S.J., Bovin N., Skehel J.J. (2013) Recognition of sulphated and fucosylated receptor sialosides by A/Vietnam/1194/2004 (H5N1) influenza virus. *Virus Res,* **178**: 12-4.

15 Hiono T., Okamatsu M., Igarashi M., Mcbride R., De Vries R.P., Peng W., Paulson J.C., Sakoda Y., Kida H. (2016) Amino acid residues at positions 222 and 227 of the hemagglutinin together with the neuraminidase determine binding of H5 avian influenza viruses to sialyl Lewis X. *Arch Virol,* **161**: 307-16.

16 Xu R., De Vries R.P., Zhu X., Nycholat C.M., Mcbride R., Yu W., Paulson J.C., Wilson I.A. (2013) Preferential recognition of avian-like receptors in human influenza A H7N9 viruses. *Science,* **342**: 1230-5.

17 Couceiro J.N., Paulson J.C., Baum L.G. (1993) Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res,* **29**: 155-65.

18 Ito T., Suzuki Y., Suzuki T., Takada A., Horimoto T., Wells K., Kida H., Otsuki K., Kiso M., Ishida H., Kawaoka Y. (2000) Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. *J Virol,* **74**: 9300-5.

19 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**: 6108-13.

20 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**: 2275-89.

457 21 Soda K., Asakura S., Okamatsu M., Sakoda Y., Kida H. (2011) H9N2 influenza
458 virus acquires intravenous pathogenicity on the introduction of a pair of di-basic
459 amino acid residues at the cleavage site of the hemagglutinin and consecutive
460 passages in chickens. *Virol J,* **8**: 64.
461 22 De Vries R.P., De Vries E., Bosch B.J., De Groot R.J., Rottier P.J., De Haan C.A.
462 (2010) The influenza A virus hemagglutinin glycosylation state affects receptor-
463 binding specificity. *Virology,* **403**: 17-25.
464 23 Ni F., Kondrashkina E., Wang Q. (2015) Structural and Functional Studies of
465 Influenza Virus A/H6 Hemagglutinin. *PLoS One,* **10**: e0134576.
466 24 Stipkovits L., Egyed L., Palfi V., Beres A., Pitlik E., Somogyi M., Szathmary S.,
467 Denes B. (2012) Effect of low-pathogenicity influenza virus H3N8 infection on
468 Mycoplasma gallisepticum infection of chickens. *Avian Pathol,* **41**: 51-7.
469 25 Hatta M., Gao P., Halfmann P., Kawaoka Y. (2001) Molecular basis for high
470 virulence of Hong Kong H5N1 influenza A viruses. *Science,* **293**: 1840-2.
471 26 Twu K.Y., Kuo R.L., Marklund J., Krug R.M. (2007) The H5N1 influenza virus
472 NS genes selected after 1998 enhance virus replication in mammalian cells. *J*
473 *Virol,* **81**: 8112-21.
474 27 Sorrell E.M., Perez D.R. (2007) Adaptation of influenza
475 A/Mallard/Potsdam/178-4/83 H2N2 virus in Japanese quail leads to infection
476 and transmission in chickens. *Avian Dis,* **51**: 264-8.
477
478

List of Abbreviations: AIV, avian influenza virus; Ck/Tainan, A/chicken/Tainan/V156/1999 (H6N1); BSA, bovine serum albumin; Dk/HK, A/duck/Hong Kong/960/1980 (H6N2); DMEM, Dulbecco's modified Eagle's medium; d.p.i., days post inoculation; FCS, fetal calf serum; GnTI, N-acetyl-glucosaminyltransferase I; HA, hemagglutinin; HEK, human embryonic kidney; Le^x, Lewis X; MDCK, Madin-Darby canine kidney; MEM, Minimum Essential Medium; PBST, PBS containing 0.05% Tween 20; PFU, plaque forming unit; RFU, relative fluorescence unit; rHA, recombinant HA; SAα2,3Gal, sialic acid linked α2,3 to galactose; SAα2,6Gal, sialic acid linked α2,6 to galactose; SE, standard error.

489 **List of Tables and Figures**

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491 Virus recovery from chickens intranasally inoculated with chicken and duck H6

492 influenza viruses.

493

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497

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500 derived from A/duck/Hong Kong/960/1980 (H6N2).

501

502 **Fig. 1.**

503 Glycan-binding specificity of the soluble trimeric recombinant hemagglutinins (rHAs).

504 The glycan-binding specificities of rHAs from Ck/Tainan (A) and Dk/HK (B) were

505 analyzed using a glycan microarray. Nonsialylated controls (glycans1 and 2), sulfated

506 α2,3 sialylated glycans (glycans 3–7), nonsulfated α2,3 sialylated glycans (glycans 8–

507 35), and α2,6 sialylated glycans (glycans 36–58) were printed on the array. The data are

508 presented as the mean ± SE of triplicate experiments. The structures of glycans that were
509 preferentially bound (glycans 3, 4, 7, 14, and 24–26) are shown in Fig. 2.
510
511 **Fig. 2.**
512 Structures of glycans that were bound by rHAs on the array. Each symbol indicates sialic
513 acid (purple diamonds), galactose (yellow circles), *N*-acetylglucosamine (blue squares),
514 *N*-acetylgalactosamine (yellow squares), fucose (red triangles) and sulfo (S). The
515 schematic structures of the glycans that yielded significant signals in the glycan
516 microarray analysis with rHA from Ck/Tainan and Dk/HK (Fig. 1) are shown in this
517 figure.
518
519 **Fig. 3.**
520 Glycan-binding specificity of wild-type and mutant rHAs of Ck/Tainan. The glycan-
521 binding specificities of rHAs of Ck/Tainan (A), Ck/Tainan-HA190E (B), Ck/Tainan-
522 HA192N (C), and Ck/Tainan-HA190E/192N (D) to sialylglycoconjugates containing
523 SAα2,3Gal-bio (closed circles) and Su-SAα2,3Gal-bio (open circles) were investigated
524 using solid-phase direct binding assays. The data are presented as the mean ± SE of
525 triplicate experiments.
526

Fig. 4.

Glycan-binding specificity of wild-type and mutant rHAs of Dk/HK. The glycan-binding specificities of rHAs of Dk/HK (A), Dk/HK-HA190V (B), Dk/HK-HA192D (C) and Dk/HK-HA190V/192D (D) to sialylglycoconjugates containing SAα2,3Gal-bio (closed circles) and Su-SAα2,3Gal-bio (open circles) were investigated using solid-phase direct binding assays. The data are presented as the mean ± SE of triplicate experiments.

Supplemental Table 1

Primer information for single-site mutagenesis.

Supplemental Table 2

Printed glycan list for the array.

Supplemental Table 3

Result of glycan microarray for rHAs of Ck/Tainan and Dk/HK

Supporting Figure Legend

545 Genome structure of mutant viruses in this study. The gene constellation and amino acid
546 substitutions of parental and mutant viruses generated by reverse genetics were
547 illustrated.
548

Table 1 Virus recovery from chickens intranasally inoculated with chicken and duck H6 influenza viruses.

Viruses	Swabs (log PFU/ml)			
	Oral		Cloacal	
	3 dpi	5 dpi	3 dpi	5 dpi
Ck/Tainan	5.3	2.3	-*	-
	2.7	2.8	-	-
	3.4	-	-	3.2
Dk/HK	-	-	-	-
	-	-	-	-
	-	-	-	-

* Dash (-) indicates <1.0
dpi: days post inoculation
Ck/Tainan: A/chicken/Tainan/V156/1999 (H6N2)
Dk/HK: A/duck/Hong Kong/960/1980 (H6N2)

Table 2 Amino acid residues comparison of 190/192 motifs in HA of H6 chicken and duck influenza viruses.

Host	Amino acid residue		Number of strains
	190	192	
Chicken (101 strains)	E	N	42
	V	**D**	32
	A	N	8
	V	N	4
	E	E	4
	E	D	3
	G	N	2
	E	A	2
	L	E	2
	L	N	1
	R	N	1
Duck (537 strains)	**E**	**N**	454
	E	E	51
	E	A	20
	A	N	5
	E	D	4
	V	N	1
	V	D	1
	L	D	1

Amino acid sequence of H6 HA were obtained from GenBank.
Amino acid motifs of Ck/Tainan and Dk/HK are shown in bold.

Table 3 Virus recovery from chickens intranasally inoculated with wild type and mutant viruses derived from A/duck/Hong Kong/960/1980 (H6N2)

Viruses	Swabs (log PFU/ml)		Tissues (log PFU/g)		
	Tracheal	Cloacal	Trachea	Lung	Colon
rgDk/HK	-*	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
rgDk/HK-HA190V	-	-	-	-	-
	1.4	-	-	1.7	-
	1.7	-	-	-	-
rgDk/HK-HA192D	1.7	-	-	-	-
	1.5	-	-	-	-
	-	-	-	-	-
rgDk/HK-HA190V/192D	2.4	-	2.6	-	-
	2.3	-	2.5	1.8	-
	4.3	-	4.4	3.3	-

* Dash (-) indicates <1.0

rgDk/HK: A/duck/Hong Kong/960/1980 (H6N2) generated by reverse genetics

Samples were collected at 3 dpi.

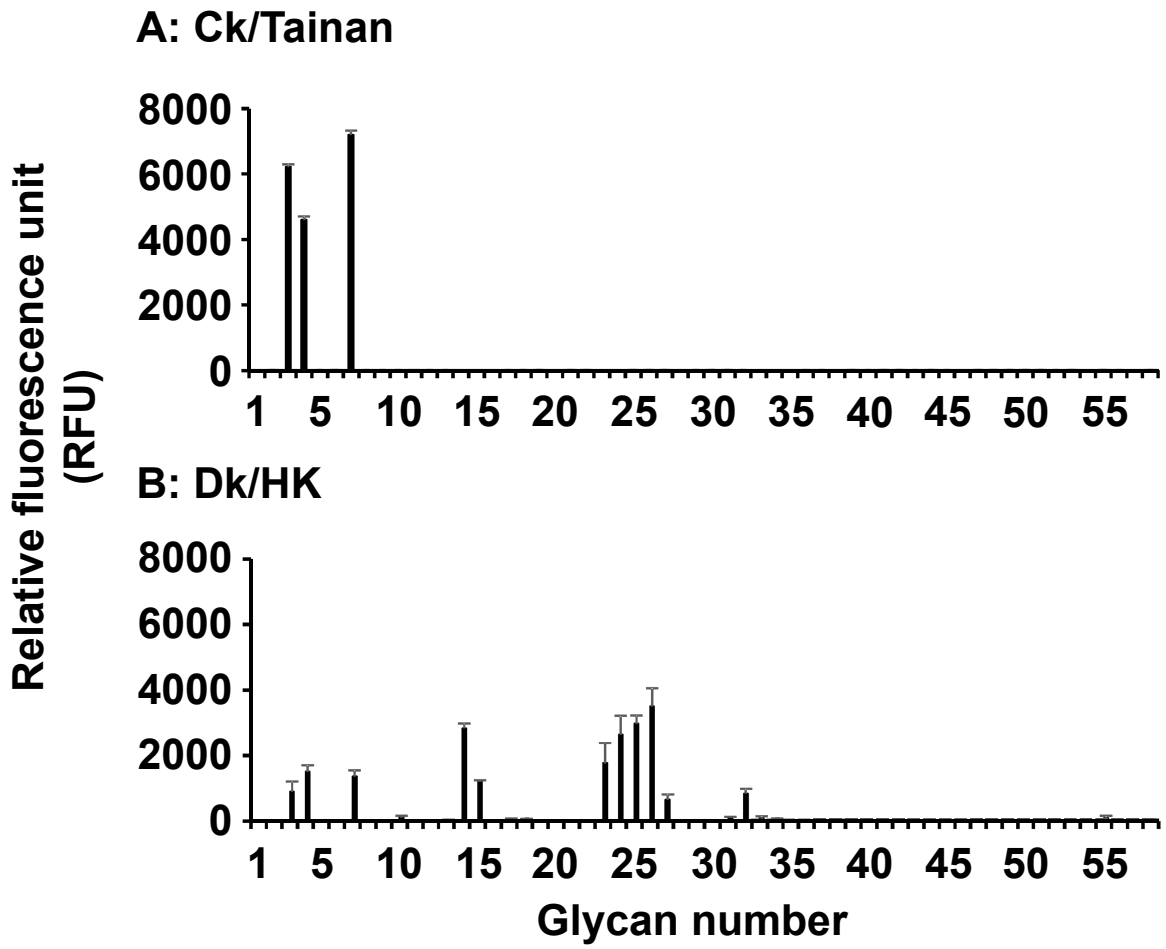


Fig. 1. Kikutani *et al.*

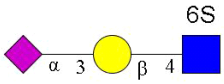
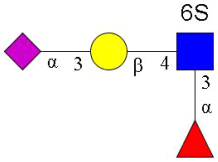
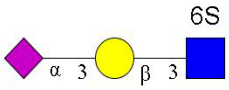
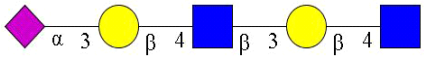
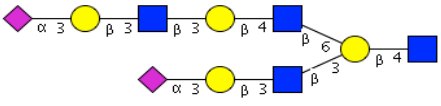
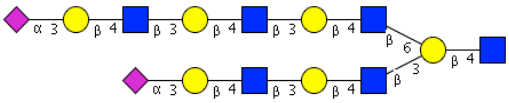
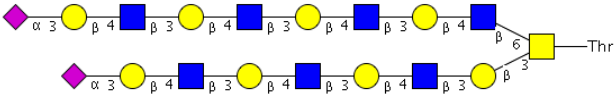
No	Structures
3	
4	
7	
14	
24	
25	
26	

Fig. 2 Kikutani *et al.*

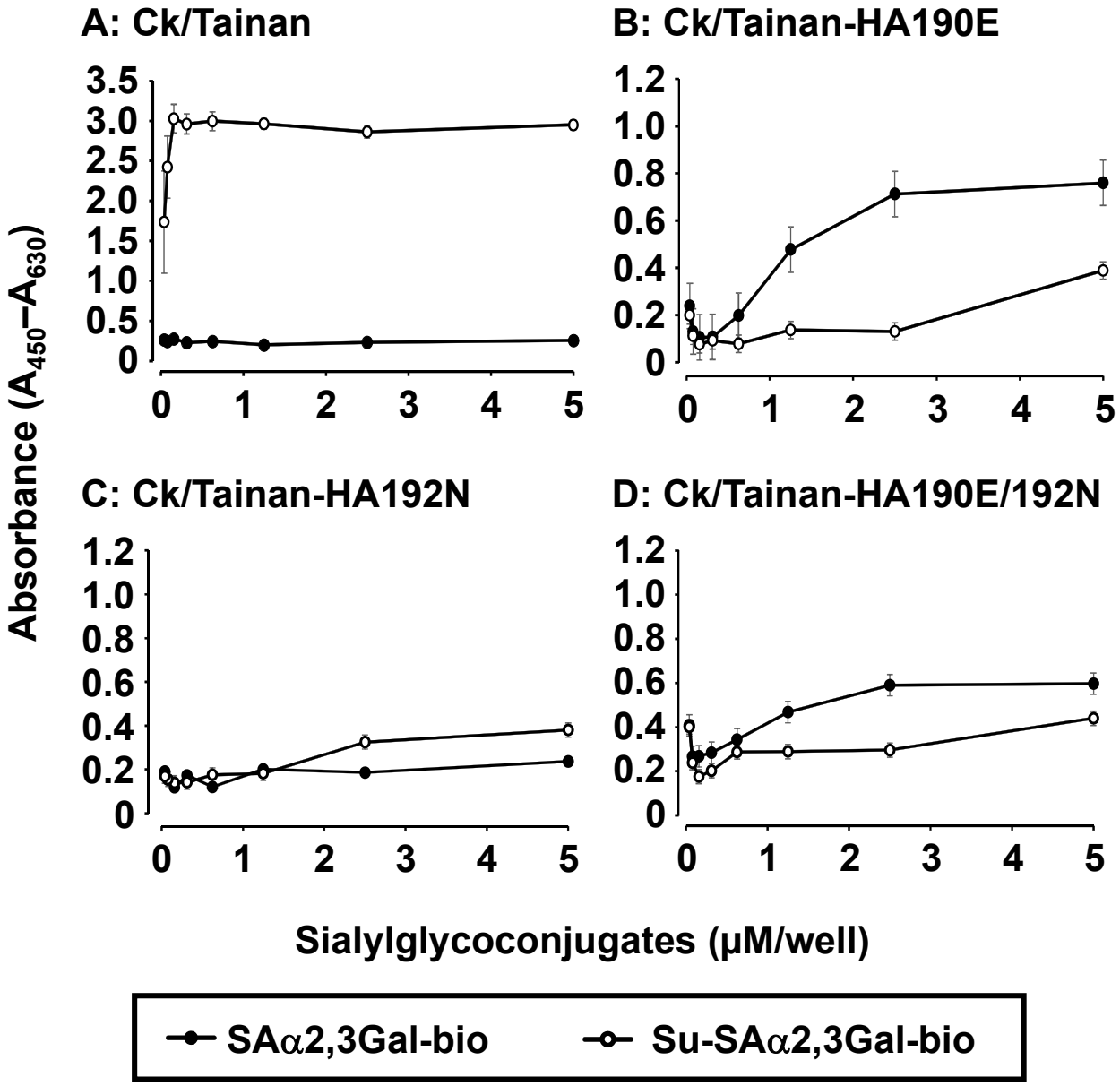
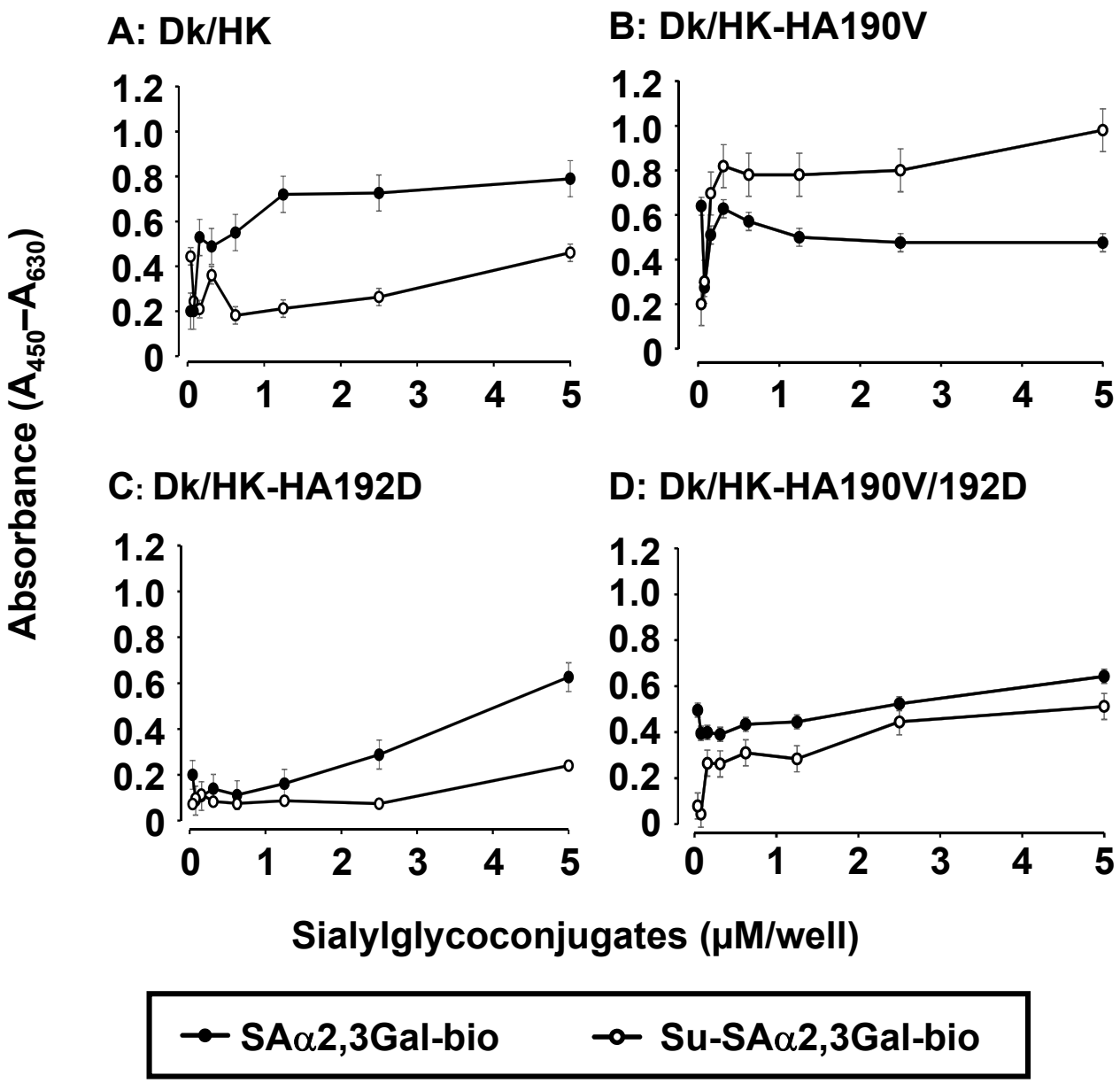


Fig. 3. Kikutani *et al.*



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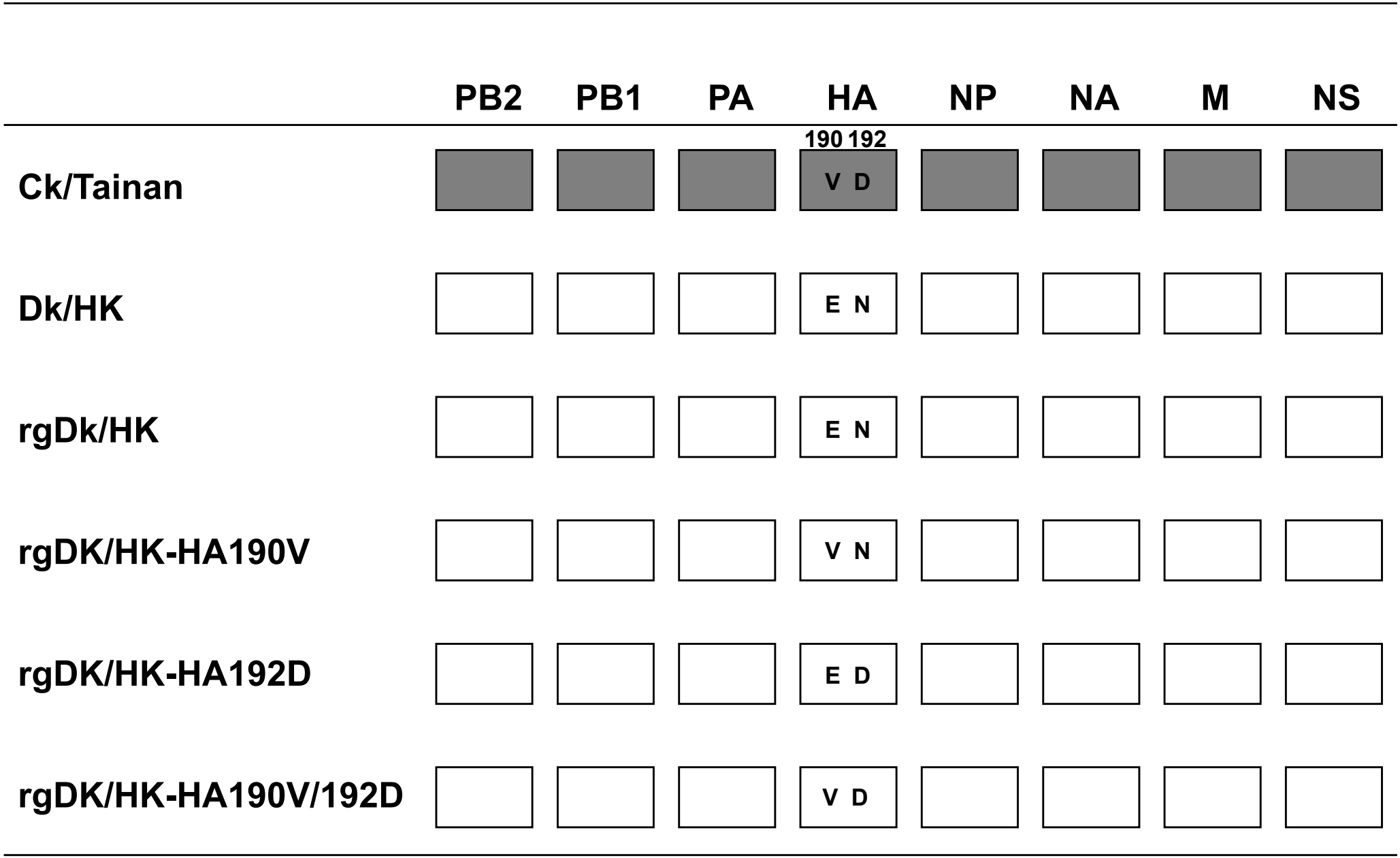

Ck/Tainan: A/chicken/Tainan/V156/1999 (H6N1), Dk/HK:A/duck/Hong Kong/960/1980 (H6N2)

Supplemental figure. Genome structure of mutant viruses in this study