1 **Experimental infection of highly and low pathogenic avian influenza viruses to chickens,**
2 **ducks, tree sparrows, jungle crows, and black rats for the evaluation of their roles in virus**
3 **transmission**

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24

25 **Abstract**

26 Highly pathogenic avian influenza viruses (HPAIVs) have spread in both poultry and wild
27 birds. Determining transmission routes of these viruses during an outbreak is essential for the
28 control of avian influenza. It has been widely postulated that migratory ducks play crucial
29 roles in the widespread dissemination of HPAIVs in poultry by carrying viruses along with their
30 migrations; however close contacts between wild migratory ducks and poultry are less likely in
31 modern industrial poultry farming settings. Therefore, we conducted experimental infections
32 of HPAIVs and low pathogenic avian influenza viruses (LPAIVs) to chickens, domestic ducks,
33 tree sparrows, jungle crows, and black rats to evaluate their roles in virus transmission. The
34 results showed that chickens, ducks, sparrows, and crows were highly susceptible to HPAIV
35 infection. Significant titers of virus were recovered from the sparrows and crows infected with
36 HPAIVs, which suggests that they potentially play roles of transmission of HPAIVs to poultry.
37 In contrast, the growth of LPAIVs was limited in each of the animals tested compared with that
38 of HPAIVs. The present results indicate that these common synanthropes play some roles in
39 influenza virus transmission from wild birds to poultry.

40

41 **Keywords:** influenza virus; sparrows; crows; black rats; pathogenicity

42

43 **1. Introduction**

44 Influenza A viruses are widely distributed in mammals and birds. Influenza A viruses of
45 each of the known subtypes (H1–16 and N1–9) have been isolated from water birds, particularly
46 from migratory ducks (Kida et al., 1979; Fouchier et al., 2005). Therefore, migratory ducks
47 are the natural hosts for influenza A viruses (Webster et al., 1992; Kida, 2008). Since late 2003,
48 H5N1 highly pathogenic avian influenza viruses (HPAIVs) have seriously affected poultry in
49 Eurasia and Africa (OIE website,
50 <http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/>). HPAIVs are
51 generated when non-pathogenic viruses circulating among water birds are transmitted to
52 chickens via domestic water and terrestrial birds, where they acquire pathogenicity in chickens
53 via multiple infection and replication in the chicken population (Ito et al., 2001). After 2005,
54 H5N1 HPAIVs were isolated from dead migratory water birds on the way back to their nesting
55 lakes in Siberia in May (Liu et al., 2005; Okamatsu et al., 2010; Sakoda et al., 2010). The
56 pathogenicity of HPAIVs to migratory ducks varies depending on the virus strain
57 (Sturm-Ramirez et al., 2003; Sakoda et al., 2010; Kajihara et al., 2013); in general, HPAIVs are
58 less pathogenic to ducks compared with chickens (Kishida et al., 2005). Indeed, infected
59 ducks shed viruses without showing any clinical signs (Kida et al., 1980). Thus, it has been
60 widely postulated that migratory ducks play crucial roles in the widespread dissemination of
61 HPAIVs in poultry by carrying viruses along with their migrations.

62 Recently, HPAIVs of various genetic clades derived from A/goose/Guangdong/1/1996
63 (H5N1) have been circulating in Asian countries (Donis et al., 2015). These HPAIVs have
64 evolved to be genetically and antigenically divergent (Shichinohe et al., 2013; Hiono et al.,
65 2015). HPAIVs of clade 1.1.1 have been detected in Mekong River Delta, 2.1.3 in Indonesia,
66 and 2.2.1 in Egypt. In addition, HPAIVs of clades 2.3.2.1 and 2.3.4 have widely spread mainly
67 in East and Southeast Asia. Moreover, HPAIVs of clade 2.3.4.4 have spread to North
68 American and European continents along with migration of wild ducks (Hall et al., 2015). It is
69 therefore imperative to prepare for the future outbreaks caused by HPAIVs of various clades.

70 In 2010, two H5N1 HPAIVs of clade 2.3.2.1 were isolated from fecal samples of ducks on
71 the migration flyway from Siberia to the south in Hokkaido, which was followed by 63 cases of
72 HPAIV infections in wild birds and 24 sporadic cases in poultry (Kajihara et al., 2011; Sakoda
73 et al., 2012). Monitoring avian influenza viruses in wild bird populations is highly beneficial
74 to prepare for outbreaks of highly pathogenic avian influenza (HPAI). On the other hand, close
75 contacts between wild migratory ducks and poultry are less likely in modern industrial poultry
76 farming settings. Accordingly, there should be some other factors involved in the transmission
77 of HPAIVs from wild migratory ducks to poultry.

78 Unveiling the routes of virus transmission that cause outbreaks is essential for controlling
79 avian influenza in poultry. Viruses might invade poultry houses via newly introduced birds,
80 feed, equipment, and wild animals. Tree sparrows (*Passer montanus*), jungle crows (*Corvus*
81 *macrorhynchos*), and black rats (*Rattus rattus*) are characterized as synanthropes, and they are

82 found commonly around poultry houses in Japan. In the present study, we selected seven
83 influenza viruses, which comprised of five HPAIVs isolated recently in Asia, and two low
84 pathogenic avian influenza viruses (LPAIVs) including an H7N9 influenza virus isolated in
85 China, and a non-pathogenic H5N1 strain. We investigated their pathogenicity to these wild
86 animals to evaluate their potential risk for carrying viruses into poultry houses.

87

88 **2. Materials and methods**

89 *2.1. Viruses*

90 A/muscovy duck/Vietnam/OIE-559/2011 (H5N1) (VN/559) was isolated from a muscovy
91 duck in a live bird market of Vietnam in our previous study (Okamatsu et al., 2013).
92 A/whooper swan/Hokkaido/4/2011 (H5N1) (Hok/4) was isolated from dead whooper swan in
93 2011 (Sakoda et al., 2012). A/peregrine falcon/Hong Kong/810/2009 (H5N1) (HK/810) was
94 kindly provided by Dr. Luk S.M. Geraldine of the Tai Lung Veterinary Laboratory, Hong Kong
95 SAR. A/chicken/Kumamoto/1-7/2014 (H5N8) (Km/1-7) was kindly provided by Dr. Takehiko
96 Saito of the National Institute of Animal Health, Japan (Kanehira et al., 2015).
97 A/chicken/Taiwan/0502/2012 (H5N2) (Tw/0502), which is a classical Taiwanese HPAIV, was
98 kindly provided by Animal Health Institute, Taiwan. A/Anhui/1/2013 (H7N9) (Ah/1) was
99 kindly provided by Dr. Masato Tashiro of the National Institute of Infectious Diseases, Japan
100 (Gao et al., 2013). A/duck/Hokkaido/Vac-1/2004 (H5N1) (Hok/Vac1), which is a reassortant
101 virus from non-pathogenic H5N2 and H7N1 viruses was previously established in our

laboratory and used as a representative strain of non-pathogenic avian influenza viruses circulating in wild migratory ducks. (Soda et al., 2008). The viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 36–48 h, and the infectious allantoic fluids were used as virus stocks (Table 1).

2.2. Animal experiments

Four-week-old chickens (*Gallus gallus*, Julia) were obtained from Hokkai Starchick, Hokkaido, Japan. Four-week-old domestic ducks (*Anas platyrhynchos* var. *domesticus*, Cherry Valley) were obtained from Takikawa Shinseien, Hokkaido, Japan. Tree sparrows (*Passer montanus*) were captured in Azumino, Nagano, Japan. Jungle crows (*Corvus macrorhynchos*) were captured in Yubari, Hokkaido, Japan. Black rats (*Rattus rattus*) were bred and raised at IKARI Institute of Technology, Chiba, Japan. The sera of chickens, ducks, and crows were collected before the challenge. The absence of specific antibodies against the challenge virus was confirmed by hemagglutination-inhibition (HI) test with 25 µl of collected sera according to the standard protocol. Because of technical problem, serological status of sparrows and black rats were not able to be monitored before the challenge. Eight of each animal were used in the present study and they were randomly divided into two groups. All chickens, ducks, and crows were intranasally inoculated with 100 µl of virus solution containing $10^{6.0}$ 50% egg infectious dose (EID_{50}) of either VN/559, Hok/4, HK/810, Km/1-7, Tw/0502, Ah/1, or Hok/Vac1. Sparrows were intranasally inoculated with 30 µl of virus solution containing $10^{6.0}$

122 EID₅₀ of each virus. Black rats were intranasally inoculated with 30 µl of virus solution
123 containing 10^{6.0} EID₅₀ of each virus in anesthetized conditions. At 3 dpi, four individuals of
124 each type of inoculated animals were euthanized, and oral and cloacal swabs (chickens, ducks,
125 sparrows, and crows), blood samples (ducks, sparrows, crows, and black rats), as well as brain
126 (all animals), lung (all animals), kidney (chickens, ducks, crows, and black rats), colon
127 (chickens, ducks, and crows), large intestine (sparrows and black rats), and feces (black rats)
128 were collected. Since all of chickens inoculated with VN/559 or HK/810 died at 2 dpi, swabs
129 and tissue samples were collected from the dead birds. To prepare a 10% suspension with
130 Minimum Essential Medium (MEM; Nissui Pharmaceutical, Tokyo, Japan), tissue samples and
131 feces were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The
132 infectivity titers for swabs, blood samples, tissue samples, and feces were calculated by plaque
133 assays or TCID₅₀. The other four animals were observed clinically for 14 days after
134 inoculation. The Kaplan–Meier method with log-rank test was applied to compare survival
135 curves. For the evaluation of immune response, specific antibodies against each of challenge
136 viruses were detected using the HI test with 25 µl of collected sera according to the standard
137 protocol. All of the infected animals were kept in self-contained isolator units (Tokiwa
138 Kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary
139 Medicine, Hokkaido University, Japan.

140

141 2.3. Virus titration

142 Madin-Darby canine kidney (MDCK) cells were maintained in MEM supplemented with
143 0.3 mg/ml L-glutamine, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 8 µg/ml gentamicin and
144 10% calf serum. Tenfold dilutions of viruses were inoculated onto confluent monolayers of
145 MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were removed, and the cells
146 were then washed with phosphate-buffered saline (PBS). The cells were then overlaid with
147 MEM containing 1% Bacto-agar (Life Technologies, Carlsbad, CA, USA) in the presence of
148 5 µg/ml trypsin acetylated (Sigma Aldrich, St. Louis, MO, USA) both in assays with HPAIVs
149 and LPAIVs. After 48 h of incubation at 35 °C, the cells were stained with 0.005% neutral red.
150 After 24 h, visible plaques were counted. Since Tw/0502 did not form visible plaque in
151 MDCK cells, virus titer of Tw/0502 was measured based on the 50% tissue culture infectious
152 dose (TCID₅₀) using MDCK cells. In brief, tenfold dilutions of viruses were inoculated onto
153 confluent monolayers of MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were
154 removed, and the cells were then washed with PBS. Then MEM containing 5 µg/ml trypsin
155 acetylated was added to each well. After 72 h of incubation at 35 °C, the cytopathic effects of
156 cells were observed. Titers were calculated by the method of Reed and Muench (1938).
157 Considering a Poisson distribution, 1 TCID₅₀ of virus would be expected to be equivalent to
158 0.69 PFU of virus. Thereby, the theoretical value for PFU titer of Tw/0502 was calculated by
159 multiplying the measured TCID₅₀ titer by 0.69.

160

161 2.4. Ethics statements

162 All of the animal experiments were authorized by the Institutional Animal Care and Use
163 Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval
164 numbers: 11-0152, 13-0109). All of the experiments were performed according to the
165 guidelines of the committee.

166

167 **3. Results**

168 *3.1. Pathogenicity of HPAIVs and LPAIVs to chickens*

169 To examine the pathogenicity of HPAIVs and an H7N9 influenza virus recently isolated
170 in Asia to chickens, five HPAIVs, namely VN/559 of clade 1.1, Hok/4 of clade 2.3.2.1, HK/810
171 of clade 2.3.4, Km/1-7 of clade 2.3.4.4, and Tw/0502 of a classical Taiwanese HPAIV, as well as
172 a Chinese H7N9 influenza virus isolated from a diseased woman, Ah/1 and non-pathogenic
173 H5N1 strain Hok/Vac1 were intranasally inoculated into 4 chickens (Fig. 1a). All of the
174 chickens inoculated with VN/559, Hok/4, HK/810, or Tw/0502 died within 4 days post
175 inoculation (dpi). Three out of four chickens inoculated with Km/1-7 died within 4 dpi;
176 however one chicken survived for 14 days without exhibiting any clinical signs. It was notable
177 that this surviving chicken did not seroconvert after 14 dpi (Table 2). All of the chickens
178 inoculated with Ah/1 or Hok/Vac1 survived for 14 days. Other 4 chickens inoculated with
179 each of viruses were euthanized at 3 dpi, and virus titers in tissue samples were determined
180 (Table 3). Virus was recovered from each of the samples infected with VN/559, Hok/4,
181 HK/810, and Tw/0502, indicating that these viruses caused systemic infection. Virus recovery

182 was positive for 3 out of 4 chickens inoculated with Km/1-7 but the virus was not recovered
183 from the remaining chicken. Low titers of virus were recovered from the respiratory tracts of
184 chickens inoculated with Ah/1. No virus was recovered from the chickens inoculated with
185 Hok/Vac1.

186

187 *3.2. Pathogenicity of HPAIVs and LPAIVs to domestic ducks*

188 To examine the pathogenicity of these HPAIVs and LPAIVs to water fowls, the viruses
189 were intranasally inoculated into 4 domestic ducks (Fig. 1b). One out of four ducks inoculated
190 with VN/559 died at 5 dpi. One duck survived for 14 days and it exhibited depression from 5
191 to 8 dpi. The remaining two ducks did not exhibit any clinical signs. Two out of four ducks
192 inoculated with Hok/4 died at 5 dpi. One duck, which survived for 14 days, showed
193 depression from 5 to 7 dpi. The remaining one did not exhibit any clinical signs. One out of
194 four ducks inoculated with HK/810 died at 4 dpi. One duck that survived showed significant
195 neurological signs from 6 dpi until the end. The remaining two ducks survived without
196 showing any clinical signs. Ducks inoculated with the other viruses survived for 14 days
197 without showing any clinical signs. Interestingly, all of the ducks inoculated with Km/1-7
198 seroconverted after 14 dpi, whereas none of the ducks inoculated with Tw/0502 seroconverted
199 (Table 2). Other 4 ducks inoculated with each of the viruses were euthanized at 3 dpi, and
200 virus titers in tissue samples were determined (Table 4). Viruses were recovered from each of
201 the ducks inoculated with VN/559, Hok/4, or Km/1-7. Viruses were also recovered from the

202 brain samples of some ducks inoculated with these viruses. Viruses were recovered from only
203 one duck inoculated with HK/810; however viruses were recovered from all of the swabs and
204 tissue samples from this bird. Low titers of virus were recovered from the trachea of the ducks
205 infected with Ah/1. No virus was recovered from the ducks inoculated with Tw/0502 or
206 Hok/Vac1.

207

208 *3.3. Pathogenicity of HPAIVs and LPAIVs to tree sparrows*

209 All of the sparrows inoculated with VN/559, Hok/4, HK/810, or Km/1-7 died within 10
210 dpi (Fig. 1c). Notably, the sparrows died suddenly without exhibiting detectable clinical signs
211 until 1 or 0.5 days before death. Immediately before death, these sparrows exhibited
212 depression and astasia. Only one sparrow inoculated with Tw/0502 died at 12 dpi; whereas the
213 remaining sparrows survived throughout observation period without any clinical signs. All of
214 the sparrows inoculated with Ah/1 or Hok/Vac1 survived for 14 days and some of them
215 seroconverted at 14 dpi, thereby suggesting that the sparrows were infected with these virus
216 strains (Table 2). Viruses were recovered from the blood or brain samples of all the sparrows
217 infected with VN/559, Hok/4, or HK/810 at 3 dpi (Table 5). However, viruses were recovered
218 from the blood or brain samples of 2 out of 4 sparrows inoculated with Km/1-7 although viruses
219 were recovered from each of the four sparrows inoculated. Growth of the Tw/0502 was
220 limited in the respiratory and intestinal tracts, which are the primary replication sites of
221 influenza viruses, suggesting that these cases were not systemic infections. No virus was

222 recovered from sparrows inoculated with Ah/1 or Hok/Vac1.

223

224 *3.4. Pathogenicity of HPAIVs and LPAIVs to jungle crows*

225 All of the jungle crows inoculated with HK/810 died within 7 days (Fig. 1d).

226 Remarkably, these crows did not exhibit clear clinical signs 2 days before death, and they

227 started to show clear clinical signs such as depression, anorexia, and neurological signs up to 1

228 or 0.5 days before death. Half of the crows inoculated with VN/559 or Km/1-7 died. Crows

229 inoculated with VN/559 exhibited depression. On the other hand, crows infected with Km/1-7

230 died without showing detectable clinical signs. One crow infected with Hok/4 exhibited slight

231 clinical signs of anorexia from 5 to 8 dpi. Crows inoculated with Tw/0502 and Ah/1 survived

232 for 14 days without showing any clinical signs; however all of the surviving crows

233 seroconverted at 14 dpi (Table 2). No crow was seroconverted after inoculation with

234 Hok/Vac1 at 14 dpi, which suggests that this virus did not infect the crows. Viruses were

235 recovered from the brains of crows infected with HK/810 or Km/1-7 (Table 6). In contrast,

236 only low titers of viruses were recovered from the lungs of the crows infected with VN/559.

237 Viruses were recovered from only 1 out of 4 crows inoculated with Hok/4. Low titers of virus

238 were recovered from the oral swabs of 2 out of 4 crows infected with Ah/1. No virus was

239 recovered from crows inoculated with Tw/0502 or Hok/Vac1.

240

241 *3.5. Pathogenicity of HPAIVs and an H7N9 influenza virus to black rats*

242 All of the black rats inoculated with VN/559, Hok/4, HK/810, Km/1-7, Tw/0502, Ah/1, or
243 Hok/Vac1 survived throughout the 14 day observation period without showing any visible
244 clinical signs (Fig. 1e). Black rats inoculated with these viruses seroconverted at 14 dpi,
245 except those with Km/1-7 and Hok/Vac1 (Table 2). Only low titers of virus were recovered
246 from the kidney and colon of one of four black rats inoculated with Hok/4 (Table 7). No virus
247 was recovered from the other black rats inoculated with VN559, HK/810, Km/1-7, Tw/0502,
248 Ah/1, or Hok/Vac1.

249

250 **4. Discussion**

251 In the present study, we analyzed the susceptibility of chickens and ducks, as well as
252 common synanthropes, namely tree sparrows, jungle crows, and black rats, to infection with
253 influenza viruses to assess the potential roles of these animals in influenza virus transmission
254 from wild migratory ducks to domestic poultry. VN/559, Hok/4, HK/810, and Tw/0502 caused
255 lethal infections to all chickens inoculated with $10^{6.0}$ EID₅₀ of each virus (Fig. 1a). In contrast,
256 1 out of 4 chickens survived for 14 days after the inoculation with Km/1-7. The lack of any
257 detectable antibody response in this chicken suggests that the infectivity of Km/1-7 to chickens
258 is relatively low compared with other HPAIVs (Table 2). This finding is well consistent with a
259 previous report by Kanehira et al. (2015). A previous study demonstrated that 50% lethal dose
260 of Km/1-7 to chickens was $10^{5.8}$ EID₅₀, which is comparable to challenge dose in the present
261 study (Gamoh et al., 2015). Thus the chicken survived without detectable antibody response.

262 On the other hand, the remaining 3 chickens died by 4 dpi, thereby indicating that chickens are
263 going to die in case where virus infection occurs. In contrast, ducks were highly susceptible to
264 infection with Km/1-7 (Table 4), which suggests that this virus strain is readapted to water fowls,
265 and thus it has decreased infectivity to chickens. Notably, none of the ducks infected with
266 Km/1-7 exhibited clinical signs. These findings suggest that ducks infected with this virus
267 strain can transfer viruses during their migration, resulting in the widespread dissemination of
268 clade 2.3.4.4 viruses (Hall et al., 2015). None of the ducks seroconverted after infection with
269 Tw/0502, which suggests that ducks were not infected with this virus strain (Table 2).
270 Classical Taiwanese HPAIVs, where the HA and NA genes belong to the North American
271 lineage, are endemic in Taiwan, whereas no HPAI outbreaks caused by this strain have been
272 reported outside Taiwan (Lee et al., 2014). In contrast to the viruses of clade 2.3.4.4, the low
273 susceptibility of water fowls to this strain might help to explain the limited dissemination of
274 classical Taiwanese HPAIVs.

275 Tree sparrows were highly susceptible to infection with VN/559, Hok/4, HK/810, and
276 Km/1-7 (Fig. 1c, Table 5). The natural infections of tree sparrows with H5N1 HPAIVs have
277 been previously reported. For example, an H5N1 HPAIV was isolated from a dead sparrow
278 found in a park within the quarantine area during an HPAI outbreak in Hong Kong in 2002
279 (Ellis et al., 2004). In addition, H5N1 HPAIVs were isolated from sparrows found near
280 traditional duck farms in Indonesia during 2010 (Poetranto et al., 2011). These findings
281 suggest that tree sparrows are susceptible to HPAIV infections in natural settings. A previous

282 study also demonstrated that sparrows infected with $10^{6.0}$ EID₅₀ of HPAIVs of clade 2.2 or 2.3.2
283 shed $10^{2.5}$ – $10^{6.5}$ EID₅₀/ml of viruses from oral swabs, which is sufficient to transmit viruses to
284 contact chickens (Yamamoto et al., 2013). It is noteworthy that the majority of the dead
285 sparrows had not shown any detectable clinical signs until they died. In addition, sparrows
286 inoculated with VN/559, Hok/4, HK/810, or Tw/0502 survived for significantly longer period
287 than chickens ($p < 0.05$; Fig. 1a, c). Considering that high titer of viruses were recovered from
288 the dead birds, it is possible that sparrows infected with HPAIVs may shed viruses without
289 showing clinical signs during their slightly longer incubation period. This suggests that
290 sparrows infected with HPAIVs may play a role as a vector in virus transmission from wild
291 migratory ducks to chickens. Interestingly, virus growth of Tw/0502 in sparrows was limited
292 in the respiratory and intestinal tracts, which is common in avian influenza virus infections
293 (Table 5). This was also the case even in the dead sparrow at 12 dpi when infected with
294 Tw/0502, where viruses were recovered from oral and cloacal swabs as well as the colon (data
295 not shown). The virus and host factors involved in systemic infection in these small birds are
296 not known. On the other hand, evidence of systemic infections in small birds such as tree
297 sparrows is a risk factor for virus invasion into poultry houses via these birds. Thus, further
298 studies should determine the factors related to virus pathogenicity to these birds.

299 The pathogenicity of HPAIVs to jungle crows depended on the virus strains (Fig. 1d). In
300 general, virus growth in crows was less drastic than that in sparrows (Table 5, 6); however virus
301 titers in tissue samples of dead crows infected with HK/810 reached at most $10^{7.6}$ plaque

302 forming units (PFU)/g in the brain (data not shown). In 2004, HPAIVs belonging to clade 2.5
303 were isolated from dead crows in Japan (Tanimura et al., 2006). The crows were found at 30
304 km distant from the outbreak site and approximately 1 month later than the last outbreak. In
305 addition, a crow die-off in crow roosts was reported in 2011 during an HPAI outbreak caused by
306 clade 2.3.2.1 viruses in Bangladesh (Khan et al., 2014). These facts suggest that HPAIVs
307 continued to circulate among crow population for a certain period. Similar to sparrows, crows
308 infected with VN/559, Hok/4, HK/810, or Tw/0502 survived for significantly longer period than
309 chickens ($p < 0.05$; Fig. 1a, d). Accordingly, preventing direct contact between these birds and
310 poultry is important for reducing the risk of HPAIV invasions in poultry houses.

311 Compared with sparrows and crows, black rats were less susceptible to HPAIV infections
312 (Fig. 1e, Table 7). In Japan, three species of rodents are recognized as synanthropes: house
313 mice (*Mus musculus*), brown rats (*Rattus norvegicus*), and black rats (*Rattus rattus*). Among
314 them, black rats are found most commonly in poultry houses. Although a previous study
315 demonstrated that HI antibodies against H5 influenza virus was detected from wild rats collected
316 in swage system in Egypt (El-Sayed et al., 2013), there is no report, to our knowledge, on the
317 isolation of HPAIVs from black rats. In addition, experimental infection of HPAIVs in
318 laboratory rats, which originated from brown rats, demonstrated that virus growth is limited
319 (Shortridge et al., 1998). These facts suggest that *Rattus* species, which is the most common
320 small animal observed in poultry houses, are resistant to HPAIV infections.

321 Growth of Ah/1, which is designated as an LPAIV, was highly limited in each animal

322 compared with that of the HPAIVs (Tables 3-7). Previously, a natural infection with a Chinese
323 H7N9 viruses in a tree sparrow was reported; however, the dissemination of these viruses in
324 wild birds is limited (Zhao et al., 2014). This may be related to the fact that the distribution of
325 this virus strain is limited to the southeast part of mainland China (Lam et al., 2015).
326 Nevertheless, multiple replication and the transmission of H5 and H7 LPAIVs in chicken
327 populations may result in generation of HPAIVs. Thus, the continued monitoring of H5 and
328 H7 LPAIVs in wild and domestic animals is recommended for the control of avian influenza.

329 Hok/Vac1, which is an H5N1 vaccine strain licensed in Japan is a reassortant virus between
330 non-pathogenic H5N2 and H7N1 viruses isolated from fecal samples of migratory ducks (Soda
331 et al., 2008). Although Hok/Vac1 is expected to have similar character to viruses circulating
332 among migratory ducks, no virus was recovered from ducks inoculated with Hok/Vac1 (Table 4).
333 On the other hand, ducks inoculated with this virus seroconverted at 14 dpi (Table 2). This
334 indicates that Hok/Vac1 infected the ducks and might slightly replicate; however, virus growth
335 of Hok/Vac1 was not brisk in these ducks, progeny viruses were not recovered from the tissue
336 samples.

337 In the present study, animals were inoculated with $10^{6.0}$ EID₅₀ of each virus. Serological
338 analyses indicates that 50% infectious doses of each HPAIV for each animal are $10^{6.0}$ EID₅₀ or
339 less except those of Tw/0502 for ducks and Km/1-7 for black rats. Chickens infected with
340 VN/559 shed up to $10^{9.1}$ PFU/ml of viruses from swabs and ducks infected with Hok/4 shed up
341 to $10^{5.9}$ PFU/ml of viruses at 3 dpi (data not shown). Considering that these titers are

comparable to 50% infectious doses of each HPAIV for each animal, HPAIVs may transmit from chickens and ducks to sparrows, crows, and black rats in natural settings. On the other hand, although animals were seroconverted after the infection with $10^{6.0}$ EID₅₀ of Ah/1 or Hok/Vac1, none of animals inoculated with Hok/Vac1 shed virus (Table 3-7) and virus shedding from animals infected with Ah/1 was at most $10^{1.7}$ PFU/ml in an oral swab sample of a crow (data not shown). Accordingly it cannot be assumed that these LPAIVs are successfully transmitted from a flock to another via sparrows, crows, and black rats.

The present results illustrate the importance of wild synanthropes for virus invasion into poultry houses in HPAIV transmission from wild migratory ducks to chickens. The infectivity of HPAIVs to chickens varies depending on the strains; generally 10^3 – 10^4 EID₅₀ is estimated as the 50% lethal dose in chickens (Shichinohe et al., 2013). In sparrows and crows, higher titers of virus were recovered when compared with the lethal dose in chickens. On the other hand, virus growth was less drastic in black rats. Accordingly, avian synanthropes have a higher risk of introducing viruses into poultry houses. Although these birds play a role in the transmission of HPAIVs, the biosecurity system which protects poultry houses from virus invasion along with human activities is fundamental for the prevention of HPAI outbreaks. Also, the early detection of outbreaks and a stamping out policy will reduce the possibility that viruses spill out into wild animals, which leads secondary outbreaks. Thus, these golden standard countermeasures for the prevention of HPAI outbreaks must be essential for the successful eradication of HPAIVs.

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363

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515

516

517 **Table 1.** Influenza viruses used in the present study

Viruses	Abbreviations	Genetic clades	References
A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)	VN/559	1.1	Okamatsu et al., 2013
A/whooper swan/Hokkaido/4/2011 (H5N1)	Hok/4	2.3.2.1	Sakoda et al., 2012
A/peregrine falcon/Hong Kong/810/2009 (H5N1)	HK/810	2.3.4	Shichinohe et al., 2013
A/chicken/Kumamoto/1-7/2014 (H5N8)	Km/1-7	2.3.4.4	Kanehira et al., 2015
A/chicken/Taiwan/0502/2012 (H5N2)	Tw/0502	NA	-
A/Anhui/1/2013 (H7N9)	Ah/1	NA	Gao et al., 2013
A/duck/Hokkaido/Vac-1/2004 (H5N1)	Hok/Vac1	NA	Soda et al., 2008

NA: clade definition is not applicable.

-: references are not available. DDBJ/EMBL/GenBank accession numbers are KJ720208- KJ720215.

518

519

520 **Table 2.** HI titers in serum of animals inoculated with each virus at 14 dpi

521	Viruses	Animals				
		Chickens	Ducks	Sparrows	Crows	Black rats
522	VN/559	-	128	-	512	64
		-	64	-	256	32
		-	32	-	-	16
		-	-	-	-	16
	Hok/4	-	64	-	1,024	64
		-	<2	-	512	32
		-	-	-	16	16
		-	-	-	4	16
	HK/810	-	64	-	-	32
		-	<2	-	-	32
		-	<2	-	-	16
		-	-	-	-	16
	Km/1-7	<2	64	-	64	<2
		-	32	-	<2	<2
		-	32	-	-	<2
		-	16	-	-	<2
	Tw/0502	-	<2	16	128	32
		-	<2	4	128	16
		-	<2	<2	32	16
		-	<2	-	8	16
	Ah/1	8	<2	16	512	64
		2	<2	8	64	64
		<2	<2	4	64	64
		<2	<2	<2	32	32
	Hok/Vac1	<2	128	16	<2	<2
		<2	128	2	<2	<2
		<2	64	<2	<2	<2
		<2	64	<2	<2	<2

-: the animal died before 14 dpi.

Each of four animals were inoculated with each virus strain.

523 **Table 3.** Virus recovery from chickens inoculated with each virus at 3 dpi

Viruses	No. of animals from which each virus was recovered [GM value of the virus titer (log 10)]						
	Swabs (log PFU/ml)		Tissue samples (log PFU/g)				
	Oral	Cloacal	Brain	Trachea	Lung	Kidney	Colon
VN/559 ^a	4 (6.7)	4 (6.8)	4 (7.6)	4 (7.5)	4 (7.6)	4 (8.0)	4 (7.7)
Hok/4	4 (6.9)	4 (6.1)	4 (7.7)	4 (8.4)	4 (8.9)	4 (8.7)	4 (6.9)
HK/810 ^a	4 (6.0)	4 (5.2)	4 (8.6)	4 (8.9)	4 (8.6)	4 (8.2)	4 (6.9)
Km/1-7	1 (2.4)	1 (2.0)	3 (4.2)	3 (4.7)	3 (4.9)	3 (4.9)	3 (5.4)
Tw/0502	4 (3.6)	4 (1.2)	4 (5.9)	4 (5.7)	4 (5.6)	4 (7.3)	4 (4.6)
Ah/1	4 (1.3)	0	0	2 (2.5)	0	0	0
Hok/Vac1	0	0	0	0	0	0	0

Virus titers are represented as the geometric mean (GM) value based on positive samples.

Each of four animals was inoculated with each virus strain.

^aSwabs and tissue samples were collected from dead birds at 2 dpi.

524

525 **Table 4.** Virus recovery from domestic ducks inoculated with each virus at 3 dpi

Viruses	No. of animals from which each virus was recovered [GM value of the virus titer (log 10)]							
	Swabs (log PFU/ml)		Blood (log PFU/ml)	Tissue samples (log PFU/g)				
	Oral	Cloacal		Brain	Trachea	Lung	Kidney	Colon
VN/559	1 (2.1)	1 (1.0)	0	3 (3.9)	3 (2.5)	4 (4.5)	4 (4.1)	4 (4.5)
Hok/4	4 (5.7)	1 (3.3)	1 (2.5)	2 (5.0)	4 (5.3)	3 (6.3)	4 (5.3)	4 (6.4)
HK/810	1 (5.6)	1 (3.7)	1 (6.1)	1 (7.4)	1 (7.4)	1 (7.5)	1 (9.8)	1 (7.4)
Km/1-7	4 (3.0)	2 (2.5)	3 (3.9)	3 (5.0)	4 (5.1)	4 (4.4)	4 (3.5)	4 (2.5)
Tw/0502	0	0	0	0	0	0	0	0
Ah/1	0	0	0	0	3 (2.4)	0	0	0
Hok/Vac1	0	0	0	0	0	0	0	0

Virus titers are represented as the GM value based on positive samples.

Each of four animals was inoculated with each virus strain.

526

527

528 **Table 5.** Virus recovery from tree sparrows inoculated with each virus at 3 dpi

Viruses	No. of animals from which each virus was recovered [GM value of the virus titer (log 10)]					
	Swabs (log PFU/ml)		Blood (log PFU/ml)	Tissue samples (log PFU/g)		
	Oral	Cloacal		Brain	Lung	Intestine
VN/559	0	0	4 (1.9)	4 (5.0)	4 (4.8)	4 (3.8)
Hok/4	2 (3.8)	0	3 (5.3)	4 (3.9)	4 (5.2)	4 (3.9)
HK/810	1 (1.2)	0	4 (3.0)	3 (4.3)	2 (4.7)	4 (3.1)
Km/1-7	2 (1.8)	0	2 (2.6)	1 (2.9)	3 (3.6)	1 (2.3)
Tw/0502	4 (1.6)	4 (1.4)	0	0	2 (2.4)	3 (2.7)
Ah/1	0	0	0	0	0	0
Hok/Vac1	0	0	0	0	0	0

Virus titers are represented as the GM value based on positive samples.

Each of four animals was inoculated with each virus strain.

529

530 **Table 6.** Virus recovery from jungle crows inoculated with each virus at 3 dpi

Viruses	No. of animals from which each virus was recovered [GM value of the virus titer (log 10)]							
	Swabs (log PFU/ml)		Blood (log PFU/ml)	Tissue samples (log PFU/g)				
	Oral	Cloacal		Brain	Trachea	Lung	Kidney	Colon
VN/559	0	0	0	0	0	2 (2.7)	0	0
Hok/4	0	0	0	0	0	1 (4.5)	1 (2.9)	1 (3.1)
HK/810	2 (1.8)	1 (3.6)	0	3 (4.2)	4 (4.0)	4 (5.4)	3 (4.9)	4 (4.3)
Km/1-7	3 (2.4)	4 (1.8)	2 (1.4)	2 (3.1)	1 (2.6)	2 (2.6)	0	0
Tw/0502	0	0	0	0	0	0	0	0
Ah/1	2 (1.6)	0	0	0	0	0	0	0
Hok/Vac1	0	0	0	0	0	0	0	0

Virus titers are represented as the GM value based on positive samples.

Each of four animals was inoculated with each virus strain.

531

532

533 **Table 7.** Virus recovery from black rats inoculated with each virus at 3 dpi

Viruses	No. of animals from which each virus was recovered					
	[GM value of the virus titer (log 10)]					
	Blood	Tissue samples (log PFU/g)				Feces
	(log PFU/ml)	Brain	Lung	Kidney	Intestine	(log PFU/g)
VN/559	0	0	0	0	0	0
Hok/4	0	0	0	1 (3.0)	1 (3.0)	0
HK/810	0	0	0	0	0	0
Km/1-7	0	0	0	0	0	0
Tw/0502	0	0	0	0	0	0
Ah/1	0	0	0	0	0	0
Hok/Vac1	0	0	0	0	0	0

Virus titers are represented as the GM value based on positive samples.

Each of four animals was inoculated with each virus strain.

534

535

536 **Figure legends**

537 **Figure 1.** Survival of animals inoculated with each virus. Chickens (a), ducks (b), tree
538 sparrows (c), jungle crows (d), and black rats (e) were inoculated with $10^{6.0}$ EID₅₀ of VN/559
539 (white circle with solid line), Hok/4 (black circle with solid line), HK/810 (white triangle with
540 solid line), Km/1-7 (black triangle with solid line), Tw/0502 (white diamond with solid line),
541 Ah/1 (black circle with dashed line), or Hok/Vac1 (white circle with dashed line). Inoculated
542 animals were observed to detect clinical signs for 14 days after the challenge.