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

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Abstract

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

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

1. Introduction

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

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

2. Materials and methods

2.1. Viruses

A/muscovy duck/Vietnam/OIE-559/2011 (H5N1) (VN/559) was isolated from a muscovy duck in a live bird market of Vietnam in our previous study (Okamatsu et al., 2013). A/whooper swan/Hokkaido/4/2011 (H5N1) (Hok/4) was isolated from dead whooper swan in 2011 (Sakoda et al., 2012). A/peregrine falcon/Hong Kong/810/2009 (H5N1) (HK/810) was kindly provided by Dr. Luk S.M. Geraldine of the Tai Lung Veterinary Laboratory, Hong Kong SAR. A/chicken/Kumamoto/1-7/2014 (H5N8) (Km/1-7) was kindly provided by Dr. Takehiko Saito of the National Institute of Animal Health, Japan (Kanehira et al., 2015). A/chicken/Taiwan/0502/2012 (H5N2) (Tw/0502), which is a classical Taiwanese HPAIV, was kindly provided by Animal Health Institute, Taiwan. A/Anhui/1/2013 (H7N9) (Ah/1) was kindly provided by Dr. Masato Tashiro of the National Institute of Infectious Diseases, Japan (Gao et al., 2013). A/duck/Hokkaido/Vac-1/2004 (H5N1) (Hok/Vac1), which is a reassortant 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 intranassally 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 intranassally inoculated with 30 μl of virus solution containing $10^{6.0}$

EID_{50} of each virus. Black rats were intranasally inoculated with 30 μl of virus solution containing $10^{6.0}$ EID_{50} of each virus in anesthetized conditions. At 3 dpi, four individuals of each type of inoculated animals were euthanized, and oral and cloacal swabs (chickens, ducks, sparrows, and crows), blood samples (ducks, sparrows, crows, and black rats), as well as brain (all animals), lung (all animals), kidney (chickens, ducks, crows, and black rats), colon (chickens, ducks, and crows), large intestine (sparrows and black rats), and feces (black rats) were collected. Since all of chickens inoculated with VN/559 or HK/810 died at 2 dpi, swabs and tissue samples were collected from the dead birds. To prepare a 10% suspension with Minimum Essential Medium (MEM; Nissui Pharmaceutical, Tokyo, Japan), tissue samples and feces were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The infectivity titers for swabs, blood samples, tissue samples, and feces were calculated by plaque assays or $TCID_{50}$. The other four animals were observed clinically for 14 days after inoculation. The Kaplan–Meier method with log-rank test was applied to compare survival curves. For the evaluation of immune response, specific antibodies against each of challenge viruses were detected using the HI test with 25 μl of collected sera according to the standard protocol. All of the infected animals were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

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 ($TCID_{50}$) 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 $TCID_{50}$ 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 $TCID_{50}$ titer by 0.69.
160
161 *2.4. Ethics statements*

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

3. Results

3.1. Pathogenicity of HPAIVs and LPAIVs to chickens

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

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

3.2. Pathogenicity of HPAIVs and LPAIVs to domestic ducks

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

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

4. Discussion

In the present study, we analyzed the susceptibility of chickens and ducks, as well as common synanthropes, namely tree sparrows, jungle crows, and black rats, to infection with influenza viruses to assess the potential roles of these animals in influenza virus transmission from wild migratory ducks to domestic poultry. VN/559, Hok/4, HK/810, and Tw/0502 caused lethal infections to all chickens inoculated with $10^{6.0}$ EID_{50} of each virus (Fig. 1a). In contrast, 1 out of 4 chickens survived for 14 days after the inoculation with Km/1-7. The lack of any detectable antibody response in this chicken suggests that the infectivity of Km/1-7 to chickens is relatively low compared with other HPAIVs (Table 2). This finding is well consistent with a previous report by Kanehira et al. (2015). A previous study demonstrated that 50% lethal dose of Km/1-7 to chickens was $10^{5.8}$ EID_{50}, which is comparable to challenge dose in the present 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

study also demonstrated that sparrows infected with $10^{6.0}$ EID_{50} of HPAIVs of clade 2.2 or 2.3.2 shed $10^{2.5}$–$10^{6.5}$ EID_{50}/ml of viruses from oral swabs, which is sufficient to transmit viruses to contact chickens (Yamamoto et al., 2013). It is noteworthy that the majority of the dead sparrows had not shown any detectable clinical signs until they died. In addition, sparrows inoculated with VN/559, Hok/4, HK/810, or Tw/0502 survived for significantly longer period than chickens ($p < 0.05$; Fig. 1a, c). Considering that high titer of viruses were recovered from the dead birds, it is possible that sparrows infected with HPAIVs may shed viruses without showing clinical signs during their slightly longer incubation period. This suggests that sparrows infected with HPAIVs may play a role as a vector in virus transmission from wild migratory ducks to chickens. Interestingly, virus growth of Tw/0502 in sparrows was limited in the respiratory and intestinal tracts, which is common in avian influenza virus infections (Table 5). This was also the case even in the dead sparrow at 12 dpi when infected with Tw/0502, where viruses were recovered from oral and cloacal swabs as well as the colon (data not shown). The virus and host factors involved in systemic infection in these small birds are not known. On the other hand, evidence of systemic infections in small birds such as tree sparrows is a risk factor for virus invasion into poultry houses via these birds. Thus, further studies should determine the factors related to virus pathogenicity to these birds.

The pathogenicity of HPAIVs to jungle crows depended on the virus strains (Fig. 1d). In general, virus growth in crows was less drastic than that in sparrows (Table 5, 6); however virus 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 rom 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 $10^{6.0}$ EID_{50} of each virus. Serological
338 analyses indicates that 50% infectious doses of each HPAIV for each animal are $10^{6.0}$ EID_{50} 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

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

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

Conflict of interest statement

None.

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518 **Tables**

519 **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.

520

521

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

523

Viruses	Animals				
	Chickens	Ducks	Sparrows	Crows	Black rats
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

524

-: the animal died before 14 dpi.

Each of four animals were inoculated with each virus strain.

525 **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.
[a]Swabs and tissue samples were collected from dead birds at 2 dpi.

526

527 **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	Tissue samples (log PFU/g)				
	Oral	Cloacal	(log PFU/ml)	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.

528

529

530 **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	Tissue samples (log PFU/g)		
	Oral	Cloacal	(log PFU/ml)	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.

531

532 **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	Tissue samples (log PFU/g)				
	Oral	Cloacal	(log PFU/ml)	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.

533

534

535 **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

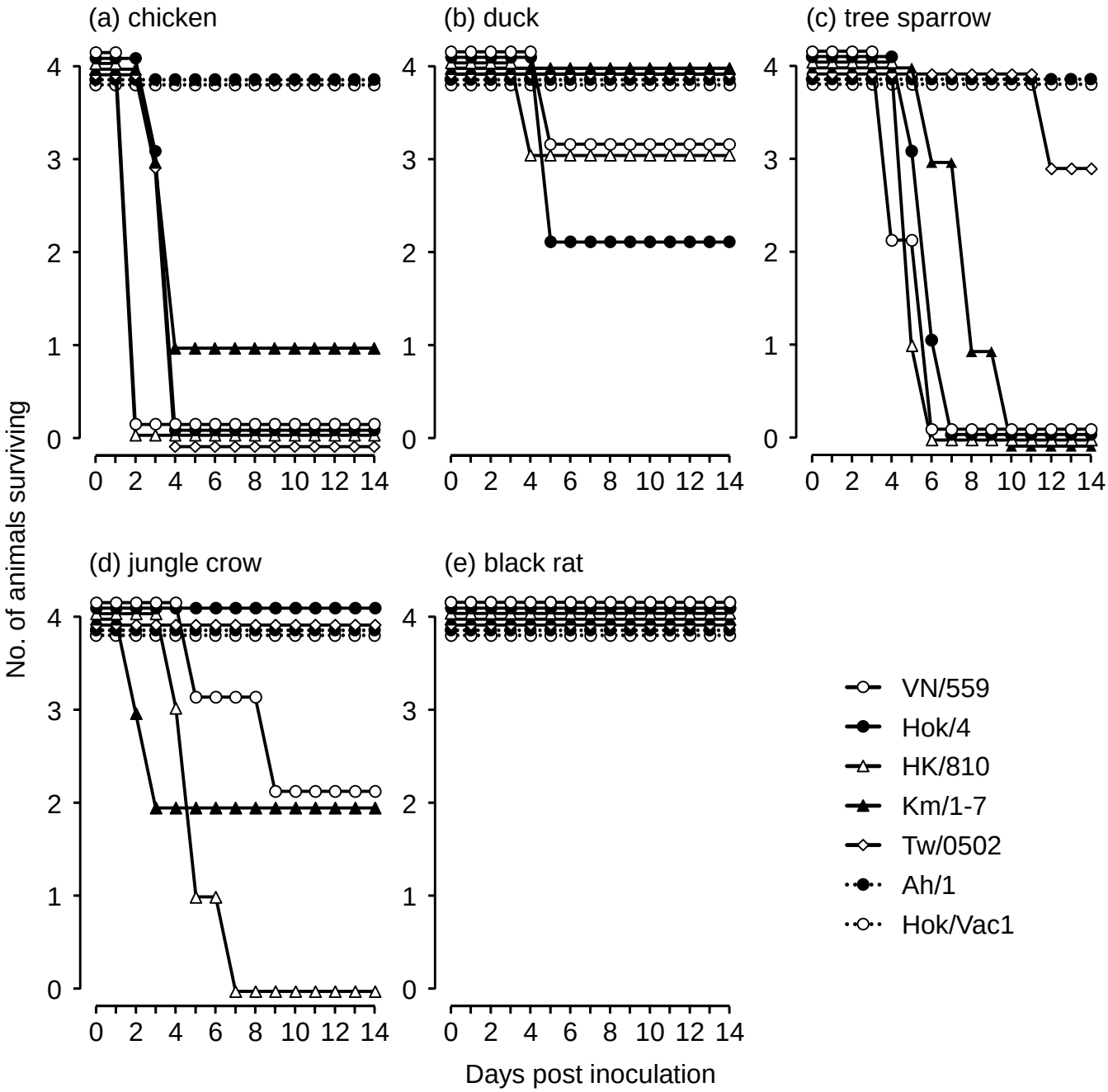
Virus titers are represented as the GM value based on positive samples.
Each of four animals was inoculated with each virus strain.

536

537

538 **Figure legends**

539 **Figure 1.** Survival of animals inoculated with each virus. Chickens (a), ducks (b), tree
540 sparrows (c), jungle crows (d), and black rats (e) were inoculated with $10^{6.0}$ EID_{50} of VN/559
541 (white circle with solid line), Hok/4 (black circle with solid line), HK/810 (white triangle with
542 solid line), Km/1-7 (black triangle with solid line), Tw/0502 (white diamond with solid line),
543 Ah/1 (black circle with dashed line), or Hok/Vac1 (white circle with dashed line). Inoculated
544 animals were observed to detect clinical signs for 14 days after the challenge.



Hiono *et al.* Figure 1