Phenotypes influencing the transmissibility of highly pathogenic avian influenza viruses in chickens

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To identify critical phenotypes that affect avian influenza virus transmission in chickens, we compared the transmissibility of three H5N1 highly pathogenic viruses of different pathogenicity in chickens by monitoring the exact time of death using wireless thermo-sensors. This study showed that, despite quick deaths, the most virulent H5N1 A/chicken/Yamaguchi/7/2004 transmitted quickly in chickens via contact and airborne routes. Intermediate virulent H5N1 A/chicken/Miyazaki/K11/2007 spread moderately and less virulent H5N1 A/duck/Yokohama/aq10/2003, causing severe clinical signs and a long period to death, spread slowly among the animals. The transmissibility was correlated with virus titres of oropharyngeal and cloacal swabs, and the time for swab virus titres to reach 50 % chicken infective dose affected the transmission speed. These results demonstrate that peak virus titres excreted and the time required for virus titres to reach a minimal chicken infectious dose may be the critical phenotypes influencing the transmissibility of highly pathogenicity avian influenza viruses in chickens.

The incidence of outbreaks of low-pathogenicity (LP) and high-pathogenicity (HP) avian influenza (AI) in domestic poultry has increased worldwide (Alexander, 2007b; Senne, 2007; Swayne & Halvorso, 2003), resulting in great economic losses in the poultry industry. The AI outbreaks in poultry also raise concerns about food safety and public health. From 2003 to March 2010, the H5N1 HPAI viruses caused 291 human deaths in 15 countries. Understanding the phenotypic determinants of the transmissibility of AI viruses in chickens is a prerequisite for better control of AI outbreaks in poultry farms. Previous studies have provided valuable insights into the transmission mechanisms of AI viruses in chickens (Alexander et al., 1986; Okamatsu et al., 2007; Swayne & Slemons, 2008; Tsukamoto et al., 2007; van der Goot et al., 2003; Westbury et al., 1981). These studies suggest that the important phenotypic determinants of the virus are pathogenicity, amount of virus shed into the environment, duration of virus shedding and chicken infectious doses. However, the phenotypic determinants are still obscure because multiple factors affect transmissibility of AI viruses in chickens such as virus strain (adaptation or pathogenicity) (Forman et al., 1986; Swayne & Slemons, 2008; van der Goot et al., 2003), bird infectious dose (Swayne & Slemons, 2008), age and weight of chickens (Tsukamoto et al., 2007), air circulation and the rearing system of a chicken house. Also ambient humidity and temperature affect transmission of influenza viruses to a greater extent than initially thought (Lowen et al., 2006, 2007, 2008). In a guinea pig model, both cold and dry conditions favour aerosol transmission, whereas high temperature (30 °C) or high humidity (80 %) blocks the transmission.

Currently, it is thought that LP viruses can transmit to chickens more efficiently than HPAI viruses because chickens infected with HPAI viruses have early deaths and the virus has a short excretion time (Alexander, 2007a). Westbury et al. (1981) reported that HP viruses spread slowly and failed to infect all chickens that had contact, whereas LP viruses spread quickly and infected all chickens that had contact. Our previous studies also support this hypothesis that transmission of a H5N1 HPAI virus is inefficient when a small number of infected chickens are used (Tsukamoto et al., 2007), while LPAI virus transmits efficiently (Okamatsu et al., 2007). Before HPAI outbreaks, the LPAI viruses usually spread broadly in the field. On the other hand, results from one paper clearly indicated that the HPAI virus transmits more efficiently than the LPAI virus (van der Goot et al., 2003). Studies on
the association between virus excretion kinetics and the transmissibility in chickens infected with AI viruses are limited (Forman et al., 1986). Therefore, further studies on the transmission mechanisms in chickens are necessary.

A wireless thermo-sensor is a tool to monitor fever and the exact time of death of each chicken infected with the HPAI virus (Suzuki et al., 2009). In this study, we used the wireless thermo-sensor to compare the transmissibility of three H5N1 HPAI viruses, of varied pathogenicity, in chickens by precisely monitoring the body temperature kinetics or the time of death of inoculated and control chickens.

Three H5N1 HPAI viruses were used in this study: A/chicken/Yamaguchi/7/2004 (H5N1) (genotype V, clade 2-5) (CkYM7), A/chicken/Miyazaki/K11/2007 (genotype Z, clade 2-2) (H5N1) (CkMZ11) and A/duck/Yokohama/aq 10/2003 (H5N1) (clade 6) (DkYK10). The most virulent virus, CkYM7, isolated from a chicken during the outbreak in Japan (Mase et al., 2005b) causes only severe depression and no fever with a short time to death (Suzuki et al., 2009). The intermediate virulent virus, CkMZ11, isolated from a chicken farm, induces slight gross lesions with an intermediate time to death. The least virulent virus, DkYK10, isolated in Japan from duck meat imported from China (Mase et al., 2005a), causes severe clinical signs, severe gross lesions, and high fever with a long time to death (Suzuki et al., 2009). The amino acid sequences at the haemagglutinin cleavage sites of CkYM7, CkMZ11 and DkYK10 are PQRERRKKR, PQGERRRKKR and PQRRERRRKKR, respectively. These viruses were propagated in the allantoic membrane of 10-day-old embryonated chicken eggs, and the 50 % egg infective dose (EID50) was determined by the method of Reed and Muench (1938). The HPAI viruses were handled in a biosafety level (BSL) 3 facility, following the biosafety manual for HPAI viruses. All animal experiments were approved by the Animal Ethics Committee of our institute.

The 50 % chicken infective dose (CID50) is the minimal infectious dose required for chicken infection and determined by the method of Reed and Muench (1938). Since those of CkYM7 and DkYK10 are described previously (Suzuki et al., 2009), the CID50 of CkMZ11 was determined. Serial 10-fold dilutions of CkMZ11, containing 101.0–106.0 EID50 0.1 ml−1, were inoculated intranasally into four 4-week-old specific-pathogen-free (SPF) chickens wearing a wireless thermo-sensor. The sensor developed for this study was composed of a sensor (1.2 g) and battery (1.8 g), and was attached to the surface of the abdominal skin of each chicken using sports tape. The sensor signal was continuously recorded by a computer every 20 s from 1 day before virus inoculation to the end of the experiment (7 days). Body temperature and time of death of each chicken were estimated as described previously (Suzuki et al., 2009). The presence of clinical signs and gross lesions was observed three times a day until all of the chickens died or for 7 days. Infection of each chicken was defined based on observations of clinical signs and gross lesions, kinetic curves of body temperature, and the time of death to prevent the artificial transmission by handling chickens for viral sampling. The average fevers and mean time of death (MTD) of each group are summarized in Table 1, and the CID50 of CkMZ11 was determined to be 102.5 EID50, which was the same as CkYM7 and DkYK10 (Suzuki et al., 2009). Fever in chickens infected with CkYM7 and CkMZ11 was slight, whereas it was high in those infected with DkYK10. MTD became shorter in proportion with increasing virus inoculum, while fever was consistent in each virus strain and not influenced by the virus amount. All of the surviving chickens were shown to be free from haemagglutination inhibition antibodies to CkMZ11 and no virus was isolated from lungs by using embryonated chicken eggs.

Transmissibility from inoculated chickens to control contact or separated chickens was compared among three HPAI viruses in a negative-pressure isolator. The isolator was divided into compartments A and B by a stainless wire mesh (1 cm square) to prevent direct contact of chickens (Tsukamoto et al., 2007). A total of 18 4-week-old chickens per strain wearing the sensor was divided into inoculated, contact and separated groups. The five inoculated and five contact chickens were placed in compartment A, while eight separated chickens were put in compartment B. The ambient temperature and humidity of the BSL3 room was 25 °C and 40 %, respectively, and feed and water were supplied automatically. To maintain the chance of virus transmission, corrugated paper was set on a mesh floor in each compartment to prevent the dropping of faeces to the bottom floor of the isolator. The five chickens in the inoculated group were intranasally administered with 106.0 EID50 0.1 ml−1 of each virus and returned to compartment A. Then, the isolators were kept closed throughout the experimental period (20 days) to prevent the artificial or accidental transmission of the virus to chickens by handling. Also, dead chickens were left in the isolators throughout the experimental period to keep the transmission condition the same as field outbreaks. Time of death of each chicken was determined as described previously (Suzuki et al., 2009), but in case of a broken sensor the time of death was estimated roughly by the clinical observations (∗ in Fig. 1).

Transmissibility in chickens was clearly different among the three HPAI viruses, and all of the chickens from the CkYM7, CkMZ11 and DkYK10 groups were dead within four, seven and 16 days after inoculation, respectively (Fig. 1). Observations of clinical signs and gross lesions and fever kinetic curves analysis confirmed that all chicken deaths were caused by the infection with these HPAI viruses and not associated with accidental death. CkYM7 transmitted the quickest of the three viruses to contact and separated chickens (Fig. 1a). The MTDs of the inoculated, contact and separated groups were 33.0 ± 5.2,
Table 1. MTD and fever of 4-week-old chickens after inoculation with different amounts of HPAI viruses

The four 4-week-old SPF chickens in each isolator were attached with a wireless thermo-sensor and inoculated intranasally with each dose of virus. These chickens were observed three times a day for 7 days. Sera collected from the veins of the wing of surviving chickens did not contain antibodies to AI viruses, as shown by an agar gel precipitation test.

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Amount of virus inoculated in each chicken (EID50)</th>
<th>CID50†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^1</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>Fever‡</td>
<td>CkYM7</td>
<td>0±0.3</td>
<td>0±0.5</td>
</tr>
<tr>
<td></td>
<td>CkMZ11</td>
<td>0±0.2</td>
<td>0±0.2</td>
</tr>
<tr>
<td></td>
<td>DkYK10</td>
<td>0±0.2</td>
<td>0±0.2</td>
</tr>
<tr>
<td>MTD§</td>
<td>CkYM7</td>
<td>&gt;168</td>
<td>&gt;168</td>
</tr>
<tr>
<td></td>
<td>CkMZ11</td>
<td>&gt;168</td>
<td>&gt;168</td>
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<tr>
<td></td>
<td>DkYK10</td>
<td>&gt;168</td>
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</tbody>
</table>

*CkYM7, A/chicken/Yamaguchi/7/2004 (H5N1); CkMZ11, A/chicken/Miyazaki/K11/2007 (H5N1); DkYK10, A/duck/Yokohama/aq10/2003 (H5N1).
†CID50, 50% chicken infectious dose.
‡Fever, difference in degrees of body temperature before and after virus inoculation in each chicken was calculated and the average of four chickens is shown above.
§MTD, mean time of death given in hours after virus inoculation.

64.9±7.7 and 82.8±14.2 h, respectively. Airborne transmission may have occurred in three of eight separated chickens († in Fig. 1a), since the MTD of the three chickens (66.2 h) was similar to that of the five contact chickens (64.9 h). The MTD of the five remaining separated chickens was 92.8 h. Transmission to contact chickens or separated chickens occurred all at once in CkYM7 and the transmission interval was around 30 h.

CkMZ11 spread at a moderate speed, and the MTDs of the inoculated, contact and separated groups were 51.4±3.3, 101.6±9.6 and 147.4±25.1 h, respectively (Fig. 1b). One separated chicken died at 98.3 h post-inoculation, probably through airborne transmission († in Fig. 1b), whereas the other five separated chickens died later (MTD = 155.6 h). The CkMZ11 also transmitted simultaneously to contact or separated chickens, and the transmission interval was around 50 h.

On the other hand, a different pattern of transmission emerged for DkYK10 (Fig. 1c). The death times varied widely among chickens, especially in separated chickens. The MTDs of the inoculated, contact and separated groups were 84.4±17.2, 165.2±26.1 and 285.5±67.3 h, respectively. One separated chicken († in Fig. 1c) died earlier than others, probably through airborne transmission because the time of death (166.3 h) was similar to that of contact chickens (165.2 h). We found that some chickens in the group pecked the feathers, skin or blood of dead chickens, suggesting these behaviours may contribute to the spread of DkYK10 to chickens.

To determine the association between virus excretion and transmissibility in chickens, we compared the virus excretion kinetics from chickens infected with one of the three H5N1 HPAI viruses. Five chickens were inoculated intranasally with CkYM7, CkMZ11 or DkYK10 at 10^{6.0} EID50 ml⁻¹, and oropharyngeal and cloacal swabs were collected at each time point and titrated for virus infectivity with embryonated chicken eggs. The titres were expressed as EID50 ml⁻¹ by the method of Reed and Muench (1938). The virus titres of both oropharyngeal and cloacal swabs continuously increased after inoculation and peaked at the time of death in all three strains (Fig. 2). The peak virus yields in both swabs differed considerably among virus strains, and those from oropharyngeal swabs were 10^{5.8±1.0} EID50 ml⁻¹ for CkYM7, 10^{4.7±0.2} EID50 ml⁻¹ for CkMZ11 and 10^{5.3±1.7} EID50 ml⁻¹ for DkYK10. In addition, the time for virus titres of both swabs to reach to CID50 (10^{-5} EID50 in all three viruses) was earliest in CkYM7 followed by CkMZ11 and then DkYK10.

The swab sampling to determine the infection of control birds made the chickens panic, which resulted in stirring up the dust in the isolator, leading to artificial transmission. Also, the basic reproduction number (R0) serves as an indicator to determine how many control animals are infected from one infected animal. If R0 is >1 disease can spread, while if R0 is <1 transmission chains will fade out. However, this method is not applicable when infection does not occur from one infected chicken as shown in CkYM7 (Tsukamoto et al., 2007) or all control animals are infected because the R0 is ∞. In contrast, the sensor system
is useful to compare the transmissibility of HPAI viruses by monitoring the exact time of death of each chicken with minimum artificial or accidental transmission. Our study demonstrates that transmissibility of HPAI viruses in chickens was directly associated with the time taken for the virus titre in excreted body fluids to reach the minimal infectious dose and the amount of virus excreted, but not with virus shedding time or MTD. Despite the early death of infected chickens, the most virulent CkYM7 was quickly and largely excreted from chickens and resulted in quick transmission to chickens compared with the other two HPAI viruses. Also, the CID_{50} serves as a phenotypic determinant for transmissibility in chickens (Swayne & Slemons, 2008). Since three HPAI virus strains tested had the same CID_{50}, the present study shows that two phenotypes identified in this study may play a key role in transmissibility of HPAI viruses in chickens. Our study also shows that a swab virus kinetic or swab virus titration at the time of death, in addition to CID_{50} of the strain, are appropriate methods to determine the transmissibility of AI viruses in chickens.

The present study suggests that HPAI viruses may transmit more efficiently than LPAI viruses in chickens; however, this conclusion seems inconsistent with the current understanding of transmission (Alexander, 2007a, b; Alexander et al., 1986; Swayne & Halvorso, 2003). The HPAI virus, A/duck/Victoria/75 (H7N6), fails to infect all contact chickens, whereas the LPAI virus, A/duck/Victoria/76 (H7N6), spreads to all contact birds (Westbury et al., 1981). This discrepancy may be explained by the difference in tissue tropism between the two AI viruses. Some HPAI viruses can replicate more efficiently in the brain and/or heart rather than in respiratory and/or digestive tracts (Swayne, 1997, 2007), whereas LPAI viruses replicate only in these tracts. Such HPAI viruses cause early death of chickens in spite of low virus shedding from tracts. The swab virus titre of DkYK10, which was equivalent to that of...
LPAl viruses, was maintained at less than CID50 just before the time of death, but DkYK10 caused early death of chickens probably by the efficient replication in the brain (unpublished data). Since the MTD of A/duck/Victoria/75 (7 days) (Westbury et al., 1981) is similar to DkYK10 (5.6 days) (data not shown), it is likely that A/duck/Victoria/75 (HPAl virus) may not replicate well in the respiratory and/or digestive tracts. Thus, tissue tropism of HPAl viruses may influence transmissibility. Also, the transmission mechanisms of AI viruses in chickens may be more complex than the mammalian model, in which there is positive association between virus excretion and transmissibility in the animals (Lowen et al., 2006, 2007, 2008; Maines et al., 2006).

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