Antigenicity and transmissibility of a novel clade 2.3.2.1 avian influenza H5N1 virus

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A genetic variant of the H5N1 influenza virus, termed subclade 2.3.2.1, was first identified in Bulgaria in 2010 and has subsequently been found in Vietnam and Laos. Several cases of human infections with this virus have been identified. Thus, it is important to understand the antigenic properties and transmissibility of this variant. Our results showed that, although it is phylogenetically closely related to other previously characterized clade 2.3 viruses, this novel variant exhibited distinct antigenic properties and showed little cross-reactivity to sera raised against other H5N1 viruses. Like other H5N1 viruses, this variant bound preferentially to avian-type receptors, but contained substitutions at positions 190 and 158 of the haemagglutinin (HA) protein that have been postulated to facilitate HA binding to human-type receptors and to enhance viral transmissibility among mammals, respectively. However, this virus did not appear to have acquired the capacity for airborne transmission between ferrets. These findings highlight the challenges in selecting vaccine candidates for H5N1 influenza because these viruses continue to evolve rapidly in the field. It is important to note that some variants have obtained mutations that may gain transmissibility between model animals, and close surveillance of H5N1 viruses in poultry is warranted.

INTRODUCTION

Highly pathogenic avian influenza H5N1 viruses continue to spread globally among birds, resulting in occasional transmission of the virus from infected poultry to humans. Genetic analyses have indicated that multiple genotypes of the H5N1 virus have evolved since 1997 (Guan et al., 2002). It is not clear whether the genetic variations in the HA protein are a result of immune escape or are associated with host adaptation (Yamada et al., 2006). It is therefore difficult to predict the best H5N1 epitopes to target with vaccines without understanding the antigenicity of emerging strains. As H5N1 influenza viruses have expanded their geographical and host ranges, it has become increasingly important to determine whether these viruses have acquired characteristics that allow efficient transmission between humans. To date, the transmission of H5N1 viruses to humans has been inefficient, occurring only through close contact with infected poultry or through the consumption of undercooked meat or blood from infected birds (Beigel et al., 2005). In addition, there have been limited reports of cases of probable human-to-human transmission (Beigel et al., 2005; Tran et al., 2004; Ungchusak et al., 2005). However, the potential routes and determinants required for H5N1 influenza virus transmission between humans are poorly understood. The receptor distribution in the human airway has been proposed to restrict the interhuman transmission of H5N1 influenza viruses (Shinya et al., 2006). Human influenza viruses specifically recognize α2,6-linked sialic acid receptors, which are dominant on epithelial cells in the upper respiratory tract. In contrast, avian influenza viruses specifically recognize α2,3-linked sialic acid receptors, which are located in the lower respiratory tract (Shinya et al., 2006; van Riel et al., 2006, 2007) and are not easily reached by the large droplets (diameter >10 μm) produced

by coughing or sneezing (Bridge et al., 2003). However, a small number of H5N1 virus isolates from humans exhibit limited binding to human-type receptors (Auweraraku et al., 2007; Gambryan et al., 2006; Stevens et al., 2008; Wang et al., 2010; Watanabe et al., 2011; Yamada et al., 2006), a property conferred by several amino acid changes in the haemagglutinin (HA) protein. To date, none of the natural H5N1 viruses that have been tested have demonstrated efficient transmission in the ferret model (Belser et al., 2007; Jackson et al., 2009; Maines et al., 2006; Yan et al., 2007), although in 2012, Kawaoka and Fouchier and co-workers showed that the A/Indonesia/5/2005 H5N1 virus required as few as five amino acid substitutions to become transmissible between ferrets via respiratory droplets (Herfst et al., 2012), and the A/Vietnam/1203/2004 strain required four substitutions and reassortment (Imai et al., 2012).

On 15 December 2011, a novel H5N1 virus was isolated from a dead wild bird in Hong Kong. On 31 December 2011, a male 39-year-old bus driver from Shenzhen, China, which is adjacent to Hong Kong, died from avian influenza. This man lived close to the Hong Kong Wetland Park, and therefore it is possible that he had contact with wild birds and became infected. The sequencing results showed that there was high homology between the viruses isolated from the deceased patient and the dead bird. This case suggests that the continued endemicity of the H5N1 influenza virus in wild birds increases the likelihood of the introduction of the virus into human populations.

In this study, we first performed a phylogenetic analysis of this novel H5N1 strain. We found that this wild bird-derived virus belongs to clade 2.3.2.1. Because there have been only a few published studies investigating this virus clade (Choi et al., 2013; Hu et al., 2013; Marinova-Petkova et al., 2012; Tung et al., 2013), we next systematically examined the antigenicity, transmissibility, receptor-binding specificity, and antiviral sensitivity of this novel virus. This type of comprehensive characterization of emerging viruses is necessary to understand the evolution of H5N1 viruses and provides information that is important for preventing and controlling potential pandemics in the future.

**RESULTS**

**Phylogenetic and sequence analysis**

Phylogenetic analysis based on the HA protein showed that A/wild bird/Hong Kong/07035−1/2011 virus belongs to clade 2.3.2.1 (Fig. 1), as defined by the World Health Organization (WHO) H5N1 Evolution Working Group (WHO, 2013).

The sequences of the HA and neuraminidase (NA) genes of A/wild bird/Hong Kong/07035−1/2011 virus were determined. The amino acid sequence of the HA cleavage site in this virus was RRRRRK/GLF. This site contains six basic amino acids, a feature that is characteristic of highly pathogenic influenza viruses (Callan et al., 1997). The critical amino acid sites in the receptor-binding pocket of the HA protein were G225, Q226, S227 and G228 (H3 numbering), which suggested that this virus may preferentially bind to the avian α2,3-linked sialic acid receptors (Imai & Kawaoka, 2012) (Table 1). However, the virus also exhibited substitutions at positions 190 and 158 of HA that have been postulated to enhance binding to human-type receptors and to facilitate viral transmissibility among mammals, respectively (Auweraraku et al., 2007; Imai et al., 2012). None of the established markers of NA inhibitor resistance (E119A, H274Y and N294S; N2 numbering) were present in the NA protein of this virus, suggesting that this virus is probably still sensitive to antiviral drugs. However, all of these viral characteristics predicted based on amino acid sequences must be verified experimentally.

**Antigenicity evaluation**

Ferret antiserum is commonly used for the evaluation of influenza virus antigenicity (Smith et al., 2004; WHO, 2013). The novel virus A/wild bird/Hong Kong/07035−1/2011 was tested for reactivity to antisera raised against A/Vietnam/1194/2004 (clade 1) and A/Shenzhen/406H/2006 (clade 2.3.4). Both ferret antisera raised against other clades reacted poorly (titre <10) to the A/wild bird/Hong Kong/07035−1/2011 virus. Ferret antiserum against A/wild bird/Hong Kong/07035−1/2011 was also produced, and its cross-reactivity with the A/Vietnam/1194/2004 and A/Shenzhen/406H/2006 viruses was assessed using a haemagglutination inhibition (HI) assay. Consistent with the other cross-reactivity results, A/wild bird/Hong Kong/07035−1/2011 virus-specific ferret antiserum showed little cross-reactivity (titre <40) with the clade 1 and 2.3.4 viruses (Table 2).

**Oseltamivir resistance test**

To determine whether the novel H5N1 virus has retained its sensitivity to antiviral drugs, we determined the IC50 value of oseltamivir for this virus, according to the method of Jain et al. (2009). The results showed that A/wild bird/Hong Kong/07035−1/2011 virus was sensitive to the antiviral drug oseltamivir (Table 3).

**Receptor-binding properties**

To characterize further the receptor-binding properties of the A/wild bird/Hong Kong/07035−1/2011 virus, we used solid-phase binding assays in which the virus was incubated with sialyglycopolymer-coated plates. The results showed that A/wild bird/Hong Kong/07035−1/2011 virus bound to the avian-type 3’SLN and 3’Di-LN receptors (Fig. 2a, b) but not the 6’SLN and 6’Di-LN receptors (Fig. 2c, d) (see Methods). The two control viruses, A/Shenzhen/406H/2006 and A/Brisbane/10/2007 (H3N2), selectively bound to the 3’ and 6’ substrates, respectively,
confirming the specificity and validity of the solid-phase binding assay.

**Pathogenicity and transmissibility in ferrets**

Two groups of three ferrets were inoculated intranasally with $10^4$ TCID$_{50}$ virus: one group was inoculated with A/wild bird/Hong Kong/07035-1/2011 virus and the other group with A/Vietnam/1194/2004 virus. The animals were observed for clinical signs and were weighed daily as an indicator of disease. Both viruses caused sneezing, ruffled fur, lethargy and decreased appetite in the ferrets. One ferret that had been inoculated with A/Vietnam/1194/2004 virus died at 4 days post-inoculation (p.i.); none of the ferrets that had been inoculated with A/wild bird/Hong Kong/07035-1/2011 virus died. The mean maximum weight loss was 10.3% for animals inoculated with A/wild bird/Hong Kong/07035-1/2011 virus and 27.8% for those inoculated with A/Vietnam/1194/2004 virus over the course of 9 days (Table 4, Fig. 3a). Virus shedding from the upper respiratory tract began at day 1 p.i. and continued until day 7 p.i. for both viruses. The levels of virus shedding in the upper respiratory tract, detected using nasal and throat swabs, were significantly higher in animals inoculated with A/Vietnam/1194/2004 virus than in animals inoculated with A/wild bird/Hong Kong/07035-1/2011 virus at all time points ($P<0.01$) (Fig. 3b). However, neither virus was transmitted from the inoculated ferrets to the naïve ferrets via respiratory droplets. No virus could be detected in either the nasal or throat swabs from the ferrets in the airborne contact group (Fig. 3b), and the convalescent sera (21 days post-exposure) of the contact ferrets was negative for virus-specific antibodies according to the HI assay.

**DISCUSSION**

The continued endemicity of highly pathogenic avian influenza H5N1 viruses in wild birds increases the likelihood of the introduction of the virus into human populations. In response to the rapid evolution of the Eurasian H5N1 viruses, the WHO/OIE/FAO H5N1 Evolution Working Group designed a nomenclature system for these viruses based on their phylogenetic characteristics and the nucleotide sequence divergence of the HA gene (WHO/OIE/FAO H5N1 Evolution Working Group, 2012). Since the ancestor virus was first identified in China in 1996 (Xu et al., 1999), 10 genotypes (clades 0–9) of H5N1 viruses have been isolated, and these viruses have further diverged into second-, third- and fourth-order clades. Several of these genotypes are still circulating and evolving, whereas others have been replaced by novel genetic variants (Chen et al., 2006; Salzberg et al., 2007; WHO/OIE/FAO H5N1 Evolution Working Group, 2012). In this study, a novel H5N1 virus isolated from a dead wild bird in Hong Kong was phylogenetically analysed. The results showed that this virus belonged to clade 2.3.2.1. Clade 2.3.2 is widely distributed throughout Asia, particularly in China, Hong Kong, Korea, Vietnam, Laos, Bangladesh, Nepal, Mongolia and the Tyva Republic. It is also found in Eastern Europe, primarily in Romania and Bulgaria. However, little is known about the antigenicity, pathogenesis, transmissibility, receptor-binding specificity and antiviral sensitivity of this clade. Therefore, we systematically examined the biological characteristics of the dead wild bird-derived clade 2.3.2.1 H5N1 virus in this study.

Antigenic drift is a continuous process in influenza A viruses in which point mutations arise in the viral genome during the replication cycle. This process is evident in all of the gene products of influenza; however, it is most pronounced in the antibody-binding sites of the HA and NA glycoproteins. Because of the lack of a proofreading mechanism, these mutations cannot be repaired, and the resulting accumulation of amino acid sequence changes alters these antigenic sites such that they are less recognizable by the host antibody response. Thus, these virus variants, which are no longer neutralized by host antibodies, are naturally selected for, because they lead to increased viral fitness (Boni, 2008; Treanor, 2004).

It is critical to determine whether the currently available vaccines and drugs can provide efficient protection or antiviral effects against this clade 2.3.2.1 virus. Antigenic drift prediction is one tool that can be used to increase the likelihood that a vaccine will match the circulating strain (Bush et al., 1999; Smith et al., 2004). The traditional approach, using antisera raised in immunized ferrets, is the gold standard for evaluating antigenic changes in human influenza viruses (Smith et al., 2004), and a similar methodology has been adopted for H5N1 virus, as recommended by the WHO (Chen et al., 2006; Smith et al., 2006; WHO, 2013). In this study, we immunized two ferrets twice with live A/wild bird/Hong Kong/07035-1/2011 virus, collected the produced antisera and analysed their cross-reactivity with the A/Vietnam/1194/2004 (clade 1) and A/Shenzhen/406H/2006 (clade 2.3.4) viruses. A/wild bird/Hong Kong/07035-1/2011 virus was also tested for cross-reactivity with antisera raised against A/Vietnam/1194/2004 and A/Shenzhen/406H/2006 viruses. Both experiments showed that there was little antibody cross-reactivity (titre <40) between the clade 2.3.2.1 virus and clade 1 and 2.3.4 viruses, demonstrating that there is
significant antigenic divergence between this novel H5N1 virus and conventional vaccine candidates. The vaccine candidate for clade 2.3.2.1 [A/Hubei/1/2010(H5N1)-PR8-IDCPC-RG30] has been available from the WHO since January 2012. However, its antigenic properties showed little reactivity (HI titre of only 5) with p.i. ferret antiserum against A/Anhui/1/2005 (clade 2.3.4) virus (WHO, 2012). Meanwhile, according to a report by Creanga et al. (2013), phylogenetic analysis showed that A/wild bird/Hong Kong/07035-1/2011 virus belongs to clade 2.3.2.1b (A/barn swallow/Hong Kong/1161/2010-like), and it has been shown that the NIBRG-14 vaccine (clade 1; A/Vietnam/1194/2004) can protect birds from clade 1 and clade 2.3.2.1a (A/Hubei/1/2010-like) infections but does not confer protection against clade 2.3.2.1b challenge virus (Tung et al., 2013). All the above data are consistent with our antigenicity results.

One promising result is that A/wild bird/Hong Kong/07035-1/2011 virus still displayed sensitivity to the antiviral drug oseltamivir. Sequence analysis of this virus also showed that none of the established markers of NA inhibitor resistance (E119A, H274Y and N294S; N2 numbering) is present in the NA protein of this virus.

Many events of direct transmission of H5N1 viruses from infected domestic or wild aquatic birds to mammalian hosts (humans, felids and stone martens) have been reported (Abdel-Ghafar et al., 2008; Beigel et al., 2005; Michaelis et al., 2009; Wang et al., 2008; Yingst et al., 2006). These direct bird-to-mammal transmission events may allow H5N1 viruses to gradually become adapted to and spread among mammalian species, including humans. The host restriction of the replication and transmission of influenza A viruses is partly determined by the specific sialic acid receptors on the surfaces of susceptible cells. The differences in receptor distribution between human and avian species are thought to be responsible for the host restriction of influenza A viruses. A switch in receptor specificity from avian a2,3-linked sialic acid receptors to human a2,6-linked sialic acid receptors is thought to be necessary for an avian virus to become transmissible between humans and thus gain the potential to cause a pandemic in humans (Herfst et al., 2012). To evaluate whether the clade 2.3.2.1 H5N1 virus isolated from the dead wild bird had the potential to be transmitted among humans through the upper respiratory tract, we examined the receptor-binding properties of this virus using a solid-phase binding assay. This assay showed that this clade 2.3.2.1 virus bound only to avian-type 3SLN or 3'S-Di-LN receptors and not to 6'SLN and 6'S-Di-LN receptors.

Sequence analysis showed that the critical amino acid sites in the receptor-binding pocket of the HA protein of A/wild bird/Hong Kong/07035-1/2011 virus are G225, Q226, S227 and G228 (H3 numbering), indicating that this virus probably binds preferentially to avian a2,3-linked sialic acid receptors (Imai & Kawaoka, 2012), consistent with our glycan assay results. However, the virus also displayed substitutions at positions 190 and 158 of HA that are postulated to facilitate binding to human-type HA receptors and to enhance viral transmissibility among mammals, respectively (Auewarakul et al., 2007; Imai et al., 2012). It is worth noting that neither of these two mutations has been reported in any other strains of clade 2.3.2.1 H5N1 viruses. The receptor-binding domain (RBD) of HA is formed by the 190-helix at the top of HA, the 220-loop at the edge of the globular head and the 130-loop at the other edge of the globular head. Amino acid changes in and around the RBD dramatically alter the receptor-binding preferences of influenza viruses. The Q226L and G228S substitutions in the H2 and H3 HAs confer a complete switch from a2,3-linked to a2,6-linked glycan binding (Harvey et al., 2004; Herfst et al., 2012; Imai et al., 2012; Vines et al., 1998). In the case of H1 HAs, the E190D and D225G mutations are critical for the shift from a2,3-linked to a2,6-linked glycan recognition (Gamblin et al., 2004; Glaser et al., 2005; Stevens et al., 2006). These amino acid changes (E190D, D225G, Q226L and G228S) have not

### Table 1. Amino acids changes in the HA protein that confer human-type receptor recognition to H5N1 influenza viruses

Substitutions of N190D and E158D have been postulated to facilitate HA binding to human-type receptors and to enhance viral transmissibility among mammals, respectively.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acid position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>158</td>
</tr>
<tr>
<td>A/Vietnam/1194/2004 (clade 1)*</td>
<td>N</td>
</tr>
<tr>
<td>A/Indonesia/5/2005 (clade 2.1)†</td>
<td>N</td>
</tr>
<tr>
<td>A/Shenzhen/4061H/2006 (clade 2.3.4)‡</td>
<td>N</td>
</tr>
<tr>
<td>A/wild bird/Hong Kong/07035-1/2011 (clade 2.3.2.1)#</td>
<td>D</td>
</tr>
<tr>
<td>Human-type receptor-specific viruses</td>
<td>D</td>
</tr>
</tbody>
</table>

*GenBank accession no. for the HA gene: EF541402.
†GenBank accession no. for the HA gene: CY116646.
‡GenBank accession no. for the HA gene: EF137706.
#GenBank accession no. for the HA gene: KF572434.
been observed among avian H5N1 viruses isolated from humans in previous studies (Gambaryan et al., 2006; Harvey et al., 2004; Stevens et al., 2006; Yamada et al., 2006). However, in our study, the E190D substitution was found in this clade 2.3.2.1 H5N1 virus. It remains to be determined experimentally whether this virus has obtained the ability to be transmitted among mammals. Choi et al. (2013) reported that two Korean clade 2.3.2.1 H5N1 viruses isolated from mandarin duck and Eurasian eagle owl, respectively, have virulence markers for mallard ducks, such as 436Y in PB1 and 515T in the PA protein (Hulse-Post et al., 2007), and 51M, 56V and 87E in PB1–F2 (Marjuki et al., 2010). Both of these viruses exhibited 226Q and 228G in the RBD, and no mammalian virulence markers were found (Choi et al., 2013). Marinova-Petkova et al. (2012) reported that the NA protein of a clade 2.3.2.1 H5N1 virus isolated from common buzzards in Bulgaria had a 20-residue deletion in the stalk region (residues 49–68), which is thought to be required for influenza viruses to adapt from wild aquatic birds to domestic chickens (Matrosovich et al., 1999). This deletion in the NA protein was also found in another two clade 2.3.2.1 H5N1 viruses isolated from chicken and goose in Jiangsu province of China (Hu et al., 2013). The genome polymorphism of clade 2.3.2.1 H5N1 viruses, especially those molecular markers in host and virulence determinant domains, and their relationship with virus pathogenicity and transmissibility, needs to be further studied.

Table 2. Antigenicity of A/wild bird/Hong Kong/07035-1/2011 virus analysed by HI test using ferret antiserum

This novel virus exhibited little cross-reactivity to sera raised against other H5N1 viruses, and this virus-specific ferret antiserum also showed little cross-reactivity with other H5N1 viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ferret antiserum titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Vietnam/1194/2004 (clade 1)</td>
<td>A/Shenzhen/406H/2006 (clade 2.3.4)</td>
</tr>
<tr>
<td>A/Vietnam/1194/2004</td>
<td>160</td>
</tr>
<tr>
<td>A/Shenzhen/406H/2006</td>
<td>NT</td>
</tr>
<tr>
<td>A/wild bird/Hong Kong/07035-1/2011</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

NT, not tested.

Human-to-human transmission of influenza viruses can occur through direct contact, indirect contact via fomites (i.e. contaminated environmental surfaces) and/or airborne transmission via small aerosols or large respiratory droplets. The pandemic and epidemic influenza viruses that have circulated in humans throughout the past century were all transmitted via the airborne route, in contrast to many other respiratory viruses that are transmitted exclusively via contact (Herfst et al., 2012). In this study, we observed that this clade 2.3.2.1 H5N1 virus could not be transmitted between ferrets via respiratory droplets, consistent with results published for almost all naturally isolated H5N1 strains that have been studied to date. Ferrets are a suitable animal model for influenza virus infection in humans because they are susceptible to natural infection and develop respiratory disease and lung pathology similar to that in humans infected with seasonal, avian or pandemic influenza viruses (Maher & DeStefano, 2004). The patterns of influenza virus attachment to cells in the trachea and lower respiratory tract are similar in ferrets and humans (van Riel et al., 2007). Therefore, the ferret model is the best choice for studies of virus transmission via direct contact, aerosols or respiratory droplets (Herfst et al., 2012; Imai et al., 2012; Maines et al., 2009; Munster et al., 2009; Sorrell et al., 2009; Yen et al., 2007). Since 2012, some reports about the pathogenesis of clade 2.3.2.1 H5N1 viruses in different animal species have arisen (Choi et al., 2013; Hu et al., 2013; Marinova-Petkova et al., 2012; Tung et al., 2013). Choi et al. (2013) assessed the pathogenic properties of two clade 2.3.2.1 H5N1 viruses isolated from a mandarin duck and a dead Eurasian eagle owl, and found that the two Korean isolates produced highly pathogenic characteristics in chickens, ducks and mice without pre-adaptation. Meanwhile, both viruses were transmitted efficiently to un inoculated contact ducks, although virus titres were relatively lower compared with those challenged ducks. Hu et al. (2013) evaluated the pathogenicity of two clade 2.3.2.1 H5N1 viruses, which were isolated from chicken and goose in Jiangsu province of China, in chickens, ducks, mice and guinea pigs. They found that, although the two viruses showed distinct
pathogenicity in mice and ducks, they were both lethal to chickens and guinea pigs. However, Marinova-Petkova et al. (2012) isolated a clade 2.3.2.1 H5N1 virus from a common buzzard (Buteo buteo) in Bulgaria, and found that this virus was highly pathogenic in chickens but had low pathogenicity in mice and ferrets, and had no molecular markers of increased pathogenicity in mammals. All these data indicate that the pathogenicity of clade 2.3.2.1 H5N1 viruses varies in avian and mammal species, ranging from asymptomatic to lethal according to the virus strain, infection dose, route and host factors. However, no studies have reported the transmissibility of clade 2.3.2.1 H5N1 viruses in mammalian species.

In summary, in this study, an H5N1 virus isolated from a dead wild bird was assessed to evaluate its genetic and pathobiological characteristics, and the transmissibility of this virus to naïve ferrets was also evaluated. This virus was

![Fig. 2. Direct binding assay with synthetic sialylglycopolymers. (a) Affinity to synthetic 3’SLN. (b) Affinity to synthetic 3’S-Di-LN. (c) Affinity to synthetic 6’SLN. (d) Affinity to synthetic 6’S-Di-LN.](image)

**Table 4. Clinical signs of infection and virus shedding of A/wild bird/Hong Kong/07035-1/2011 virus in ferrets**

Results for clinical signs are given as number of animals with sign/total number inoculated. For weight loss, the mean maximum percentage after inoculation is shown in parentheses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Weight loss (%)</th>
<th>Sneezing</th>
<th>Lethality</th>
<th>Virus shedding from the upper respiratory tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/wild bird/Hong Kong/07035-1/2011</td>
<td>3/3 (10.3 %)</td>
<td>3/3</td>
<td>0/3</td>
<td>Onset  Peak titre*  Duration (days p.i.)</td>
</tr>
<tr>
<td>A/Vietnam/1194/2004</td>
<td>3/3 (27.8 %)</td>
<td>3/3</td>
<td>1/3</td>
<td>1      3.49  1–7</td>
</tr>
</tbody>
</table>

*Calculated as log_{10} TCID_{50} (ml nasal or throat swab)^{−1}.
Characteristics of clade 2.3.2.1 of H5N1 virus

Little antibody cross-reactivity was found between this virus and clade 1 and 2.3.4 viruses, indicating that there has been significant antigenic divergence of the clade 2.3.2.1 H5N1 virus from conventional vaccine candidates. However, classified phylogenetically into clade 2.3.2.1, which is widely distributed in many Asian and European countries and has caused numerous deaths in wild birds and tremendous economic losses in the poultry industry.

Fig. 3. Pathogenicity and transmissibility of A/wild bird/Hong Kong/07035-1/2011 virus and A/Vietnam/1194/2004 virus in ferrets. (a) Body weight changes of inoculated ferrets. The data are expressed as the mean body weight (%) ± SD of each group of ferrets relative to the value before inoculation (100%). (b) Viral shedding by inoculated or airborne contact group ferrets as assessed by nasal and throat swabs. The data are presented as the mean ± SD of the log10 TCID50 ml−1 for each group of ferrets. The virus detection limit was 10 TCID50 ml−1 (dashed line).
this virus still exhibited sensitivity to the antiviral drug oseltamivir. Although the receptor-binding experiments showed that this clade 2.3.2.1 H5N1 virus has avian-like receptor-binding avidity but not human-like receptor-binding avidity, it is worth noting that this virus also possesses the N158D and E190D substitutions in the HA protein that have been postulated to facilitate HA binding to human-type receptors and to enhance viral transmissibility among mammals. However, this virus could not be transmitted between ferrets via respiratory droplets. All the results reported herein provide a better understanding of the evolution of H5N1 viruses and information that is important for preventing and controlling potential pandemics in the future.

METHODS

Viruses and cells. The H5N1 virus was isolated from the trachea of a dead wild bird on 21 December 2011, in Hong Kong and was subsequently inoculated into the allantoic cavities of 10-day-old chicken eggs, which were incubated at 37 °C for 48 h. Aliquots of the virus were stored at −80 °C. This virus was named A/wild bird/Hong Kong/07035-1/2011. The A/Vietnam/1194/2004 and A/Shenzhen/406H/2006 viruses were propagated in Madin–Darby canine kidney (MDCK) cells. The TCID50 was determined by serial titration of the virus in MDCK cells, and titres were calculated according to the method of Reed & Muench (1938). All experiments involving H5N1 influenza viruses were conducted under Biosafety Level 3 conditions.

DNA sequencing and phylogenetic analysis. The HA and NA genes of A/wild bird/Hong Kong/07035-1/2011 virus were amplified from inoculated allantoic fluid by high-fidelity PCR (KOD plus DNA polymerase; Toyobo). The PCR products were purified and sequenced (Invitrogen). A neighbour-joining phylogenetic tree based on the amino acid sequence of the HA protein was constructed in MEGA5 using the Jones–Taylor–Thornton amino acid replacement model with 1000 bootstrap replicates.

Ferret antiserum production. Ferrets (Mustela putorius furo) were obtained through a special breeding programme at the Institute of Laboratory Animal Sciences of the Chinese Academy of Medical Sciences. Animals were 5–10 months old and were seronegative for currently circulating influenza A H1N1 viruses, including the 2009 pandemic H1N1, H3N2 and H5N1 viruses, and influenza B viruses, as determined by HI tests. Six 8-month-old castrated ferrets were divided into three groups, with two animals per group. After being anaesthetized, each of the two animals was inoculated intranasally with 10^6 TCID50 A/wild bird/Hong Kong/07035-1/2011 virus, 10^5 TCID50 A/Vietnam/1194/2004 or 10^4 TCID50 A/Shenzhen/406H/2006 in a volume of 400 μl. At 14 days p.i., sera were collected and a second intranasal inoculation was given. Sera were collected again after a further 14 days. All procedures were approved by the Animal Use and Care Committee of the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (approval number ILAS-PC-2012-002).

HI assay. Ferret antiserum were pre-treated with receptor destroying enzyme (Miller et al., 2010) from Vibrio cholerae filtrate (Denka Seiken) at 37 °C for 18 h and then with 100 % turkey erythrocytes at 4 °C for 1 h to remove non-specific inhibitors and agglutinins. Serial 1:2 antiserum dilutions starting from a 1:10 initial dilution were made in PBS in a 96-well polystyrene microtitre plate, with 25 μl in each well. A 25 μl aliquot of virus suspension containing four haemagglutinin units (HAU) was then added to each well. After incubation at room temperature for 1 h, 50 μl 1 % suspension of turkey erythrocyte cells was added to each well. After incubation at room temperature for 30 min, the HI titres were determined as the reciprocal of the highest serum dilution that completely inhibited haemagglutination.

Antiviral resistance test. MDCK cells were seeded at 3 × 10^4 cells per well in 96-well plates and cultured overnight. Virus stocks were diluted to 1 × 10^3 TCID50 ml⁻¹ and added to cells at a volume of 100 μl per well. After incubation for 1 h at 37 °C, 100 μl of serially diluted oseltamivir carboxylate was added (range 170 pM–50 μM) in four replicates. After 3 days of incubation at 37 °C, the presence of the virus in the supernatants of the cell cultures was assayed using the agglutination of turkey erythrocytes. The level of agglutination was used as an indicator of virus replication in the cells to calculate the IC50. The 2009 pandemic H1N1 strain A/Hong Kong/423432/2009 (H1N1) with the H275Y mutation in the NA protein was used as a positive control for oseltamivir resistance.

Solid-phase binding assay. Synthetic Neu5Ac2-3Galβ1-4GlcNAcβ1-Sp (3′SLN), Neu5Ac2-3Galβ1-4GlcNAcβ1-3fβ-Sp (3′S-Di-LN), Neu5Ac2-6Galβ1-4GlcNAcβ1-Sp (6′SLN) and Neu5Ac2-6Galβ1-4GlcNAcβ1-3fβ-Sp (6′S-Di-LN) were obtained through the resource request programme of the Consortium of Functional Glycomics. Flat-bottom polystyrene 96-well plates were incubated with these sialylglycopolymers in PBS at 4 °C overnight. The glycopolymer solution was then removed, and the plates were blocked with 0.15 ml PBS containing 4 % BSA at room temperature for 1 h. After four successive washes with ice-cold PBS, the plates were incubated with a solution containing the virus (32 HAU) at 4 °C overnight. The plates were then washed as described above and incubated for 2 h at 4 °C with a mAb against the influenza A virus nucleoprotein (1:1000 dilution; IRR Ltd) at 4 °C. The plates were then washed again as before and incubated with HRP-conjugated goat anti-mouse IgG antisera for 2 h at 4 °C. After another washing step, the plates were incubated with tetramethylbenzidine substrate solution, and the absorbance at 450 nm (A450) was measured. A/Shenzhen/406H/2006 and A/Brisbane/10/2007 (H3N2) viruses were used as positive controls for α2,3 and α2,6 binding, respectively.

Airborne transmission experiment in ferrets. Castrated adult ferrets, 6–12 months of age and seronegative for currently circulating influenza viruses according to an HI assay, were used in this study. Transmission experiments were conducted in cages that were designed to prevent direct contact between animals but allow airflow between an inoculated ferret and a neighbouring naïve ferret. Two groups of three ferrets were inoculated intranasally with 10^6 TCID50 of virus 400 μl; one group was inoculated with A/wild bird/Hong Kong/07035-1/2011 virus and the other group with A/Vietnam/1194/2004 virus. At 24 h p.i., each inoculated animal was housed individually with a naïve ferret in a transmission cage to test the transmissibility of the virus. All animals were observed for clinical signs and were weighed daily as an indicator of disease. Nasal and throat swabs were collected from inoculated and naïve animals at 1, 3, 5, 7 and 9 days p.i. or post-exposure, respectively, and transferred to 1 ml PBS. The virus titre was determined by end-point titration in MDCK cells.

Statistical analysis. Data are expressed as the mean±sd. The differences among the different groups were analysed by one-way ANOVA followed by post-hoc Bonferroni correction, and the differences between the two groups were analysed by Student’s t-test. All statistical analysis was performed using spss 11.5 software. P values <0.05 were considered statistically significant.

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