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Enhanced virulence of clade 2.3.2.1 highly pathogenic avian influenza A H5N1 viruses in ferrets

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Abstract

Sporadic avian to human transmission of highly pathogenic avian influenza (HPAI) A(H5N1) viruses necessitates the analysis of currently circulating and evolving clades to assess their potential risk. Following the spread and sustained circulation of clade 2 viruses across multiple continents, numerous subclades and genotypes have been described. To better understand the pathogenesis associated with the continued diversification of clade 2 A(H5N1) influenza viruses, we investigated the relative virulence of eleven human and poultry isolates collected from 2006 to 2013 by determining their ability to cause disease in the ferret model. Numerous clade 2 viruses, including a clade 2.2 avian isolate, a 2.2.2.1 human isolate, and two 2.2.1 human isolates, were found to be of low virulence in the ferret model, though lethality was detected following infection with one 2.2.1 human isolate. In contrast, three of six clade 2.3.2.1 avian isolates tested led to severe disease and death among infected ferrets. Clade 2.3.2.1b and 2.3.2.1c isolates, but not 2.3.2.1a isolates, were associated with ferret lethality. All A(H5N1) viruses replicated efficiently in the respiratory tract of ferrets regardless of their virulence and lethality. However, lethal isolates were characterized by systemic viral dissemination, including detection in the brain and enhanced histopathology in lung tissues. The finding of disparate virulence phenotypes between clade 2 A(H5N1) viruses, notably differences between subclades of 2.3.2.1 viruses, suggests there are distinct molecular determinants present within the established subclades, the identification of which will assist in molecular-based surveillance and public health efforts against A(H5N1) viruses.

Keywords

Pathogenesis; Ferrets; Influenza H5N1

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1. Introduction

Since the re-emergence of A(H5N1) highly pathogenic avian influenza (HPAI) viruses in 2003, this subtype has spread and become endemic in bird populations in over 70 countries of Asia, Europe, the Middle East and Africa. Active A(H5N1) virus outbreaks in poultry continue to occur in Bangladesh, Cambodia, China, Egypt, India, Indonesia, Republic of Korea, Vietnam, and several countries in West Africa (WHO, 2016a). To date, avian-to-human transmission of A(H5N1) viruses has resulted in > 850 human cases, causing death in > 450 of them; the majority of human cases and fatalities have been reported in Egypt followed by Indonesia and Vietnam (WHO, 2016b). Human infection has primarily occurred via close contact with infected poultry although limited human-to-human transmission has been reported (Abdel-Ghafar et al., 2008). The continued evolution of A(H5N1) viruses poses a direct threat to public health should mutation allow for increased zoonotic transmission events or sustained human-to-human transmission (Rejmanek et al., 2015).

Established surveillance and analysis of currently circulating viruses in poultry have continually tracked and identified genetically and antigenically distinct A(H5N1) lineages (Creanga et al., 2013; Gerloff et al., 2014; Thor et al., 2015). While genetic analysis of the hemagglutinin (HA) gene of these lineages has resulted in the classification of isolates into multiple clades and subclades, only viruses from clades 0, 1, 2, and 7 have crossed the species barrier to cause disease and death in humans (Abdel-Ghafar et al., 2008). The persistent geographic spread and genetic evolution of select clades of A(H5N1) viruses, specifically those within the 2.2 and 2.3 clusters, have required further classification into third and fourth-tiered clades (Creanga et al., 2013; WHO/OIE/FAO, 2014; Younan et al., 2013). In Egypt, only clade 2.2 viruses have been found to circulate in poultry with all confirmed human infections between 2009 and 2016 caused by 2.2.1 and 2.2.1.2 viruses (Refaey et al., 2015; Younan et al., 2013). In Vietnam, based on the available surveillance data, clade 1 viruses were gradually replaced by clades 2.3.2 and 2.3.4 viruses beginning as early as 2005. Clade 2.3.2.1c and clade 2.3.4.4 (H5N6) have subsequently become dominant throughout much of the country since 2009 (Creanga et al., 2013; Nguyen et al., 2016). The continued HA evolution of clade 2.3.2.1 viruses has resulted in three subclade classifications as of 2012 termed clade 2.3.2.1a, 2.3.2.1b and 2.3.2.1c (WHO/OIE/FAO, 2014). In China, ongoing circulation of clade 2.3.2.1c and 2.3.4.4 viruses in poultry has also been a concern due to the expanding diversity of these viruses and sporadic detection of human infections, many of which were fatal (WHO, 2016a).

Here, we use the ferret model to evaluate the virulence of a panel of clade 2 HPAI A(H5N1) viruses isolated from humans or avian species from 2006 to 2013. Ferrets are an indispensable small animal model used to assess the pathogenicity of influenza A viruses as they are naturally susceptible to influenza virus infection, present with signs of infection that closely mimic those found in human infections, and have a similar profile of sialic acid moieties of the viral receptors that are found in the human respiratory tract (Belser et al., 2011b). Our analysis of eleven clade 2 A(H5N1) avian and human isolates revealed distinct virulence profiles in the ferret model. Our results demonstrate that isolates from clade 2.2, 2.2.1, and 2.2.2.1 are generally of low virulence and not lethal in ferrets, whereas clade 2.3.2.1 display a more diverse phenotype, with 2.3.2.1a viruses possessing reduced virulence

compared with 2.3.2.1b and 2.3.2.1c. As A(H5N1) influenza viruses continue to evolve, a detailed analysis of the actively emerging 2.3.2.1 clade compared to other 2.2 clades could identify clade and subclade specific molecular determinants of virulence.

2. Materials and methods

2.1. Viruses

A(H5N1) viruses used in this study are listed in Table 1. The stock viruses were grown using the same substrate as that from previous passages, either eggs or cell cultures. Viruses were propagated in Madin-Darby Canine Kidney (MDCK) cells (EG/2321, EG/3072, BnSw/HK/10, BD/5487, Dk/VN/672, Dk/VN/1206, Dk/VN/1232) or the allantoic cavity of ten-day-old embryonating hens' eggs at 37 °C for 24–26 h (Ck/KR/06, EG/4935, Dk/VN/0004, Dk/VN/2848) as previously described (Maines et al., 2005, 2009). Pooled allantoic fluid or cell supernatant was clarified by centrifugation and aliquots were stored at –70 °C until use. Stocks were titrated in MDCK cells using standard plaque assay methods for determination of plaque forming unit (PFU) titer (Zeng et al., 2007). A 50% egg infectious dose (EID₅₀/mL) for egg grown stocks was determined using standard methods (Reed and Muench, 1938). All experiments were performed in biosafety level 3 containment laboratories with enhancements as required by the Animal and Plant Health Inspection Service (US Department of Agriculture) and the National Select Agent Program (Department of Health and Human Services) (Chosewood and Wilson, 2009).

2.2. Ferret pathogenesis experiments

Male Fitch ferrets (Triple F Farms, Sayre, PA), 6–12 months of age and serologically negative by hemagglutination inhibition assay for currently circulating influenza viruses, were used in this study. Ferrets were housed for the duration of each experiment in a Duo-Flo BioClean environmental enclosure (Lab Products, Seaford, DE). Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Ferrets were anesthetized prior to procedures with an intramuscular injection of ketamine hydrochloride cocktail (Gustin et al., 2011). Six ferrets per experiment were intranasally (i.n.) administered 10⁶ or 10⁷ PFU or EID₅₀ of virus diluted in PBS in a 1 mL volume. Ferrets were monitored daily for clinical signs of infection and nasal wash (NW) samples were collected on alternate days post-inoculation (p.i.) for virus titration in eggs as previously described (Maines et al., 2005). Ferrets that lost more than 25% total body weight or displayed signs of neurological dysfunction were euthanized. Three days p.i., three ferrets from each virus infected group were euthanized and tissues were collected for virus titration as previously described (Maines et al., 2005). The following tissues were aseptically collected in this order: intestines, spleen, kidneys, liver, trachea, lungs, brain-posterior, brain-anterior, olfactory bulb, and nasal turbinates. Lungs were also collected for histopathological examination. Statistics between titers obtained from high or low virulence viruses was performed by Student's *t*-test.

2.3. Histopathology and immunohistochemistry (IHC)

Necropsies were performed on three ferrets from each group on day 3 p.i. Three 5-mm lung sections of each lung lobe were collected for histopathology. Lung sections were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μ m and were stained with hematoxylin and eosin (HE). A duplicate 5- μ m section was immunohistochemically stained for detection of influenza A virus nucleoprotein as described previously (Perkins and Swayne, 2001).

2.4. Virus sequencing and molecular analysis

Following virus isolation, allantoic fluid was harvested and viral RNA was isolated with the Qiagen RNeasy extraction kit (Qiagen) or Magna Pure LC system (Roche). Influenza viral RNA was amplified using the Access Quick one-step RT-PCR kit (Promega) and A(H5N1)-specific primers (sequence available upon request). The 400–600 bp amplicons were sequenced by an Applied Biosystem 3730xl system using cycle sequencing dye terminator chemistry (Life Technologies). Full-length open reading frames with at least triple coverage were generated using Sequencer 5.0 (Gene Codes). Genome sequences can be found in the GISAID and/or GenBank sequence databases. A(H5N1) virus clades were identified for each virus using the LABEL tool (Shepard et al., 2014). Full length coding sequences were aligned with related reference viruses using the Muscle algorithm (Edgar, 2004). For molecular analysis of the viral protein sequences, we used the full-length ORF sequences of NA and the internal genes starting with the ATG codon, whereas the analysis of the HA proteins was performed using sequences of the mature HA protein with signal peptide removed.

3. Results

3.1. Characteristics of A(H5N1) influenza viruses tested in this study

A selection of HPAI A(H5N1) viruses representing clades 2.2, 2.2.1, 2.2.2.1, and 2.3.2.1 isolated from either human or avian hosts were evaluated for their relative virulence in ferrets (Table 1). Of the clade 2.2, 2.2.1, and 2.2.2.1 viruses tested, all but one (Ck/KR/06) were isolated from human cases of infection. All 2.3.2.1 viruses were isolated from infected poultry. Clade 2.3.2.1 is subdivided into clade 2.3.2.1a (A/Hubei/1/2010-like), 2.3.2.1b (A/Barn Swallow/Hong Kong/D10-1161/10-like), and 2.3.2.1c (A/Hong Kong/6841/2010-like) viruses (WHO/OIE/FAO, 2014). All viruses replicated to high titer in the allantoic cavities of 10-d old embryonating eggs or Madin-Darby canine kidney (MDCK) cells, in excess of $10^{8.5}$ EID₅₀/mL and $10^{7.5}$ PFU/mL, respectively.

3.2. Pathogenicity of A(H5N1) viruses in ferrets

Infection with HPAI A(H5N1) viruses bearing potential genetic indicators of pathogenicity can cause mild to severe disease in mammalian models (Herfst et al., 2012; Imai et al., 2012; Maines et al., 2005; Suguitan et al., 2012; Thor et al., 2015). To evaluate the relative virulence of selected clade 2 A(H5N1) viruses, ferrets were inoculated i.n. with 10^6 – 10^7 PFU or EID₅₀ of each virus, a dose shown to consistently infect ferrets with this subtype (Table 2) (Maines et al., 2005). The relative virulence of each of the eleven A(H5N1) viruses

in ferrets was classified as either high or low virulence based on > 50% (high) or < 50% (low) mortality during the acute phase of infection (Table 2).

Among the five viruses tested from clades 2.2 (including 2.2.1 and 2.2.2.1), only one isolated from 2007 (EG/2321) was found to be lethal and of high virulence in ferrets (Table 2). Ferrets inoculated with EG/2321 virus exhibited high fever (> 2 °C above baseline) and severe morbidity (mean weight loss > 20%), warranting euthanasia of two of three ferrets by day 8 p.i. In contrast, reduced morbidity was observed following inoculation with other clade 2.2 viruses, with weight loss ranging from 8% to 10.5% through day 12 p.i. (Table 2). Nasal discharge and diarrhea were detected sporadically in approximately 25% of ferrets inoculated with these viruses.

Generally, we found the virulence of clade 2.3.2.1 viruses to be separated based on the group in which they have been classified. Both clade 2.3.2.1a viruses tested (Dk/VN/1206, Dk/VN/1232) exhibited transient fevers approximately 1.5 °C above baseline and moderate morbidity (mean weight loss approximately 10–15%). One ferret inoculated with Dk/VN/1206 virus was euthanized day 7 p.i. due to severe diarrhea with a suspected secondary bacterial infection; the virus remains classified as low virulence due to < 50% overall lethality. Inoculation of ferrets with clade 2.3.2.1b viruses (BnSw/HK/10, Dk/VN/672) resulted in pronounced morbidity, fever, persistent nasal discharge, and diarrhea, among other clinical signs of infection. The two 2.3.2.1b viruses tested were 100% lethal by day 7 p.i., with neurological complications necessitating euthanasia detected in half of all inoculated ferrets. In contrast, the two clade 2.3.2.1c viruses tested (Dk/VN/0004, Dk/VN/2848) exhibited divergent mammalian phenotypes. Dk/VN/0004 virus was found to possess high virulence in the ferret, with infected ferrets displaying severe morbidity and 100% lethality by day 7 p.i. However, Dk/VN/2848 virus exhibited a low virulence phenotype in ferrets, with moderate weight loss being the only clinical sign of infection. Dk/VN/2848 virus differed from Dk/VN/0004 virus by 8 amino acid changes in the 3 polymerase genes combined, however, none of these were identified in known markers of virulence. Three additional amino acid changes in the HA protein at positions 124, 163 and 188 were also identified. One of these, T188I, was previously found to increase binding to α 2,6-linked sialic acid receptors (Yang et al., 2007). These data demonstrate that clade 2.3.2.1 A(H5N1) viruses cause varying degrees of disease ranging from mild clinical symptoms, similar to those seen with a seasonal influenza virus infection, to pronounced disease and death.

3.3. Replication of A(H5N1) viruses in ferrets

To assess the efficiency, kinetics, and spread of virus replication in ferrets, we first analyzed nasal wash samples collected from A(H5N1) virus-inoculated ferrets on alternate days 1–7 p.i. (Fig. 1). All viruses examined replicated to high peak titers (> 10⁴ EID₅₀/mL) in nasal wash specimens, regardless of their clade or subclade, with generally comparable kinetics and magnitude between viruses of high or low virulence in the ferret model (peak mean NW titers between 10^{4.1}–10^{6.5} EID₅₀/mL and 10^{4.8}–10^{5.5} EID₅₀/mL for low and high virulence viruses, respectively). Viral clearance was evident by day 7 p.i. as titers dropped in many of ferrets to below the limit of detection (10^{1.5} EID₅₀/mL). Among low virulence A(H5N1) viruses, the clade 2.2 (Ck/KR/06) and 2.3.2.1a (Dk/VN/1232) viruses achieved the highest

titers ($10^{5.2}$ EID₅₀/mL) in NW specimens through day 3 p.i. With regard to clade 2.3.2.1 viruses tested, 2.3.2.1b viruses peaked at $10^{4.6}$ EID₅₀/mL on days 3 or 5 p.i. (both exhibiting high virulence), while 2.3.2.1a viruses peaked at $10^{4.3}$ EID₅₀/mL on days 1 or 3 p.i. (both exhibiting low virulence). Similarly, the clade 2.3.2.1c low virulence (Dk/VN/2848) virus peaked on day 1 p.i. ($10^{4.4}$ EID₅₀/mL) and the 2.3.2.1c high virulence virus (Dk/VN/0004) peaked on day 3 p.i. ($10^{5.2}$ EID₅₀/mL). Although virus replication of the 2.3.2.1 clade viruses was not always higher among those exhibiting high virulence, peak shedding was consistently observed later (3 or 5 days p.i.) compared with the low virulence viruses of the subclade that peaked on 1 or 3 days p.i. (Fig. 1).

Previous studies have used the presence or absence of replication of A(H5N1) viruses in extrapulmonary tissues as a measure of pathogenicity (Belser and Tumpey, 2013; Maines et al., 2005). Here, we analyzed the level of virus replication in ferret tissues day 3 p.i. to assess the virulence of each virus. As expected, all viruses tested replicated efficiently throughout the respiratory tissues of ferrets (nasal turbinates [NasTur], trachea, lung; Fig. 2). Highly virulent and lethal A(H5N1) viruses achieved higher mean titers (NasTur $10^{6.4}$, trachea $10^{4.8}$, lung 10^6 EID₅₀/mL) than non-lethal viruses (NasTur $10^{5.7}$, trachea $10^{3.2}$, lung $10^{4.5}$ EID₅₀/mL) in all respiratory tissues assayed; however the only significant difference between high and low virulent viruses was found in lung tissues ($p < 0.02$).

To investigate systemic spread of infection, we analyzed samples taken from brain (olfactory bulb, anterior, and posterior), spleen, and intestine tissues to quantitate virus amount (Fig. 2). Virus was detected in brain tissues analyzed for most viruses studied, demonstrating extrapulmonary spread of both lethal and non-lethal viruses. Given their proximity to upper respiratory tract tissues, the highest titers were unsurprisingly found in the olfactory bulbs (mean titer of $10^{3.9}$ and $10^{3.1}$ EID₅₀/g for lethal and non-lethal viruses, respectively). Furthermore, mean titers in the olfactory bulb were higher in 2.3.2.1 viruses compared with other clade 2 viruses tested ($10^{3.8}$ and $10^{2.9}$ EID₅₀/g, respectively), though these differences were not statistically significant. Infectious virus in both anterior and posterior sections of the brain was detected among A(H5N1) viruses of both high and low virulence. Virus replication in spleen tissue was observed in two of three ferrets infected with either Dk/VN/672 or Ck/KR/06 viruses. Similarly, infectious virus in the intestines was only sporadically detected overall, though interestingly, virus was present in ferrets inoculated with both 2.3.2.1b viruses (one of three ferrets for BnSw/HK/10, two of three ferrets for Dk/VN/672). Infectious virus was not detected from peripheral blood nor was systemic spread to the kidneys or liver detected from any viruses tested in this study (data not shown). In summary, all viruses that were analyzed showed measureable replication throughout the respiratory tract as well as varying levels of replication in extrapulmonary tissues. Generally, lethal viruses tended to replicate to higher titers in each tissue compared to non-lethal viruses, though strain-specific differences were also apparent.

3.4. Histopathologic evaluation of lung tissue samples of A(H5N1) infected ferrets

In accord with strain-specific differences observed with viral replication kinetics, magnitude, and duration, histological lesions similarly varied with individual ferrets, different viruses and different lung sections. Typically, bronchointerstitial pneumonia was identified,

affecting primarily the bronchioles, especially terminal bronchioles, and generally sparing the bronchi (Fig. 3A). These bronchioles had inflammatory infiltrates of macrophages and granulocytes with accompanied degeneration to necrosis of bronchiolar epithelial cells, and peribronchiolar edema and mononuclear cell cuffs. The areas supplied by these bronchioles had alveolitis with luminal inflammatory cells, predominantly macrophages but also granulocytes, type II pneumocyte hyperplasia, some luminal fibrin and inflammatory cell infiltrates into the septal wall. Virus was localized by immunohistochemistry most commonly to the alveolar epithelium (Fig. 3B) and macrophages (Fig. 3C), but occasionally to the bronchiolar epithelium (Fig. 3D). The most severe lung lesions were noted following infection with BnSw/HK/10 virus. The bronchointerstitial pneumonia was severe and diffuse in multiple BnSw/HK/10 virus-infected lung sections and was accompanied by multiple foci of necrotizing alveolitis and alveolar hemorrhage (Fig. 3E). Lung lesions associated with Dk/VN/672 and Dk/VN/1206 virus infection were similar to BnSw/HK/10 virus, but Dk/VN/672 virus-infected lungs had fewer foci of necrotizing alveolitis while Dk/VN/1206 lacked necrotizing alveolitis and had milder bronchiolitis. In contrast, lungs from ferrets infected with Dk/VN/1232 and BD/5487 viruses had mild to moderate interstitial pneumonia in a few lung sections with minimal to mild bronchiolar lesions (Fig. 3F). In summary, clade 2.3.2.1 viruses were capable of causing pronounced pathology in ferrets, though strain-specific differences were detected. Within this clade, 2.3.2.1b viruses possessed the most severe histopathological changes.

3.5. Molecular correlates of viral pathogenicity phenotypes in mammals

An analysis of the protein sequences of the viruses included in this study reveal a myriad of amino acid changes/substitutions that have in some instances been attributed to a specific phenotype, while the effects of others remain unknown (Tables 3, 4). Amino acid differences can be observed when comparing key molecular markers (627 K, PB1-F2, HA and NS1 PDZ) among the group of six H5N1 viruses in the 2.3.2.1 subclades and viruses in clades 2.2, 2.2.1 and 2.2.2 (Table 3). These differences are not sufficient to explain the increased viral pathogenicity observed in ferrets because these amino acid changes also occur among the non-lethal H5N1 viruses. By comparing amino acid similarities among the highly virulent H5N1 viruses (EG/2321, BnSw/HK/10 and Dk/VN/672), the polybasic HA cleavage site motif, the amino acid deletions in NA and NS1, known molecular markers of virulence (CDC, 2012), were common markers among these three H5N1 viruses. However, these amino acid substitutions can be also found in the viruses of low pathogenicity, indicating that additional unknown molecular markers for virulence exist for these H5N1 viruses.

The six viruses in the 2.3.2.1 clade used in this study span subclades 2.3.2.1a to 2.3.2.1c; each virus contains genetic features that are either commonly shared within the viruses in the same clade or unique to each virus. Both clade 2.3.2.1b viruses (BnSw/HK/10 and Dk/VN/672) caused 100% mortality in ferrets and these two viruses are very similar to each other in amino acid composition, with a total of 99.7% similarity. Conversely, the two clade 2.3.2.1a viruses (Dk/VN/1206 and Dk/VN/1232), which did not cause ferret lethality, share genome similarity of approximately 97%, due to amino acid differences that are mostly concentrated in NA (13 aa), NS1 (9 aa), and PB1-F2 (Table 3). The main molecular

differences that separates the three subclade viruses (2.3.2.1a, b, and c) from each other is found in the HA: 15 amino acids in HA1 and 5 in HA2. Although these three groups remain highly conserved at HA amino acid residues previously implicated in enhanced virulence, changes at positions 183, 188 and 189 were identified among the three subclade viruses (Table 4).

4. Discussion

HPAI A(H5N1) viruses continue to pose a public health and potential pandemic threat. While the majority of HPAI A(H5N1) influenza virus infections of poultry and humans have remained largely in China, Southeast Asia, the Middle East, and Africa, sustained human to human transmission of these viruses could result in a global pandemic. To more clearly understand the potential for divergent A(H5N1) influenza viruses to cause disease in mammals, we compared the virulence of representative clade 2 A(H5N1) viruses in the ferret animal model. With one exception, all clade 2.2, 2.2.1, and 2.2.2.1 viruses isolated from either avian or human hosts tested in this study were non-lethal and of low virulence. Our results demonstrate that clade 2.3.2.1 viruses confer a virulence phenotype depending on their genetic subgroup. Among the viruses tested here, clade 2.3.2.1a viruses possessed low virulence in the ferret model, clade 2.3.2.1b possessed high virulence, with both high and low virulence phenotypes present in 2.3.2.1 c. A single isolate, EG/2321 (clade 2.2.1) virus was also found to be of high virulence and resulted in mortality in 2 of 3 ferrets.

Despite that only 4 of 11 A(H5N1) viruses showed a high-virulence phenotype in ferrets, all viruses replicated to high titers in the respiratory tract and, with few exceptions, all viruses were capable of limited replication in brain tissues examined. Further systemic spread of these viruses was observed only sporadically in intestinal and spleen tissues, in agreement with A(H5N1) viruses from other clades (Maines et al., 2005; Yen et al., 2007). We also performed complete blood counts on acute and endpoint blood samples, as highly pathogenic A(H5N1) viruses have been shown to elicit lymphopenia following virus infection in the ferret model (Belser et al., 2011a). In summary, we found that inoculation with A(H5N1) viruses of high virulence caused significant shifts in the circulating lymphocyte, neutrophil and monocyte populations, in particular lymphopenia was most prominent during the first week of infection (data not shown). The mean percentage of lymphocytes in peripheral blood in ferrets infected with EG/2321, BnSw/HK/10, and Dk/VN/672 viruses dropped from 67–31% by day 3 p.i., persisting at < 35% by day 7 p.i. These results are comparable to the highly virulent clade 1 A(H5N1) virus, A/Vietnam/1203/04 (Belser et al., 2011a; Maines et al., 2005) and further underscores the severe disease caused by some A(H5N1) viruses in the ferret model.

Throughout the years, several studies have been performed to assess the pathogenicity of A(H5N1) viruses using mammalian animal models (reviewed in Belser and Tumpey, 2013), linking the outcomes of infection to molecular genetic factor(s) contained in these viruses. A distinct molecular characteristic of HPAI A(H5N1) viruses that is known to play role in pathogenicity is the stretch of polybasic amino acids in the HA cleavage site (Hatta et al., 2001; Steinhauser, 1999); all A(H5N1) viruses tested in this study possess this major molecular marker of HPAI viruses. Other identified markers include amino acid substitutions

in PB2 (627 K and 701N) and deletions in NA and NS1 (Hatta et al., 2001; Li et al., 2009; Matsuoka et al., 2009; Steel et al., 2009b). With respect to PB2, the amino acids 627 K and 701N are critical molecular markers of A(H5N1) virus pathogenicity in mammals (Hatta et al., 2007; Steel et al., 2009a). The 627 K mutation is one of the changes that produced a A(H5N1) transmissible in mammalian model (Herfst et al., 2012; Imai et al., 2012; Russell et al., 2012). The viruses from 2.2, 2.2.1 and 2.2.2.1 clades do possess 627 K, which is a common feature of the Qinghai Lake viruses (Chen et al., 2006), however, of the five viruses in this study that contain 627 K, only one caused lethality in ferrets (EG/2321). 627 K and 701N are not present in the clade 2.3.2.1 viruses and as such cannot explain the high pathogenicity/lethality properties of these viruses, in particular, the 2.3.2.1b viruses. This data agrees with Watanabe et al., who stated that the 627 K residue alone is not sufficient to produce a high pathogenicity phenotype in mammalian models (Watanabe et al., 2012). Alternatively, residues 147 T, 339 T and 588 T, in PB2 have been shown to be involved in the virulence of A(H5N1) viruses (Fan et al., 2014). These residues are present in all 2.3.2.1 viruses used in this study. Additional polygenic amino acids located in other viral proteins provided evidence that multiple genetic factors can modulate the virus pathogenicity in the mammalian host (Belser and Tumpey, 2013).

The HA is the viral protein with the largest number of amino acid differences separating clade 2.3.2.1a, b and c viruses. Although many of these differences are not mapped to known regions of significance, some have been reported to be associated with virus binding to 2,6 sialic acids (CDC, 2012). Importantly, several virus strains in clade 2.2.1 have been shown to contain amino acids in HA that increase binding avidity to cell receptors containing α 2,6 sialic acids (Watanabe et al., 2012). One of the molecular changes in the HA among these viruses that contributes to this increase in binding is the 154–156 loss of glycosylation, which also occurs in the viruses of 2.3.2 and 2.3.2.1 clades. Interestingly, this is one of the changes that produced a transmissible A(H5N1) virus in a mammalian model (Herfst et al., 2012; Imai et al., 2012; Russell et al., 2012). Other notable changes in the viruses studied here include one substitution, G323I, an amino acid adjacent to the cleavage site only found in the highly lethal 2.3.2.1b viruses with unknown effects, and a deletion of one polybasic amino acid in the HA cleavage site among 2.3.2.1a viruses which may be associated with a decrease in pathogenicity (Zhang et al., 2012). Because A(H5N1) variants continue to emerge from avian reservoirs, it is likely that additional markers of pathogenicity will be identified. Such molecular markers of virulence have increasingly become important features utilized in the detection and prediction of potentially pathogenic and lethal viruses.

Ferrets have been widely used as a model for influenza virus pathogenesis as well as for immunity studies (Belser et al., 2011b). It is interesting to note that among the eleven HPAI A(H5N1) viruses characterized in this study, the majority of them did not cause lethal infection in ferrets. However, as A(H5N1) viruses continue to diversify in avian species (WHO/OIE/FAO, 2014), sustained surveillance, monitoring, and assessment of these viruses is needed to examine the relative risk posed by these viruses to human health. Mammalian pathotyping of HPAI A(H5N1) influenza virus isolates permits the identification of distinct molecular determinants of virulence, which are subsequently used for public health guidance. Future experiments studying the precise contribution of individual molecular

changes present in clade 2.3.2.1 viruses as they compare to other viruses from clade 2 will provide valuable information in this regard.

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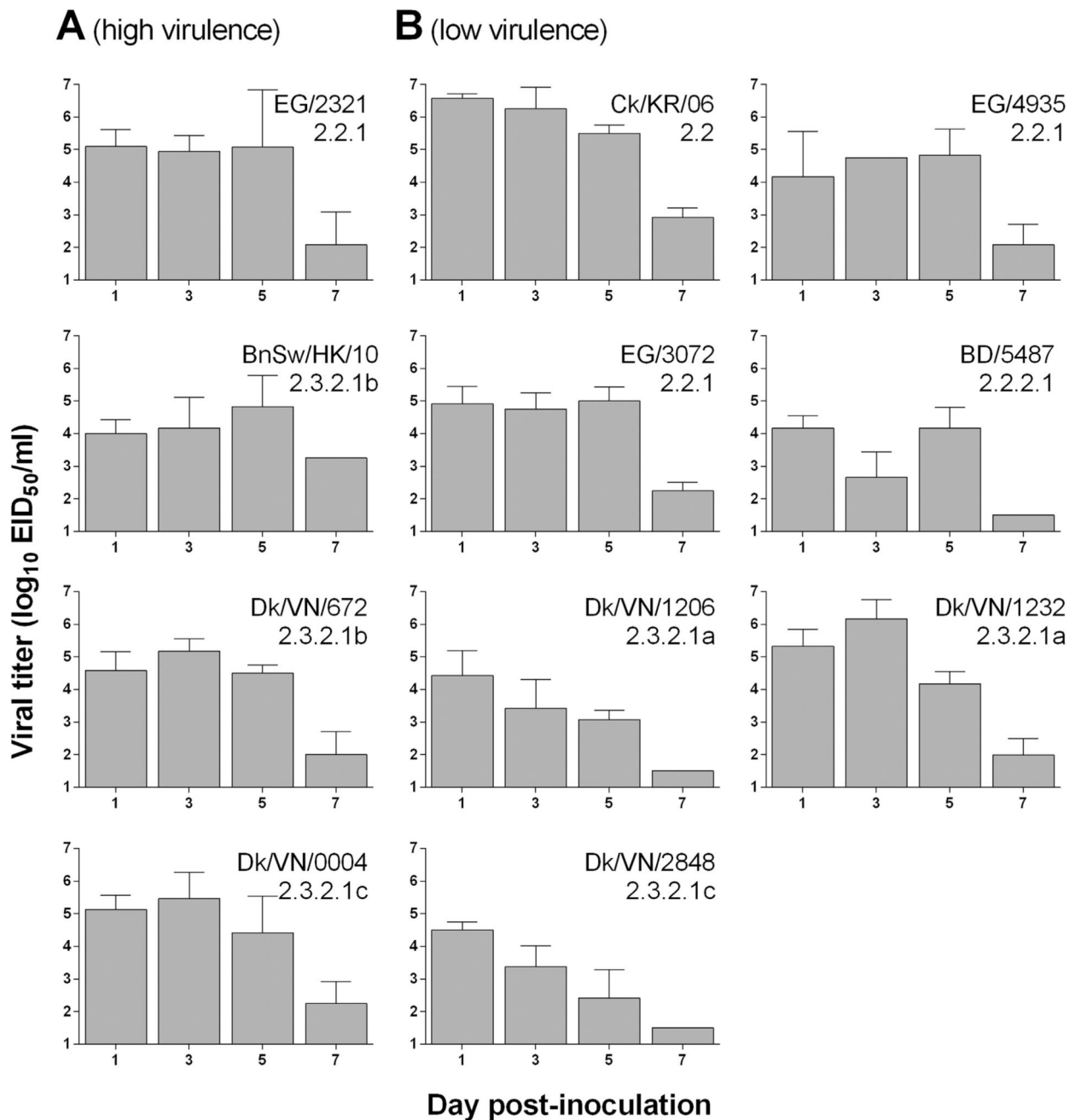


Fig. 1. Mean viral titer in nasal washes of ferrets inoculated with HPAI A(H5N1) influenza viruses

Three ferrets per group were inoculated with 10^6 or 10^7 PFU or EID₅₀ of virus. Nasal washes were collected on alternate days p.i. Mean titers for each group are shown as \log_{10} EID₅₀/mL + standard deviation (SD) from three ferrets per group unless otherwise specified. Due to virus lethality, titers day 7 p.i. reflect one (BnSw/HK/10) or two (Dk/VN/672) ferrets only. The limit of detection was $1.5 \log_{10}$ EID₅₀/mL.

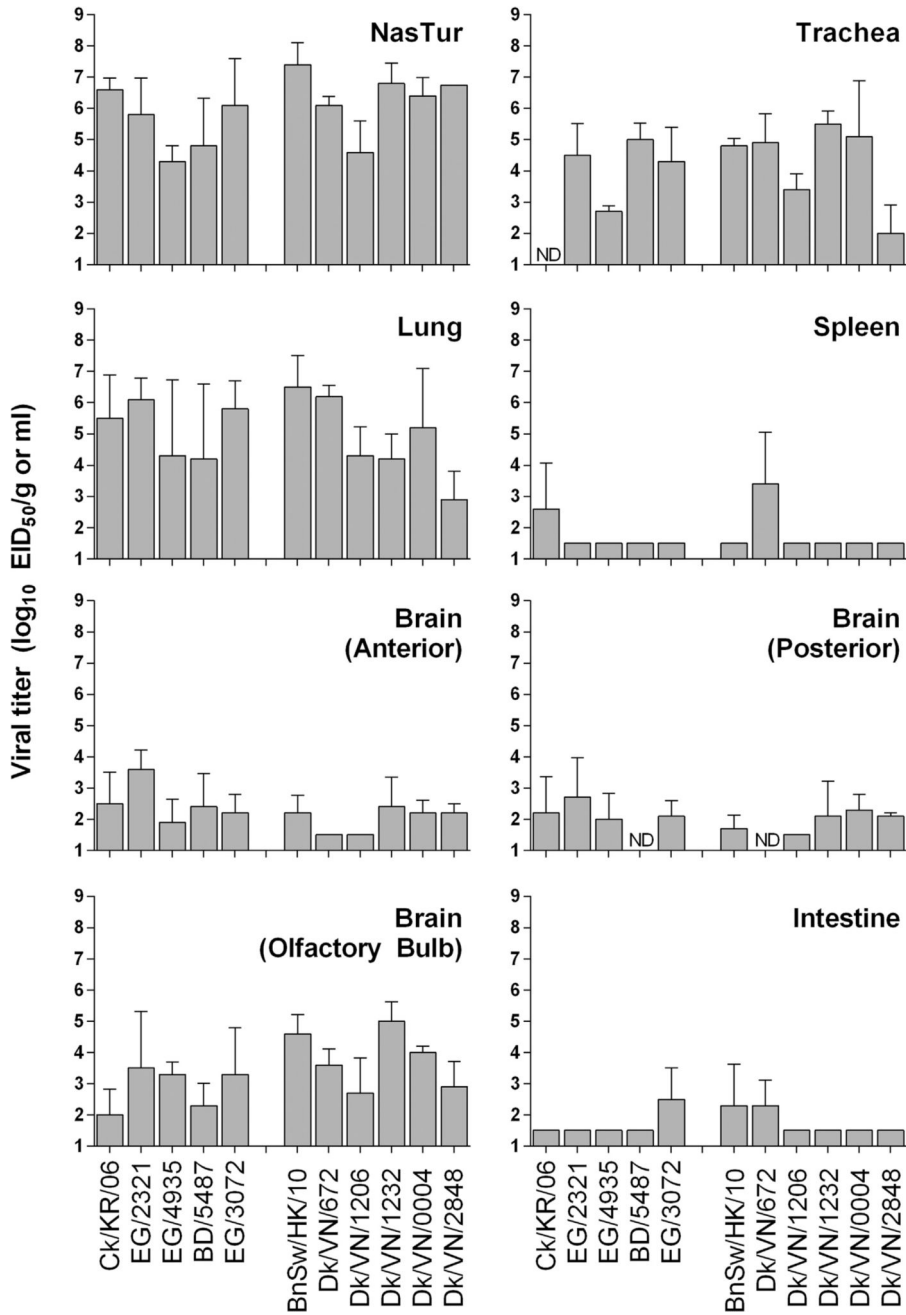


Fig. 2. Viral dissemination of A(H5N1) influenza viruses in ferrets

Three ferrets per group were inoculated with 10⁶ or 10⁷ PFU or EID₅₀ of virus and tissues were collected 3 days p.i. for virus titration. Mean titers for each group are shown as log₁₀ EID₅₀/g or mL + SD. Tissues below our limit of detection (1.5 log₁₀ EID₅₀/g or mL) were assigned a value of 1.5. ND, sample was not collected and not titered.

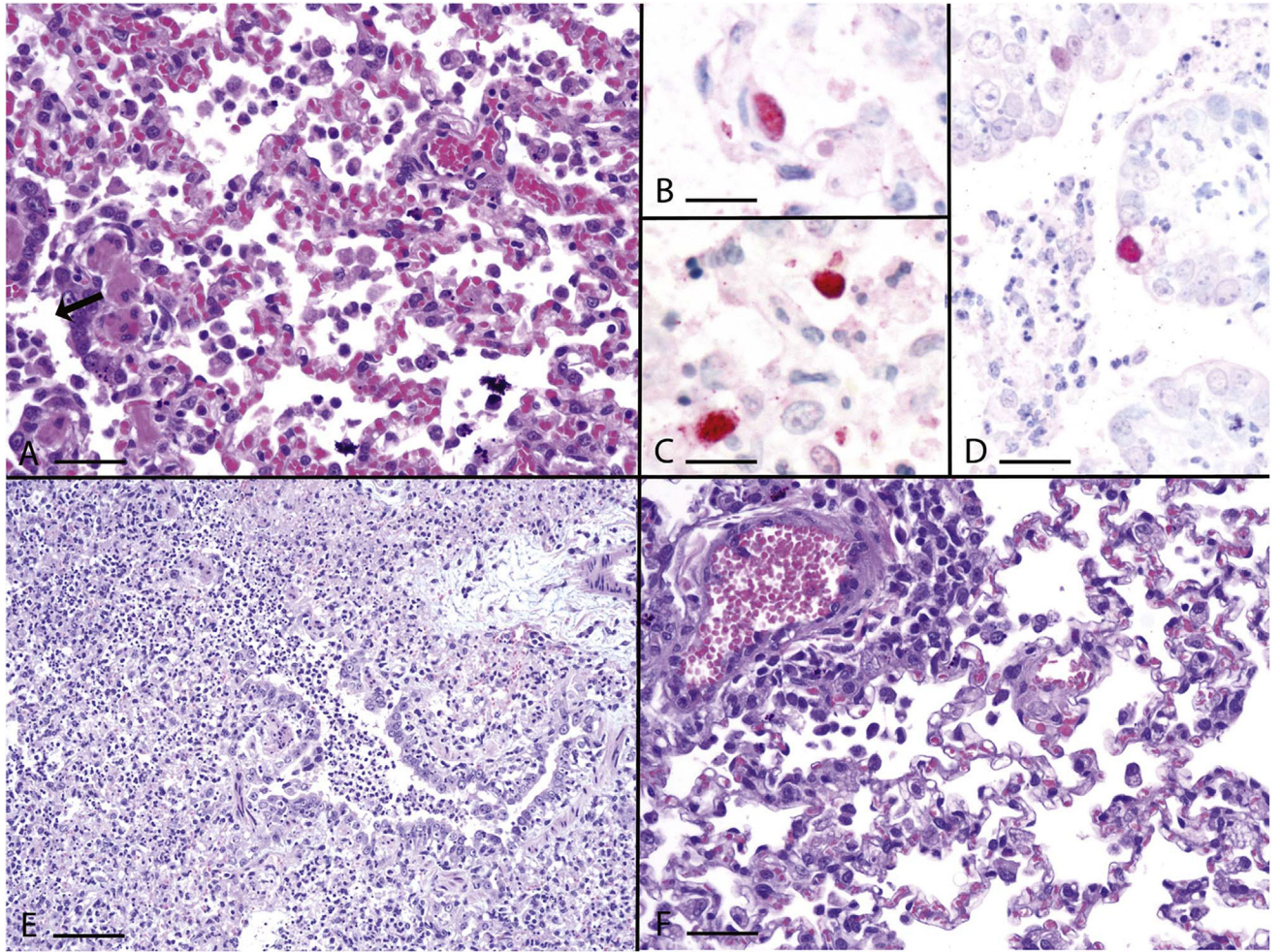


Fig. 3. Histopathologic evaluation of ferrets infected with A(H5N1) influenza viruses
 Representative photomicrographs of hematoxylin and eosin [A, E, F] and immunohistochemically [B-D] stained lung sections from influenza virus-infected ferrets collected day 3 p.i. (A) Moderate bronchointerstitial pneumonia with predominant histiocytic alveolitis and mild bronchiolitis (arrow), Dk/VN/1206, bar =40 μ m. (B) Viral antigen in type II pneumocyte, BnSw/HK/10, bar =20 μ m. (C) Viral antigen in alveolar macrophages, BnSw/HK/10, bar =20 μ m. (D) Viral antigen in bronchiolar epithelium, BD/5487, bar =35 μ m. (E) Severe diffuse bronchointerstitial pneumonia with prominent necrotizing alveolitis and severe bronchiolitis, BnSw/HK/10, bar =80 μ m. (F) Mild alveolitis with perivascular cuffing with mononuclear inflammatory cells, Dk/VN/1232, bar =40 μ m.

Table 1

A(H5N1) viruses used in this study.

Virus	Name	Clade ^a	Host	Description	Reference
A/Chicken/Korea/IS/06	Ck/KR/06	2.2	Avian	outbreak	(Lee et al., 2008)
A/Egypt/2321-NAMRU3/07	EG/2321	2.2.1	Human	recovered	(Younan et al., 2013)
A/Egypt/4935-NAMRU3/09	EG/4935	2.2.1	Human	recovered	(Younan et al., 2013)
A/Egypt/N03072/10	EG/3072	2.2.1	Human	fatal	(Younan et al., 2013)
A/Bangladesh/5487/11	BD/5487	2.2.2.1	Human	recovered	(Gerloff et al., 2014)
A/BamSwallow/HongKong/D10-1161/10	BnSw/HK/10	2.3.2.1b	Avian	detection	(WHO, 2011)
A/Duck/Vietnam/NCVD-672/11	Dk/VN/672	2.3.2.1b	Avian	outbreak	(Creanga et al., 2013)
A/Duck/Vietnam/NCVD-1206/12	Dk/VN/1206	2.3.2.1a	Avian	outbreak	(Creanga et al., 2013)
A/Duck/Vietnam/NCVD-1232/12	Dk/VN/1232	2.3.2.1a	Avian	outbreak	(Creanga et al., 2013)
A/Duck/Vietnam/NCVD-0004/13	Dk/VN/0004	2.3.2.1c	Avian	outbreak	(Nguyen et al., 2016)
A/Duck/Vietnam/NVCD-2848/13	Dk/VN/2848	2.3.2.1c	Avian	outbreak	(Nguyen et al., 2016)

^aClade subdivisions described in (WHO/OIE/FAO, 2014).

Table 2

Clinical signs and symptoms of A(H5N1) virus infection in ferrets.

Virus	Clade	% Wt loss ^a	Fever ^b	Nasal ^c	Diarrhea ^c	Neuro ^d	Lethality ^e	MD ^f	Virulence ^g
Ck/KR/06	2.2	9.4 (2–6)	2.3 (1)	2/3	0/3	0/3	0/3	–	Low
EG/2321	2.2.1	20.5 (8)	2.2 (1)	0/3	1/3	0/3	2/3	8.0	High
EG/4935	2.2.1	8.5 (3–8)	2.3 (2)	0/3	1/3	0/3	0/3	–	Low
EG/3072	2.2.1	10.5 (4–12)	1.8 (2)	0/3	0/3	0/3	0/3	–	Low
BD/5487	2.2.2.1	8.0 (2–5)	2.0 (1–2)	2/3	0/3	0/3	0/3	–	Low
BnSw/HK/10	2.3.2.1b	19.0 (6–7)	2.4 (1)	3/3	3/3	1/3	3/3	6.3	High
Dk/VN/672	2.3.2.1b	18.1 (6–7)	2.2 (1)	3/3	1/3	2/3	3/3	6.7	High
Dk/VN/1206	2.3.2.1a	15.1 (4–7)	1.4 (1)	3/3	1/3	0/3	1/3	7.0	Low
Dk/VN/1232	2.3.2.1a	10.4 (8–10)	1.6 (1)	2/3	0/3	0/3	0/3	–	Low
Dk/VN/0004	2.3.2.1c	24.9 (7)	2.8 (1)	2/3	3/3	1/3	3/3	7.0	High
Dk/VN/2848	2.3.2.1c	13.9 (3–10)	2.4 (1)	0/3	0/3	0/3	0/3	–	Low

^aPercent mean maximum weight loss. Day range p.i. of maximum weight loss in parentheses.

^bMean maximum fever reported in degrees centigrade. Day range p.i. of maximum temperature rise over baseline (37.3–39.2 °C) in parentheses.

^cIncidence of nasal discharge or diarrhea through day 14 p.i., reported as number of ferrets with symptoms/total number of ferrets.

^dNumber of ferrets which exhibited neurological symptoms/total number of ferrets. All ferrets exhibiting neurological symptoms were humanely euthanized.

^eNumber of ferrets required euthanasia due to severe disease through day 14 p.i./total number of ferrets.

^fMean day of death among all ferrets which required euthanasia due to severe disease through day 14 p.i.

^gViruses are defined as possessing high virulence when > 50% lethality is observed following infection.

Table 3

Potential genetic indicators of pathogenicity or human host preference.

Virus	Clade	PB2		PBI-F2 #aa ^b	HA # polybasic aa	NA		NS1	
		627K ^a	Y			antiviral sensitivity ^c	aa 49-68 ^d	PDZ domain ESEV	aa ^d 80-84
Ck/KR/06	2.2	Y	90	6	Y	Y	N	Y	Y
EG/2321	2.2.1	Y	90	6	Y	Y	N	Y	Y
EG/4935	2.2.1	Y	90	6	Y	Y	Y	Y	Y
EG/3072	2.2.1	Y	90	6	Y	Y	Y	Y	Y
BD/5487	2.2.2.1	Y	90	6	Y	Y	N	Y	Y
BnSw/HK/10	2.3.2.1b	N	57	6	Y	Y	Y	Y	Y
Dk/VN/672	2.3.2.1b	N	57	6	Y	Y	Y	Y	Y
Dk/VN/1206	2.3.2.1a	N	0	5	Y	Y	Y	Y	Y
Dk/VN/1232	2.3.2.1a	N	90	5	Y	Y	Y	Y	Y
Dk/VN/0004	2.3.2.1c	N	57	5	Y	Y	Y	Y	Y
Dk/VN/2848	2.3.2.1c	N	57	5	Y	Y	Y	Y	Y

^a Indicates presence of Lysine at position 627.

^b #aa: Number of amino acids indicating protein length

^c Molecular markers of antiviral resistance to oseltamivir, peramivir and zanamivir (CDC, 2012).

^d aa: Contains a deletion of amino acids at the positions shown.

Table 4

HA amino acids reported to increase binding to α 2–6 linked sialic acid receptors.

Virus	Clade	D94N	S133A	S155N	D183G ^a	T188I	K189R	P235S	E75G/ S123P ^b	L128del I151T	S133A T188I ^b	154–6 gly ^c	165–7 gly ^c
Ck/KR/06	2.2	N	S	D	D	T	R	P	E/S	S/I	S/T	Y	Y
EG/2321	2.2.1	D	S	N	D	I	R	S	E/S	S/I	S/I	Y	Y
EG/4935	2.2.1	N	S	N	D	T	R	S	E/S	del/T	S/T	N	Y
EG/3072	2.2.1	N	S	N	D	T	R	S	E/S	del/T	S/T	N	Y
BD/5487	2.2.2.1	N	S	D	D	T	K	P	E/S	S/I	S/T	Y	Y
BnSw/HK	2.3.2.1b	N	A	N	D	T	R	P	E/P	L/L	A/T	N	Y
Dk/VN672	2.3.2.1b	N	A	N	D	I	R	P	E/P	L/L	A/I	N	N
Dk/VN1206	2.3.2.1a	N	A	N	N	T	R	P	E/S	L/I	A/T	N	Y
Dk/VN1232	2.3.2.1a	N	A	N	D	T	K	P	E/S	L/I	A/T	N	Y
Dk/VN0004	2.3.2.1c	N	A	N	D	T	R	P	E/S	S/I	A/T	N	Y
Dk/VN2848	2.3.2.1c	N	A	N	D	I	R	P	E/S	S/I	A/I	N	Y

^aMarker not present but one or more viruses possesses a different amino acid at this position.

^bThis combination of amino acids is necessary for binding, but some viruses only possess one.

^cIndicates the presence (Y) or absence (N) of glycosylation at these positions.