Highly pathogenic avian influenza H5N1 clade 2.3.2.1 and clade 2.3.4 viruses do not induce a clade-specific phenotype in mallard ducks

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Abstract

Among the diverse clades of highly pathogenic avian influenza (HPAI) H5N1 viruses of the goose/Guangdong lineage, only a few have been able to spread across continents: clade 2.2 viruses spread from China to Europe and into Africa in 2005–2006, clade 2.3.2.1 viruses spread from China to Eastern Europe in 2009–2010 and clade 2.3.4.4 viruses of the H5Nx subtype spread from China to Europe and North America in 2014/2015. While the poultry trade and wild-bird migration have been implicated in the spread of HPAI H5N1 viruses, it has been proposed that robust virus-shedding by wild ducks in the absence of overt clinical signs may have contributed to the wider dissemination of the clade 2.2, 2.3.2.1 and 2.3.4.4 viruses. Here we determined the phenotype of two divergent viruses from clade 2.3.2.1, a clade that spread widely, and two divergent viruses from clade 2.3.4, a clade that was constrained to Southeast Asia, in young (ducklings) and adult (juvenile) mallard ducks. We found that the virus-shedding magnitude and duration, transmission pattern and pathogenicity of the viruses in young and adult mallard ducks were largely independent of the virus clade. A clade-specific pattern could only be detected in terms of cumulative virus shedding, which was higher with clade 2.3.2.1 than with clade 2.3.4 viruses in juvenile mallards, but not in ducklings. The ability of clade 2.3.2.1c A/common buzzard/Bulgaria/38 WB/2010-like viruses to spread cross-continentally may, therefore, have been strain-specific or independent of phenotype in wild ducks.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 of the goose/Guangdong lineage was first recognized in 1996 during an outbreak in domestic geese in southern China [1], which was followed by an outbreak of a reassortant HPAI H5N1 virus in 1997 in Hong Kong [2–4]. After several years of low-level circulation in aquatic poultry in southern China, HPAI H5N1 re-emerged with outbreaks in domestic chickens and was associated with human infections from 2003 onwards [5–9]. Due to their rapid diversification, HPAI H5N1 viruses have been classified into clades (0 to 9) and sub-clades on the basis of their haemagglutinin (HA) gene sequence [10], and also by genotypes according to their internal gene constellations [11–13].

During spring 2005 an outbreak of HPAI H5N1 was observed in wild birds at Qinghai Lake in western China and subsequently classified as clade 2.2 (various genotypes) [14–16]. From late 2005 the virus spread westward through Russia, Kazakhstan, Mongolia and Turkey to Eastern and Western Europe, the United Kingdom, the Middle East, and eventually Africa [10, 16–19]. This outbreak was eventually stamped out/ceased, with the notable exception of Egypt, where clade 2.2 viruses have become entrenched in poultry and have further diversified [16, 20]. The manner of cross-continental transmission of clade 2.2 virus remains controversial in large part due to a lack of sustained surveillance activities immediately before the first outbreaks in 2005 in many of the affected countries, but virologically and
epizootologically, the outbreak in Qinghai Lake (2005) has been linked to European and African HPAI H5N1 cases [21–24]. Wild-bird migration and poultry movement through trade have both been implicated in the cross-continental spread of the pathogen [25–29]. Among wild migratory birds, mallard ducks have been identified as possible vectors in the spread of HPAI H5N1 [30–33]. From 2008 onwards a new subclade of HPAI H5N1, clade 2.3.2.1, gained high prevalence in China and Southeast Asia [10, 34], subsequently expanding from China to Mongolia, Russia and Eastern Europe in early 2010 [35–37], and also to South Korea and Japan and into Southeast Asia (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2016/). Clade 2.3.2.1 viruses have been particularly associated with wild birds, suggesting that some inherent characteristics of this virus subgroup may enhance its ability to establish and spread in some specie(s) of the wild-bird host population [10]. This is predicated on the observation that clade 2.3.4 viruses were also widespread in Southeast Asia over the same period of time (2008–2010), but appeared to be less associated with wild birds and did not expand outside of the region [37, 38].

From 2009 onwards, reassortment events of HPAI H5N1 with LPAI viruses were detected in China, resulting in the generation of multiple subtypes, including H5N2, H5N3, H5N6 and H5N8 [39–42]. Subtypes H5N6 and H5N8 of what were now termed clade 2.3.4.4 viruses spread from China into other Asian countries in 2013/2014 [43, 44]. Further dissemination to North America and Europe followed [45–48] and additional reassortment events were documented in North America with North American LPAI lineages [45]. HPAI H5Nx viruses of clade 2.3.4.4 were detected in wild migratory birds in North America, Europe and Asia [49–51].

In this study we examined co-circulating clade 2.3.2.1 and clade 2.3.4 viruses isolated in 2008–2010 using the mallard duck model. We aimed to determine whether a general replicative advantage exists, in the absence of severe symptoms, for clade 2.3.2.1 viruses that may be consistent with their wider geographic dispersal.

RESULTS

Phylogenetic relationship between four HPAI H5N1 viruses and residue markers of interest

Two viruses each from clades 2.3.4 and 2.3.2.1 (both progeny of genotype Z.3) were selected for this study: A/chicken/Hong Kong/AP156/2008 (AP156/2.3.4), A/chicken/Lao/LH2/2010 (LH2/2.3.4.1), A/barn swallow/Hong Kong/1161/2010 (HK1161/2.3.2.1b) and A/common buzzard/Bulgaria/38 WB/2010 (Bulg/2.3.2.1c). Bulg/2.3.2.1c had been isolated from a dead wild buzzard in Eastern Europe in early 2010 and represents viruses that spread out of Asia during that time [10, 36]. Within clade 2.3.4 two divergent viruses were chosen: the HA segment of AP156/2.3.4 is situated closer to the root of the clade 2.3.4 tree than LH2/2.3.4.1, which has been classified as belonging to sub-clade 2.3.4.1 [52, 53]. Subsequent to our virus selection, clade 2.3.2.1 subcategories a, b, and c were recognized in acknowledgement of their divergent evolution. Two relatively divergent viruses of clade 2.3.2.1 were chosen: HK1161/2.3.2.1b serves as the type strain for sub-clade 2.3.2.1b, while Bulg/2.3.2.1c is closely related to the type strain of sub-clade 2.3.2.1c, A/Hong Kong/6841/2010 [54].

The multi-basic cleavage site between HA1 and HA2 contained six basic amino acids immediately preceding the cleavage position for HK1161/2.3.2.1b and five for the other three viruses. We examined the HA protein for the presence of N-linked glycosylation motifs: using the N-[-P]-[S/T]-[P] motif as the indicator of a potential N-linked glycosylation site, we detected seven sites in AP156/2.3.4 and Bulg/2.3.2.1c (H5 numbering with A/Vietnam/1203/2004 as the reference strain: positions 10 or 11, 23, 140, 165, 286, 484, 543), and eight sites in LH2/2.3.4.1 and HK1161/2.3.2.1b (positions 10 or 11, 23, 140, 165, 273, 286, 484, 543).

In the HA receptor binding site we identified the following molecular markers: 133A and 189R in AP156/2.3.4, HK1161/2.3.2.1b and Bulg/2.3.2.1c (133S and 189K for LH2/2.3.4.1), and 188I in NA, indicating sensitivity to oseltamivir and an NA stalk deletion of 20 residues (position 49–68). In the M1 protein all four viruses harboured 30D and 215A, and in M2 the 27A determinant was present in AP156/2.3.4, indicating resistance to adamantanes. LH2/2.3.4.1 and HK1161/2.3.2.1b harboured truncated PB1-F2 open reading frames with 25 and 57 residues, respectively. AP156/2.3.4 harboured the 66D marker in PB1-F2. All but LaoLH2/2.3.4.1 (25 residue length) harboured 51M and 56V. The full-length PB1-F2 proteins of Bulg/2.3.2.1c and AP156/2.3.4 harboured 87E. The open reading frame for PA-X was present in all four viruses, predicting a protein with a 252-residue length. In the NS1 protein we observed a D92E substitution for HK1161/2.3.2.1b and a five-residue deletion (positions 80–84) in AP156/2.3.4, LH2/2.3.4.1 and HK1161/2.3.2.1b. The C-terminal motif at residue positions 227–230 was GSEV for AP156/2.3.4 and ESEV for LH2/2.3.4.1, HK1161/2.3.2.1b and Bulg/2.3.2.1c.

Virulence and replication of four HPAI H5N1 in inoculated ducklings

Young (3–4-week-old) mallard ducks (ducklings) were inoculated with 10^4 EID_{50} (50% embryo infectious dose) of virus via the natural route, as described in Methods. All inoculated ducklings became productively infected as determined by virus shedding and showed clinical signs, while the survivors showed sero-conversion.

Clinical symptoms including death were observed significantly earlier for HK1161/2.3.2.1b-inoculated animals (2.8 days post-inoculation (p.i.)) compared to Bulg/2.3.2.1c-inoculated animals (4.3 days p.i.; P = 0.004) and LH2/2.3.4.1-inoculated animals (4.5 days p.i.; P = 0.005). Only one animal inoculated with AP156/2.3.4 showed clinical signs (4 days p.i.; not significantly
Table 1. Virulence and transmission of four HPAI H5N1 viruses in young mallard ducks

<table>
<thead>
<tr>
<th>Duck group*</th>
<th>Challenge virus†</th>
<th>H5 clade</th>
<th>Morbidity/ mortality†</th>
<th>Mean day of shedding onset‡</th>
<th>Mean day of symptom onset§</th>
<th>Day of death¶</th>
<th>Mean shedding duration#</th>
<th>Mean peak titre**</th>
<th>Cumulative mean virus shedding††</th>
<th>HI titre‡‡</th>
<th>Productively infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>D HK1161 2.3.2.1b</td>
<td>25/100 1.0±0.0</td>
<td>2.8±0.3</td>
<td>2.3, 3, 3, 4</td>
<td>2.0±0.4</td>
<td>7.5±0.1 (3)</td>
<td>11.4±2.5</td>
<td>NA</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulg38WB 2.3.2.1c</td>
<td>100/0 1.0±0.0</td>
<td>4.3±0.3</td>
<td>4.5±0.3</td>
<td>7.0±0.5</td>
<td>4.8±0.5 (3)</td>
<td>26.3±2.8</td>
<td>55±15 (4/4)</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaoL2 2.3.4.1</td>
<td>100/75 1.0±0.0</td>
<td>4.5±0.3</td>
<td>5.3, 7, -</td>
<td>5.1±0.7</td>
<td>6.0±0.4 (3)</td>
<td>24.2±2.2</td>
<td>80 (1/1)</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HKAP156 2.3.2.1c</td>
<td>33/33 2.0±0.7</td>
<td>4</td>
<td>3, 7, -</td>
<td>3.8±2.1</td>
<td>2.6±1.0 (3)</td>
<td>9.4±2.1</td>
<td>53±13 (3/3)</td>
<td>3/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC HK1161 2.3.2.1b</td>
<td>50/100 2.3±0.3</td>
<td>4.5±0.6</td>
<td>3, 4, 5, 7</td>
<td>2.5±0.7</td>
<td>6.1±1.4 (5)</td>
<td>12.8±3.0</td>
<td>NA</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulg38WB 2.3.2.1c</td>
<td>100/25 3.0±0.7</td>
<td>6.5±0.6</td>
<td>9, -</td>
<td>7.6±0.7</td>
<td>4.9±0.3 (7)</td>
<td>29.4±1.2</td>
<td>25±15 (2/3)</td>
<td>4/4</td>
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<td></td>
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<tr>
<td>LaoL2 2.3.4.1</td>
<td>75/75 2.5±0.5</td>
<td>6.0±0.4</td>
<td>5, 8, -</td>
<td>4.8±0.8</td>
<td>5.1±0.3 (5)</td>
<td>19.7±2.1</td>
<td>40 (1/1)</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HKAP156 2.3.4</td>
<td>25/0 3.7±0.3*</td>
<td>3</td>
<td>-</td>
<td>1.7±0.6</td>
<td>1.6±0.9 (5)</td>
<td>4.0±2.0</td>
<td>40±0 (2/4)</td>
<td>3/4</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AC HK1161 2.3.2.1b</td>
<td>25/100 3.3±0.5</td>
<td>5.3±0.5</td>
<td>4, 6, 6, 6</td>
<td>2.3±0.6</td>
<td>5.5±0.7 (6)</td>
<td>9.9±1.7</td>
<td>NA</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulg38WB 2.3.2.1c</td>
<td>75/25 4.0±1.0*</td>
<td>10.5±2.5</td>
<td>9, -</td>
<td>5.1±1.8</td>
<td>5.0±0.5 (9)</td>
<td>18.9±6.4</td>
<td>170±150 (2/3)</td>
<td>3/4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LaoL2 2.3.4.1</td>
<td>50/50 5.3±1.2</td>
<td>7.8±0.3</td>
<td>10, 14, -</td>
<td>3.4±1.1</td>
<td>4.4±0.6 (9)</td>
<td>15.1±5.0</td>
<td>40 (1/2)</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HKAP156 2.3.4</td>
<td>0/0 5.3±0.3*</td>
<td>NA</td>
<td>-</td>
<td>2.3±1.2</td>
<td>1.6±0.9 (7)</td>
<td>4.8±2.3</td>
<td>20 (1/4)</td>
<td>3/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D HK1161 2.3.2.1b</td>
<td>63/50 1.0±0.0</td>
<td>-</td>
<td>-</td>
<td>4.5±1.0</td>
<td>6.0±0.6 (3)</td>
<td>18.9±3.3</td>
<td>-</td>
<td>8/8</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Bulg38WB 2.3.2.1c</td>
<td>63/50 1.5±0.4</td>
<td>-</td>
<td>-</td>
<td>3.9±1.0</td>
<td>4.3±0.8 (3)</td>
<td>16.8±3.1</td>
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<td>7/7</td>
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<tr>
<td>LaoL2 2.3.4.1</td>
<td>50/50 3.0±0.4</td>
<td>-</td>
<td>-</td>
<td>5.1±1.1</td>
<td>4.8±0.7 (5)</td>
<td>21.1±3.5</td>
<td>-</td>
<td>8/8</td>
<td></td>
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<tr>
<td>HKAP156 2.3.4</td>
<td>50/38 3.6±0.5</td>
<td>-</td>
<td>-</td>
<td>2.9±0.8</td>
<td>3.4±0.8 (5)</td>
<td>11.9±3.3</td>
<td>-</td>
<td>7/8</td>
<td></td>
<td></td>
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<tr>
<td>DC HK1161 2.3.2.1b</td>
<td>75/63 2.6±0.4</td>
<td>-</td>
<td>-</td>
<td>5.1±1.1</td>
<td>4.8±0.7 (5)</td>
<td>21.1±3.5</td>
<td>-</td>
<td>8/8</td>
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<tr>
<td>Bulg38WB 2.3.2.1c</td>
<td>50/38 3.0±0.4</td>
<td>-</td>
<td>-</td>
<td>2.9±0.8</td>
<td>3.4±0.8 (5)</td>
<td>11.9±3.3</td>
<td>-</td>
<td>7/8</td>
<td></td>
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<tr>
<td>LaoL2 2.3.4.1</td>
<td>50/38 3.6±0.5</td>
<td>-</td>
<td>-</td>
<td>3.7±1.0</td>
<td>3.8±1.3 (9)</td>
<td>14.4±3.5</td>
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<td>7/8</td>
<td></td>
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<tr>
<td>HKAP156 2.3.4</td>
<td>25/25 5.3±0.6</td>
<td>-</td>
<td>-</td>
<td>2.3±0.7</td>
<td>2.1±0.7 (7)</td>
<td>9.9±3.2</td>
<td>-</td>
<td>7/8</td>
<td></td>
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</tr>
</tbody>
</table>

*D, donor (inoculated) animals; DC, direct-contact animals; AC, aerosol-contact animals.
†Abbreviations as in Methods.
‡In per cent; morbidity as detected via conjunctivitis, depression, or neurological symptoms (ataxia, seizure, disorientation); some animals died/were found dead without symptoms having been observed.
§Mean day p.i. ± standard error. Limit of detection was 0.75 log₁₀ EID₅₀ ml⁻¹. *one animal in the group did not shed.
‖Mean day p.i. ± standard error. For mean day of symptom onset, death was included as a symptom.
¶Either found dead in cage or euthanized due to moribund state.
#Mean day p.i. ± standard error for oropharyngeal shedding. Swabs were titrated every day or every other day; we considered that a bird that shed from day 1 to day 5 but not on day 7 and with no data for day 6 shed 4.5 days. Birds that did not shed were excluded from the shedding duration calculation.
**In log₁₀ EID₅₀ ml⁻¹. Limit of detection was 0.75 log₁₀ EID₅₀ ml⁻¹. (Day of peak titre p.i.)
††Cumulative mean virus shedding over time (area under the curve) as log₁₀ EID₅₀ of oropharyngeal swabs.
‡‡Mean haemagglutination inhibition titre to homologous antigen ± standard error (animals with HI titre ≥10/surviving animals).
§§By virus shedding and/or seroconversion.
different from any other group) (Table 1). All ducklings inoculated with Bulg/2.3.2.1c or LH2/2.3.4.1 developed conjunctivitis (4/4), as did one inoculated with AP156/2.3.4 (1/4). Neurological signs (ataxia, seizures, disorientation) developed in five of 20 inoculated ducklings, and were present in at least one bird in each virus group. Overall, 100% of donor birds inoculated with HK1161/2.3.2.1b and 75% of donor birds inoculated with LH2/2.3.4.1, succumbed to infection, while one mallard inoculated with AP156/2.3.4 died, and all birds inoculated with Bulg/2.3.2.1c survived (Tables 1 and 2).

Oropharyngeal virus shedding was higher than cloacal shedding in all groups, although bird D-4 of the AP156-shedding animals showed higher titres in the cloacal sample (Fig. 1). There was no discernable difference in the relative magnitude of oropharyngeal and cloacal shedding between any of the viruses. Oropharyngeal viral titres were used in all comparisons of virus titres and shedding duration (Fig. 1).

The mean peak titres in all virus groups were reached 3 days p.i., with HK1161/2.3.2.1b having significantly higher titres than any other group (P-values 0.008–0.026) (Table 1). Shedding onset (oropharyngeal) was rapid with virus detected 1 day p.i. and virus replication apparent by 2 days p.i. for all groups (Fig. 1). Bulg/2.3.2.1c- and LH2/2.3.4.1-inoculated birds shed virus for similar periods of time. Bulg/2.3.2.1c-inoculated birds shed virus significantly longer than those inoculated with HK1161/2.3.2.1b or AP156/2.3.4 (P-values 0.003 and 0.01, respectively). LH2/2.3.4.1-inoculated birds shed virus for significantly longer than HK1161/2.3.2.1b-inoculated birds (P-value 0.007), with the difference in shedding duration compared to AP156/2.3.4 not reaching significance (Table 1).

Overall, Bulg/2.3.2.1c and LH2/2.3.4.1 showed similar duration of virus shedding and peak virus titres in the inoculated (donor) mallards. HK1161/2.3.2.1b- and AP156/2.3.4-infected birds shed for a shorter duration and had a lower peak titre (Table 1). Using cumulative mean virus shedding over time as a measure of virus produced, we found that Bulg/2.3.2.1c and LH2/2.3.4.1 donor mallards produced significantly more virus during the course of infection than either HK1161/2.3.2.1b or AP156/2.3.4 (P-values 0.003–0.009).

All surviving ducklings exhibited haemagglutination inhibition (HI) titres >10 using chicken red-blood cells (CRBCs) and homologous antigen with no differences between groups (Table 1).

Overall, a clade-specific pattern of virus shedding or clinical symptoms was not apparent in inoculated young (donor) mallards.

Direct contact and airborne transmission of four HPAI H5N1 viruses in ducklings

Mallard ducks have been implicated in the cross-continen- tinal dissemination of HPAI H5N1 during 2005–2006 and 2009–2010 [25, 26, 28, 29], requiring multiple rounds of transmission between birds. Transmission could have been mediated through various modes: direct contact or indirect contact with infected animals, via shared food and water, through fomites, droplets, or aerosols. In order to deter- mine whether the transmission dynamics differed between the viruses under investigation, two different modes of transmission were assessed: direct contact (including shared food and water) and airborne contact (food and water not shared; birds physically separated but within the same airspace).

On 1 day p.i. one donor mallard was placed with one direct- contact and one airborne-contact mallard, with four repeti- tions for each virus (four donors, four direct contacts and four airborne contacts in total for each virus), as described in Method.

All four HPAI H5N1 viruses transmitted to direct-contact animals, with only one direct-contact mallard in the AP156/2.3.4 group remaining uninfected as determined by virus shedding, clinical signs and seroconversion (Table 1, Fig. 1).

Additionally, all four HPAI H5N1 viruses transmitted to airborne-contact animals, with only one airborne-contact mallard in the AP156/2.3.4 group and one airborne-contact mallard in the Bulg/2.3.2.1c group remaining uninfected, as determined by virus shedding, clinical signs and seroconversion (Table 1, Fig. 1).

Clinically, in the direct-contact animals, all viruses caused disease comparable to that caused in their respective inoculated donor birds. Shedding onset was rapid post-contact (1.3–2.7 days), though it was significantly delayed for AP156/2.3.4 compared to HK1161/2.3.2.1b (P-value 0.02) (Table 1).

The duration and magnitude of viral shedding was consistent between donor mallards and direct-contact mallards for each virus. Consistent with this observation, among the direct-contact mallards, Bulg/2.3.2.1c was shed for a significantly longer duration than the other three viruses [P-values of 0.001 (HK1161/2.3.2.1b), 0.03 (LH2/2.3.4.1) and 0.0002 (AP156/2.3.4)]. LH2/2.3.4.1 was shed for significantly longer than AP156/2.3.4 (P-value 0.007) (Table 1).

Among the direct-contact mallards, the AP156/2.3.4-group had statistically significantly lower mean peak titres than the LH2/2.3.4.1- and Bulg/2.3.2.1c-groups (P-values of 0.01 and 0.02, respectively) (Table 1).

Not all surviving direct-contact mallards seroconverted by HI using homologous antigen: half of the AP156/2.3.4 direct contacts did not seroconvert, and of these one did not shed detectable virus and one shed virus at one time point at the limit of detection. One of three surviving Bulg/2.3.2.1c-direct-contact mallards did not seroconvert, even though this animal shed intermediate amounts of virus. Among the mallards that did seroconvert, there were no significant differences in HI titres between donor mallards and direct contact or between different virus groups among the direct- contact mallards (Table 1).
Table 2. Virulence and replication of four HPAI H5N1 viruses in adult mallards

<table>
<thead>
<tr>
<th>Challenge virus*</th>
<th>No. ducks</th>
<th>Clade</th>
<th>Mortality/ morbidity†</th>
<th>Mean day of shedding onset‡</th>
<th>Mean day of symptom onset§</th>
<th>Day of death∥</th>
<th>Mean shedding duration¶</th>
<th>Mean shedding duration until death¶</th>
<th>Mean peak titre##</th>
<th>Cumulative mean virus shedding**</th>
<th>HI titre††</th>
<th>Productively infected‡‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK1161</td>
<td>4</td>
<td>2.3.2.1b</td>
<td>75/100</td>
<td>1.0±0.0</td>
<td>4.8±0.6</td>
<td>4, 6, 8, –</td>
<td>6.0±1.2</td>
<td>5.0±1.0</td>
<td>4.6±0.5 (5)</td>
<td>21.1±3.3</td>
<td>15 (1/1)</td>
<td>4/4</td>
</tr>
<tr>
<td>Bulg38WB</td>
<td>4</td>
<td>2.3.2.1c</td>
<td>0/50</td>
<td>1.0±0.0</td>
<td>4.5±0.5</td>
<td>–, –, –, –</td>
<td>4.8±0.5</td>
<td>NA</td>
<td>4.3±0.5 (1)</td>
<td>17.1±1.7</td>
<td>43±14 (4/4)</td>
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</tr>
<tr>
<td>LaoLH2</td>
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<td>1.0±0.0</td>
<td>NA</td>
<td>–, –, –, –</td>
<td>3.8±0.8</td>
<td>NA</td>
<td>3.5±0.5 (1)</td>
<td>11.5±3.9</td>
<td>60±12 (4/4)</td>
<td>4/4</td>
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<td>HKAP156</td>
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<td>NA</td>
<td>–, –, –, –</td>
<td>2.5±0.4</td>
<td>NA</td>
<td>2.0±1.0 (1)</td>
<td>47±2.3</td>
<td>73±7 (3/3)</td>
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<td>–</td>
<td>–</td>
<td>5.4±0.6</td>
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<td>3.5±0.4 (5)</td>
<td>19.1±1.9</td>
<td>–</td>
<td>8/8</td>
</tr>
<tr>
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<td>3.3±0.6</td>
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<td>3.3±0.4 (1)</td>
<td>8.6±2.7</td>
<td>–</td>
<td>7/7</td>
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<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Abbreviations as in Methods.
†In per cent; morbidity as detected via conjunctivitis, depression, or neurological symptoms (ataxia, seizure, disorientation). Some animals died/were found dead without symptoms having been observed.
‡Mean day p.i. ± standard error. Oropharyngeal shedding. Limit of detection was 0.75 log$_{10}$ EID$_{50}$ ml$^{-1}$. §One animal did not shed.
§Mean day p.i. ± standard error. For mean day of symptom onset, death was included as a symptom.
∥Either found dead in cage or euthanized due to moribund state.
¶Shown are mean days ± standard error of oropharyngeal shedding. Swabs were titrated every day or every other day; we considered that a bird that shed from day 1 to day 5 but not on day 7 and with no data for day 6 shed 4.5 days. The bird in the HKAP156 group that did not shed was excluded from the shedding duration calculation. NA, not applicable.
##In log$_{10}$ EID$_{50}$ ml$^{-1}$. Limit of detection was 0.75 log$_{10}$ EID$_{50}$ ml$^{-1}$. (Day of peak titre p.i.)
**Cumulative mean virus shedding over time (area under the curve) as log$_{10}$ EID$_{50}$ of oropharyngeal swabs.
††Mean haemagglutination inhibition titre to homologous antigen ± standard error (animals with HI titre ≥10/surviving animals).
‡‡By virus shedding and/or seroconversion.
Overall, no clade-specific pattern of virus replication could be detected between the four viruses. Additionally, when using combined datasets to further investigate any differences between clades 2.3.2.1 and 2.3.4 in the direct-contact animals, no differences in the magnitude or duration of virus shedding or cumulative virus shedding over time were observed.

In the airborne-contact animals the clinical symptoms and mortality rates remained largely consistent with the direct-contact and donor groups, with little difference in morbidity and mortality (Table 1). The onset of symptoms occurred significantly faster in the HK1161/2.3.2.1b group compared to the LH2/2.3.4.1 group (P-value 0.04) and the Bulg/2.3.2.1c group (P-value 0.004), while the AP156/2.3.4-group did not display any symptoms (Table 1).

In terms of shedding duration there were no significant differences between the four airborne-contact virus groups and their matched groups among direct-contact or donor mallards, but also no significant differences of shedding duration between the different virus groups (Table 1). The significant differences observed in the donor and direct-
contact groups were hence lost. There were no significant differences of peak virus titres between the airborne-contact groups and their donor and direct-contact groups (Table 1). Significantly lower mean peak titres were observed in the AP156/2.3.4 airborne contacts compared to HK1161/2.3.2.1b airborne contacts ($P$-value of 0.03) and Bulg/2.3.2.1c airborne contacts ($P$-value of 0.03), consistent with the patterns observed in the donor and direct-contact mallards.

When using combined datasets to further investigate any significant differences between clades 2.3.2.1 and 2.3.4 in the airborne-contact animals, no differences in magnitude or duration of virus shedding, or in cumulative virus shedding over time were observed (Table 1).

Not all surviving airborne-contact mallards seroconverted by HI using CRBC and homologous antigen (Table 1). Those animals that did not seroconvert tended to shed low amounts of virus or started to shed late during the experiment and therefore may have had insufficient time to seroconvert at the end of the experiment. Among the mallards that did seroconvert, there were no significant differences in HI titres between airborne contacts and donors or direct contacts, or between different virus groups among the airborne-contact mallards (Table 1).

**Virulence and replication of four HPAI H5N1 viruses in juvenile mallards**

Surveillance studies on LPAI viruses have identified first-season birds to be particularly receptive to avian influenza virus (AIV) infection, although changes of the dominant AIV subtype between different years and the infection of older adult birds have also been observed [55–58]. Mallards fledge at 50–60 days post-hatch and are considered juvenile until about 14 months of age. First-season birds or mallards during their first migration would therefore be considered juveniles.

We assessed the phenotypes of the four distinct HPAI H5N1 viruses of this investigation in AIV-naïve 3–4 month-old mallard ducks. These juvenile mallards were inoculated with $10^4$ EID$_{50}$ via the natural route as described in Methods. Overall, the clinical signs in juvenile mallards were milder than in ducklings and symptoms could only be detected in the clade 2.3.2 virus groups: three of four ducks inoculated with HK1161/2.3.2.1b developed conjunctivitis, two animals developed neurological symptoms and 75% succumbed to the infection or were euthanized due to moribund state (Table 2). These mallards showed symptoms on average 4.8 days p.i. and died on average 6 days p.i. Of the ducks inoculated with Bulg/2.3.2.1c, half developed conjunctivitis (4.5 days p.i.), but all animals had fully recovered by the end of the experiment (14 days) (Table 2). No morbidity was observed in the juvenile mallards inoculated with either AP156/2.3.4 or LH2/2.3.4.1.

Virus shedding was higher in oropharyngeal samples than in cloacal samples for all groups (Fig. 1(i,j)) and commenced early after inoculation for all virus groups (Table 2).

The mean peak titres of virus shedding were reached 1 day p.i. for all but the HK1161/2.3.2.1b-inoculated group, where the mean peak titre was reached 5 days p.i. (Table 2). There were no statistically significant differences between the mean peak titres of each virus group.

The average duration of virus shedding (oropharyngeal) was the longest for those mallards inoculated with HK1161/2.3.2.1b (6 days) and statistically similar for Bulg/2.3.2.1c (4.8 days) and LH2/2.3.4.1 (3.8 days). AP156/2.3.4 was shed on average for 2.5 days, which was statistically shorter than Bulg/2.3.2.1c ($P$-value 0.04) (Table 2).

Cumulative mean virus shedding was significantly greater in the two clade 2.3.2.1 virus groups, HK1161/2.3.2.1b and Bulg/2.3.2.1c, compared to the AP156/2.3.4 group ($P$-values 0.007 and 0.013, respectively), but with no significant difference observed compared to the LH2/2.3.4.1-group (Table 2).

We then examined virus shedding according to clade using combined datasets and found no statistical differences in the mean peak titres between the clade 2.3.2.1-inoculated and the clade 2.3.4-inoculated juvenile mallards. However, the duration of virus shedding was significantly longer in mallards inoculated with clade 2.3.2.1 viruses (5.4 days) than in clade 2.3.4 viruses (3.3 days; $P=0.038$), as was cumulative virus shedding ($P$-value=0.006). All survivors exhibited HI titres >10 using CRBC and homologous antigens (Table 2). There were no statistically significant differences in HI titres between the four groups.

**Comparison of virulence and virus replication observed in young and juvenile mallards**

Both young and juvenile mallards were exposed to the same dose of virus ($10^4$ EID$_{50}$). Clinically, ducklings displayed greater morbidity and mortality than juvenile mallards (Table S1, available in the online Supplementary Material).

Onset of virus shedding was rapid in both young and juvenile birds, with no significant differences observed (Tables 1 and 2). The mean peak titres (oropharyngeal) were significantly higher in ducklings inoculated with LH2/2.3.4.1 ($P=0.007$) and HK1161/2.3.2.1b ($P=0.009$) compared with juvenile mallards. This trend was not observed in ducklings inoculated with Bulg/2.3.2.1c and AP156/2.3.4 (Tables 1 and 2).

In terms of duration of virus shedding, ducklings inoculated with HK1161/2.3.2.1b shed for a significantly shorter period of time than the juvenile mallards ($P$-value 0.018), due to more rapid progression to death. Ducklings inoculated with Bulg/2.3.2.1c shed for a significantly longer period of time ($P$-value 0.021) compared to juvenile mallards (Tables 1 and 2; Fig. 1). LH2/2.3.4.1- or AP156/2.3.4-inoculated mallards showed no significant differences in shedding duration between young and juvenile birds (Table S1).

When comparing the cumulative virus shedding of adult and young donor mallards, ducklings inoculated with LH2/2.3.4.1 or Bulg/2.3.2.1c shed significantly more virus over time than juvenile mallards ($P$-values 0.039 and 0.038,
respectively). There was no statistical difference in cumulative virus shedding in the AP156/2.3.4 or HK1161/2.3.2.1b groups (Table S1). We then examined virus shedding according to clade using combined datasets and found no statistical differences in the cumulative virus shedding between 2.3.2.1-inoculated and 2.3.4-inoculated young and juvenile mallards.

**DISCUSSION**

The goose/Guangdong (Gs/Gd) lineage of HPAI H5N1 has become an extremely successful virus lineage since its genesis in the 1990s: different clades and sub-clades of HPAI H5N1 have disseminated over vast geographical distances and established stable host interactions with domestic waterfowl, with frequent spillover infections into poultry and wild birds [1, 12, 14, 38, 59].

To date, three large expansions of Gs/Gd-HPAI H5N1 have been observed: clade 2.2 in 2006/2007, clade 2.3.2.1 in 2009/2010 and clade 2.3.4.4 from 2014/2015 [36, 38, 48, 60]. While these viruses have all been derived from the Gs/Gd-lineage HA, their genotypic constellations have been somewhat more complicated. Genotype Z (A/duck/Guangxi/50/01-like) viruses were the most predominant by the early 2000s, eventually giving rise to genotypes Z.1 (the HA clade 2.2 precursor) and Z.3 (precursor to clades 2.1 and 2.3.4) [61]. The 2.2 lineage further evolved through mutations and was sub-divided into clades 2.2 and 2.2.1 [62]. Clade 2.3.2.1 genotypes have also evolved into 2.3.2.1a/b/c sub-clades and undergone multiple intra-2.3.2.1 reassortment events (such as in Vietnam in 2012, as described by Creanga et al. in 2013 [54]), as well as inter-clade reassortments, such as the 2.3.2.1/2.3.4.4 reassortants observed in Bangladesh in 2011–2014 [63]. A/buzzard/Bulgaria/38 WB/2010 (clade 2.3.2.1c) is a 2.3.2.1c virus harbouring an M-gene from an H5N1 clade 2.3.4 virus [64]. In addition, clade 2.3.4 viruses have further evolved into sub-sub-clades as well [62]. The 2.3.4.4 viruses have diversified through reassortment in multiple ways, including: (i) in North America in 2014–2015, where reassortment of clade 2.3.4.4 H5N8 viruses with avian viruses of the North-American lineage resulted in the generation of novel H5N2 viruses [65]; and (ii) in Europe in 2015–2017, where HPAI H5N8 viruses of clade 2.3.4.4 were generated through reassortment between Asian HPAI H5N8 viruses and Asian LPAI viruses [66]. Among the four viruses we used in the present study, A/buzzard/Bulgaria/38 WB/2010 (clade 2.3.2.1c) is actually a 2.3.2.1c virus harbouring an M-gene from H5N1 clade 2.3.4 [64], A/chicken/AP156/2008 (clade 2.3.4) contains a PB1 gene segment from H9N2 viruses [closest hit by Blast: A/chicken/Shandong/LY/2008(H9N2)], and the other two viruses harbour full 2.3.2.1 or 2.3.4 gene constellations [64].

In 2006, the HPAI H5N1 viruses of clade 2.2 were detected in Asia, Europe and Africa, and clade 2.2 became established in poultry in Egypt, disappearing from Europe and the remaining African countries [38]. In late 2009 to early 2010, clade 2.3.2.1c expanded from China to Siberia, Bangladesh, Nepal, Bhutan and eventually Eastern Europe, but not further [36, 60–63]. In August 2011 a second, seemingly independent introduction of clade 2.3.2.1c was detected in northern Iran [64]. Furthermore, clade 2.3.2.1c viruses also reached Java, Indonesia, by unknown means [65]. In 2014/2015 clade 2.3.4.4 viruses spread from China into other Asian countries, and to Europe and North America [43–48]. Outbreaks in North America and Europe seemed transient in 2014–2015, while various subtypes of clade 2.3.4.4 continued to circulate in China and Southeast Asia (EMPRES-i, http://empres-i.fao.org/, data recovered 22 March 2017, [66]). It is interesting to note that while the 2.3.4 viruses, represented by A/chicken/Hong Kong/AP156/2008 and A/chicken/Lo/LH2/2010 in this study, were localized geographically, descendants of this clade, specifically 2.3.4.4 viruses, have spread globally, aided by wild birds. Such an event is consistent with our conclusion that association with wild birds is a virus characteristic rather than a clade-specific phenomenon. It is unclear what drove the dissemination of the clade 2.3.4.4 viruses, but it is clear they have been a major influence on the impact of the Gs/Gd-lineage viruses. The circulation of clade 2.3.4.4 viruses, the majority of which did not contain the N1 gene segment predominant in the Gs/Gd-lineage HPAI H5N1 viruses, increased in China from 2010 to 2017. The Influenza Research database (https://www.fludb.org/brc/home.spg?decorator=influenza) contained 17 HA sequences of clade 2.3.4.4 H5 between 2008 and 2010 (all originating from China, all from H5N1 viruses), while 71 2.3.4.4 HA sequences were available for the 2011–2013 period (68 of them from China; 3 H5N1) and 464 were available for 2014–2017 (as of 22 March 2017; 154 of them from China; 15 H5N1 only). While it is risky to directly interpret the increase in 2.3.4.4 virus sequences in public databases as representing the actual increase in the virus in the field, it is clear that these viruses have been successful. Human cases of HPAI H5 caused by 2.3.4.4 HA viruses have all been of the H5N6 subtype, [62] and long-distance spread has been observed with H5N6 and H5N8 viruses. Both the increase in virus circulation and the changes in gene constellation may have favoured the geographical spread of clade 2.3.4.4 viruses.

In this study (carried out before 2.3.4.4 viruses spread outside Asia), we investigated the putative role of mallard ducks in the spread of one of the virus clades that has been spread intercontinentally, HPAI H5N1 clade 2.3.2.1. We hypothesized that clade 2.3.2.1 HPAI H5N1 viruses induce a phenotype in mallard ducks that would be (1) characterized by robust virus replication in the absence of overt symptoms, (2) universal for the clade and (3) readily distinguishable from the phenotype of a contemporary clade that did not spread beyond Asia (clade 2.3.4). Using two divergent viruses of clade 2.3.4 (LH2/2.3.4.1 and AP156/2.3.4) and two divergent viruses of clade 2.3.2.1 (Bulg/2.3.2.1c and HK1161/2.3.2.1b), we found that all four viruses were able to infect young (4-week-old) and adult (3-month-old) mallards efficiently. Pathogenicity varied greatly between the four viruses, from mildly pathogenic AP156/2.3.4 to lethal...
HK1161/2.3.2.1b, with ducklings experiencing more severe symptoms than juvenile mallards. A deletion in the stalk of the NA protein was present in all four viruses and has been associated with both adaptation to terrestrial poultry and increased virulence in mallards [67, 68]. This molecular signature has been a hallmark of Gs/Gd-lineage HPAI H5N1 viruses since the early 2000s [6, 38, 68].

Previous observational and experimental studies have implicated migratory waterfowl as possible vectors of HPAI viruses since the early 2000s [6, 38, 68]. Using experimental infections, several wild waterfowl species have been shown to shed different HPAI H5N1 viruses robustly in the absence of overt clinical symptoms [70, 71]. It has been proposed that this phenotype (robust shedding and few-to-no clinical signs) was necessary for migratory waterfowl to be able to contribute to the cross-continental spread of HPAI H5N1 [27, 28, 37].

Of late, the extensive spread of clade 2.3.4.4 viruses has led to several investigations of virus shedding from mallard ducks: Kang et al. [72] determined the pathogenicity of three clade 2.3.4.4 H5N8 viruses, one clade 2.2 H5N1 virus and one clade 2.3.2.1 H5N1 virus in intranasally inoculated wild-captured mallard ducks [72]. Mallard ducks showed no mortality and little morbidity when challenged with any of the study viruses. Virus replication was detected in donor mallards and in direct-contact cage mates, with virus shedding higher from the trachea than the cloaca [72]. Zhao et al. [39] reported generally mild-to-intermediate symptoms in mallard ducks inoculated intranasally with H5N5 and H5N8. Viruses were shed both from the trachea and cloacally, with higher titres detected in tracheal swabs [39].

We found that in donor (inoculated) ducklings the peak titre, duration of virus shedding and cumulative virus shedding varied between the four study viruses, but that no clade-specific phenotype could be identified. In juvenile mallards we detected significant differences between clade 2.3.2.1 and clade 2.3.4 viruses in shedding duration and cumulative virus shedding. Clade-2.3.2.1-infected birds shed for longer, resulting in greater cumulative virus shedding than their clade-2.3.4-infected counterparts. Of note, the clade 2.3.2.1c virus that represents the strains capable of cross-continental spread (Bulg/2.3.2.1c) was consistently shed for a statistically longer duration than the other viruses in both adult and young donor (inoculated) mallards and also in young direct-contact mallards. Previous studies have detected shedding of HPAI H5N1 viruses from ducks for 2–5 days p.i. and sometimes for much longer, both in the absence of overt symptoms [70, 71]. In Hulse-Post et al. [74] A/mallard/Vietnam/16D/03 and A/chicken/Vietnam/48C/04 were shed for 17 days by experimentally inoculated ducks, but direct-contact transmission was only observed with A/mallard/Vietnam/16D/03 and not with A/chicken/Vietnam/48C/04. Therefore the duration of virus shedding was not the sole determinant for virus transmission in this instance [74].

The mode of transmission of HPAI H5N1 among migratory birds necessarily has a large effect on the propensity of these hosts to spread these viruses over large distances. LPAI transmission between wild waterfowl is thought to be largely mediated by the fecal–oral route [75]. However, HPAI H5N1 is predominantly shed via the respiratory system [70] and therefore airborne transmission in addition to shared food/water may be an important mode of transmission between wild waterfowl. We therefore examined the transmission of our four viruses of interest in both a direct-contact and an airborne-contact setting.

All four study viruses were transmitted to direct-contact mallards rapidly and efficiently, with clinical symptoms and virus shedding consistent with the donor groups for each virus. Not all surviving direct-contact mallards seroconverted, which was associated with low or no detectable viral replication (AP156/2.3.4), except for one animal in the Bulg38/2.3.2.1c direct-contact group, which showed intermediate levels of virus shedding. Among the mallards that did seroconvert, there were no significant differences in HI titres between direct-contact mallards and donor mallards.

In our setting, almost all airborne-contact mallards were productively infected. Clinical symptoms and virus shedding were consistent with donor and direct-contact groups. No significant differences in magnitude or duration of virus shedding, or in cumulative virus shedding over time, were observed between clades 2.3.2.1 and 2.3.4 in the airborne group. We conclude that all four viruses were well able to transmit to airborne contacts, causing disease comparable to that in donor and direct-contact animals.

In this study we hypothesized that highly related viruses, as defined by HA clade, would display a common phenotype; in this case ability or inability to be spread via mallards. While this is a possibility, it is also possible that mixed phenotypes are present in similar viruses within a clade. In this light, a limitation of our study was that only two divergent representative viruses of each clade were examined. We were able to identify only limited evidence of a clade-specific phenotype in juvenile mallards, but not in ducklings. In the context of potential transmission during bird migration, the virus phenotype in juvenile mallards rather than in ducklings may be more informative. Furthermore, the mallard duck species may not be the optimal model system for assessing the capability of clade 2.3.2.1 or other HPAI H5N1 viruses to spread from Asia to Europe: other duck species or migratory geese may be the preferred vector for this wild-bird-mediated transmission [76–78].

Further phenotype assessment of HPAI H5N1 viruses, particularly of those more frequently isolated from wild birds, is warranted to identify fitness markers in mallards and particularly of those more frequently isolated from wild birds, and therefore airborne transmission in addition to shared food/water may be an important mode of transmission between wild waterfowl. We therefore examined the transmission of our four viruses of interest in both a direct-contact and an airborne-contact setting.

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Further phenotype assessment of HPAI H5N1 viruses, particularly of those more frequently isolated from wild birds, is warranted to identify fitness markers in mallards and potentially in other wild waterfowl species. Comparative molecular analyses between pre-2014 2.3.4, 2014–2016 2.3.4.4 (that reached Europe, Africa and North America in 2014/2015) and 2.3.2.1c gene sequences were carried out in an attempt to link phenotypes of viruses spreading over
long distances and molecular determinants, but no specific markers could be identified. Further studies of clade 2.2 viruses (that reached Europe and Africa in 2005/2006) and clade 2.3.4.4 viruses (that reached Europe, Africa and North America in 2014/2015 and 2016/2017) in comparison to other sub-clades circulating in Asia during 2005–2006 and 2014–2015, respectively, would be especially informative to better understand the factors involved in the potential for inter-continental spread of HPAI H5Nx strains by wild birds. Taken together, post-2014 2.3.4.4 HPAI H5 viruses have shown a high degree of reassortment and this ongoing evolution may indicate that the viruses have not yet reached a stable equilibrium with their host(s).

**METHODS**

**Viruses**

Four HPAI H5N1 viruses were used: clade 2.3.2.1b virus A/barn swallow/Hong Kong/1161/2010 (HK1161/2.3.2.1b), clade 2.3.2.1c virus A/buzzard/Bulgaria/38WB/2010 (Bulg/2.3.2.1c), clade 2.3.4 virus A/chicken/Hong Kong/AP156/2008 (AP156/2.3.4) and clade 2.3.4.1 virus A/duck/Lao/LH2/2.3.4.1/2010 (LH2/2.3.4.1). The viruses were propagated in 10-day-old embryonated chicken eggs. All experiments were carried out at the St Jude Children’s Research Hospital (St Jude) enhanced biosafety level 3 (ABSL3+) facility approved by the US Department of Agriculture. Viruses were sequenced by the Hartwell Center for Bioinformatics and Biotechnology at St Jude, and analysed with BioEdit and MEGA software [79, 80]. The full genome sequences are available in GenBank under the following accession numbers: CY110851 to CY110858 for Bulg/2.3.2.1c; CY098304, CY098305, KF735635 to KF735640 for LH2/2.3.4.1; KC436130, KC357320, KF735641 to KF735646 for HK1161/2.3.2.1b; and KF735634 to KF735634 for AP156/2.3.4. Before infection, virus titre was determined by calculating the 50% embryo infectious dose (EID$_{50}$) by the method of Reed and Muench [81].

**Assessment of virus pathogenicity and transmission in ducks**

Mixed-sex mallard ducks (*Anas platyrhynchos*) were either purchased as 1-day-old hatchlings from Ideal Poultry (Cameron, TX, USA) or as fertilized eggs from Duckeggs.com (Corona, CA, USA), incubated, hatched, and brought up to the required age [3–4 weeks (ducklings) or 3–4 months (juvenile)] at the Animal Resource Center at St Jude Children’s Research Hospital. The ducks were wing-banded, and provided feed and water *ad libitum*. All animal experiments were approved by the Animal Care and Use Committee of St Jude and complied with institutional, National Institutes of Health and Animal Welfare Act policies and regulations. For all experiments, mallards were inoculated by instillation of 10$^5$ EID$_{50}$ of virus in a total volume of 1 ml via the natural route (500 μL intranasal and intraocular; 500 μL oral). One day post-infection (p.i.), each inoculated (donor) bird was moved to a cage with one naïve duck [direct-contact (DC) birds], adjacent to a cage with one other naïve duck [airborne-contact (AC) birds]. Donors and DC birds were separated from AC birds by 7.5 cm (3 in.). For the adult mallard study, four donors were inoculated for each virus with the exception of AP156, which had three donors. For the studies in young birds we used four donors, four DC and four AC ducks for each virus. All birds were observed at least once daily for morbidity and mortality. Moribund state was defined as inability to feed or drink, or apparent neurological symptoms (tremors, ataxia, seizures, torticollis, disorientation). Cloacal and oropharyngeal swabs were collected daily (experiments in juvenile and ducklings) for 14 days p.i. All surviving ducks were bled 14–18 days p.i.

**Virus titration**

Virus in oropharyngeal and cloacal swabs was titrated in embryonated chicken eggs, and the log$_{10}$ EID$_{50}$ ml$^{-1}$ was calculated by the method of Reed and Muench [81]. The lower limit of virus detection was 0.75 log$_{10}$ EID$_{50}$ ml$^{-1}$. A value of zero (log$_{10}$1) was assigned to titres below the lower limit of detection.

**Serology assays**

Convalescent duck sera (sampled 14–18 days p.i.) were screened by ELISA and/or HI assay. ELISAs were performed by using the IDEXX FlockChek AI Multi-S-Scree Ab Test kit according to the manufacturer’s instructions (Westbrook, ME, USA). For HI assays, duck sera were treated with receptor-destroying enzyme (Denka Seiken Co., Japan) overnight at 37°C, heat inactivated at 56°C for 30 min, diluted 1 : 10 with PBS and tested using 0.5% packed CRBCs as previously described [82].

**Statistical analyses**

Statistically significant differences between experimental groups were determined using one-way ANOVA and Student’s t-test of means (unpaired, two tailed) with Graph Pad Prism version 5.03 or MS Office Excel version 14.3.9. P-value<0.05 was considered statistically significant.

**Prediction of N-linked glycosylation**

To determine N-linked glycosylation we used the ScanProsite server (http://prosite.expasy.org/prosite.html) on 8 June 2014 [83]. ScanProsite uses the motif N-[P]-S/T-[P], where proline is not accepted in positions 2 and 4 and either serine or threonine is accepted in position 3, to detect potential N-linked glycosylation motifs.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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