Emergence of H5N1 avian influenza viruses with reduced sensitivity to neuraminidase inhibitors and novel reassortants in Lao People’s Democratic Republic

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Pandemic influenza viruses can emerge through continuous evolution and the acquisition of specific mutations or through reassortment. This study assessed the pandemic potential of H5N1 viruses isolated from poultry outbreaks occurring from July 2006 to September 2008 in the Lao People’s Democratic Republic (PDR). We analyzed 29 viruses isolated from chickens and ducks and two from fatal human cases in 2007. Prior to 2008, all H5N1 isolates in Lao PDR were from clade 2.3.4; however, clade 2.3.2 was introduced in September 2008. Of greatest concern was the circulation of three isolates that showed reduced sensitivity to the neuraminidase (NA) inhibitor oseltamivir in an enzyme inhibition assay, each with different NA mutations – V116A, I222L and K150N, and a previously unreported S246N mutation. In addition, six isolates had an S31N mutation in the M2 protein, which conferred resistance to amantadine not previously reported in clade 2.3.4 viruses. Two H5N1 reassortants were isolated whose polymerase genes, PB1 and PB2, were homologous to those of Eurasian viruses giving rise to a novel H5N1 genotype, genotype P. All H5N1 viruses retained avian-like receptor specificity, but four had altered affinities for α2,3-linked sialic acid. This study shows that, in a genetically similar population of H5N1 viruses in Lao PDR, mutants emerged with natural resistance to antivirals and altered affinities for α2,3-linked sialic acids, together with reassortants with polymerase genes homologous to Eurasian viruses. These changes may contribute to the emergence of a pandemic influenza strain and are critical in devising surveillance strategies.

INTRODUCTION

Since the introduction of highly pathogenic H5N1 influenza viruses in 1997, H5N1 has become endemic in the southern People’s Republic of China and has spread to over 60 countries in Europe, Asia and Africa. H5N1 outbreaks have resulted in the loss of millions of poultry and in sporadic transmission to humans (Li et al., 2004; Wang et al., 2008), and have raised concerns about its pandemic potential (Chen et al., 2004; Guan et al., 2004; Peiris et al., 2007). H5N1 viruses have evolved locally from this unprecedented

The GenBank/EMBL/DDBJ accession numbers for the sequences obtained in this study are CY034685–CY034724 and CY040893–CY041049.
spread, resulting in genetically and antigenically divergent H5N1 sublineages (Chen et al., 2004) that typically originate from one isolate emerging via antigenic drift (Guan et al., 2004) or reassortment (Guan et al., 1999; Li et al., 2004) within a specific region. Evolution of the H5 haemagglutinin (HA) glycoprotein has altered the antigenic properties of H5N1 viruses and produced antigenically distinct strains among genetically similar strains (Guan et al., 2004; Horimoto et al., 2004), which are identified by ten different clade designations (Donis et al., 2008).

Monitoring the changes that influence the sensitivity of H5N1 strains to antiviral drugs is crucial for the control of emerging strains. Because several human and avian isolates are resistant to adamantanes (Cheung et al., 2006; He et al., 2008), the neuraminidase (NA) inhibitor oseltamivir is currently the main antiviral agent used to manage H5N1 infection in humans. Oseltamivir-resistant H5N1 influenza viruses can emerge under selective drug pressure in vivo (de Jong et al., 2005; Le et al., 2005). H5N1 variants with NA mutations that decrease the sensitivity to oseltamivir have been isolated in the absence of drug pressure (Rameix-Welti et al., 2006). The recent emergence and global distribution of oseltamivir-resistant H1N1 influenza viruses (Meijer et al., 2009) raise concerns that a pandemic oseltamivir-resistant H5N1 influenza virus may emerge in nature.

Retrospective genetic analyses indicate that reassortment is a major factor driving the evolution of currently circulating H5N1 influenza strains. The highly pathogenic H5N1 virus transmitted to humans in Hong Kong in 1997 originated via reassortment of the HA gene segment from A/goose/Guangdong/1/96 (H5N1) (Xu et al., 1999) and internal segments from H6N1 (Hoffmann et al., 2000) or H9N2 (Guan et al., 1999) influenza viruses. That virus was eradicated by slaughtering all poultry in Hong Kong; however, new H5N1 genotypes that emerged in 2000 and 2001 had acquired internal genes from one or more unknown avian influenza viruses of waterfowl (Guan et al., 2002a, b, 2004). Forty-four genotypes recognized in Hong Kong and mainland China have emerged by reassortment of circulating H5N1 viruses with avian viruses from other aquatic birds (Guan et al., 2002b; Li et al., 2004). These reassortments generated the Z genotype, which emerged in 2002 and was dominant in most regions of Asia until 2005 and has been replaced by the V genotype in Southeast Asia (Duan et al., 2008). Reassortment among H5N1 influenza viruses has been reported recently in Vietnam (Wan et al., 2008) and Nigeria (Owoade et al., 2008), but reassortment between H5N1 influenza viruses and other avian influenza subtypes has not been identified outside the People’s Republic of China (Vijaykrishna et al., 2008).

Despite extensive surveillance programmes worldwide, short-term regional evolution of H5N1 viruses within domestic bird populations is not well understood. In this study, we showed that, within a genetically similar population of H5N1 influenza viruses in Lao People’s Democratic Republic (PDR), different phenotypes have emerged with reduced susceptibility to adamantanes and NA inhibitors (NAIs) and altered receptor affinities for α2,3-linked sialic acid. We also identified two novel reassortant H5N1 viruses whose PB1 and PB2 genes are derived from viruses unrelated to avian influenza viruses isolated in Lao PDR during 2006–2008. Our results suggest that H5N1 viruses may also evolve by mutations and reassortments in other countries where H5N1 is endemic and contribute to the emergence of a pandemic influenza strain.

RESULTS

Phylogenetic and antigenic analysis

To determine relationships among H5N1 influenza viruses circulating in Lao PDR, we analysed 29 viruses isolated from domestic birds between 2006 and 2008. Phylogenetic analysis of HA1 revealed that H5N1 viruses isolated from 2006 to early 2008 fell within clade 2.3.4 (Fig. 1), which is consistent with antigenic analysis with ferret antisera (Table 1). H5N1 viruses isolated in late 2008 were of clade 2.3.2, indicating the introduction of a new clade in Lao PDR. Although HAs of Lao H5N1 isolates were genetically similar, additional phylogenetic analyses of the remaining seven gene segments (NA, NP, NS, M, PB1, PB2 and PA) were performed to determine the source of H5N1 viruses causing widespread outbreaks in 2007 and new introductions in 2008. The phylogenetic topologies of NA, M, NP and PA (Figs 2 and 3) paralleled those of HA1 sequences. All Lao H5N1 isolates grouped together in the multiple clusters designated B, C, G, W, V, Z and Z’ as genotype V, similar to other clade 2.3.4 viruses (Duan et al., 2008). Analysis of PB1 and PB2 gene segments revealed a new H5N1 genotype not previously described. We identified two H5N1 reassortant viruses, A/ck/Laos/P0130/07 and A/dk/Laos/P0161/07, isolated from different geographical regions of Lao PDR. Each virus possessed both the PB1 and PB2 genes unrelated to other avian influenza viruses isolated in Lao PDR between 2006 and 2008. Neither gene has been identified previously in other H5N1 isolates and they clustered with other PB1 and PB2 genes from Eurasian aquatic isolates. The isolate A/mute swan/Hungary/5973/07 (H7N7) had the closest nucleotide similarity (97 %) to PB1 (Fig. 3) of the two Lao isolates, and A/duck/Hokkaido/120/01 (H6N2) had the closest nucleotide similarity (97 %) to PB2 (Fig. 3) of the Lao isolates. PB1 and PB2 were the only two of eight genes that underwent reassortment (Figs 2 and 3) generating the novel genotype P.

H5N1 isolates with reduced sensitivity to adamantanes and NAIs

Adamantane-resistant H5N1 strains are widespread throughout Asia, and the S31N mutation in the M2 protein is most prevalent in these strains (Hill et al., 2009), predominantly found in clade 1 viruses (Cheung et al., 2006). We identified six H5N1 isolates (one isolated in 2007 and five in 2008) with
the S31N mutation, which has not been reported previously in clade 2.3.4 viruses. Of the six isolates, three had reduced sensitivity to oseltamivir and zanamivir in an NA enzyme inhibition assay (Table 2). Each isolate with reduced sensitivity to oseltamivir had different mutations in NA: one had an NA mutation at position 116 (V116A), one had three mutations at positions 222, 150 and 246 (I222L, K150N and S246N) and one had a mutation at position 246 (S246N). The NA mutation V116A reduced sensitivity to both oseltamivir (18-fold) and zanamivir (10-fold). The NA mutation I222L, a framework residue that is highly conserved among all influenza A and B viruses (Colman et al., 1993), contributed to the 77-fold reduction in susceptibility to oseltamivir of isolate A/chicken/Laos/13/08. It is not known whether the NA mutation K150N located in the 150 loop (residues 147–152) of the N1 NA alters sensitivity by changing the conformation of the 150 loop. A unique, previously unreported mutation, S246N, identified in two isolates, reduced the sensitivity of isolate A/chicken/Laos/13/08 to oseltamivir by 24-fold as a single mutation. The residue at position 246 is reported to mediate hydrogen-bonded ligand contacts with sialic acids (Landon et al., 2008).

Receptor specificity

The HA1 glycoprotein had four different mutations in Lao H5N1 isolates: the K189R mutation in A/chicken/Laos/P0050/07 and A/chicken/Laos/P0130/07, the S129L mutation in A/chicken/Laos/16/08, and N154K and H179R mutations in A/chicken/Laos/17/08. To determine whether these mutations altered receptor binding, we measured the affinity of H5N1 isolates for sialic acid-α2,3-galactose (SAα2,3Gal) or acid-α2,6-galactose (SAα2,6Gal) (Auewarakul et al., 2007). Although the receptor specificity to
The affinity for SA2,3Gal in isolate A/chicken/Laos/16/08 reduced its binding affinity. HA binding needs further study. Mutations N154K and N154K in isolate A/chicken/Laos/17/08 reduced its binding affinity for SA2,3Gal. 

Pathogenicity of Lao PDR H5N1 influenza viruses

The pathogenicity of Lao H5N1 viruses was high in chickens but variable in mice. All Lao viruses possess a multibasic cleavage site in the HA that contributes to pathogenicity in chickens (Horimoto & Kawaoka, 1994). In addition, the Lao H5N1 influenza viruses had the deletion of five residues (aa 80–84) in the NS1 protein that may enhance cytokine expression by macrophages (Seo et al., 2001), and a glutamic acid residue at position 92 in NS1 associated with increased virulence in mammals (Guan et al., 2004). No Lao isolate had an E627K or D701N substitution in PB2, which would account for the high pathogenicity of certain isolates in mice (Hatta et al., 2001; Li et al., 2005). All Lao H5N1 viruses replicated to equally high titres in eggs and Madin–Darby canine kidney (MDCK) cells (Table 3) except for A/chicken/Laos/35/08 and A/chicken/Laos/37/08. No previously identified mutations in any gene segment could account for the attenuated replication in eggs and MDCK cells of these two isolates. The most pathogenic viruses in mice – A/chicken/Laos/P0020/07, A/duck/Laos/P0050/07 and A/chicken/Laos/16/08 – possessed mutations in and adjacent to the HA RBD.

Table 1. Antigenic analysis of H5N1 influenza viruses by the haemagglutination inhibition (HI) assay

<table>
<thead>
<tr>
<th>Influenza virus</th>
<th>Post-infection ferret antiserum to H5N1 viruses (clade)*</th>
<th>Goat serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HK213 (1)</td>
<td>VN1203 (1)</td>
</tr>
<tr>
<td>A/Hong Kong/213/03</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>A/Vietnam/1203/04</td>
<td>&lt;</td>
<td>160</td>
</tr>
<tr>
<td>A/ws/Mongolia/244/05</td>
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<td>80</td>
</tr>
<tr>
<td>A/cmag/Hong Kong/645/06</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>A/dk/Laos/3295/06</td>
<td>&lt;</td>
<td>40</td>
</tr>
<tr>
<td>A/dk/Laos/25/06</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>A/ck/Laos/A0464/07</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>A/ck/Laos/P0137/07</td>
<td>&lt;</td>
<td>&lt;</td>
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<tr>
<td>A/ck/Laos/P0020/07</td>
<td>&lt;</td>
<td>&lt;</td>
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<tr>
<td>A/ck/Laos/P0050/07</td>
<td>&lt;</td>
<td>&lt;</td>
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<tr>
<td>A/ck/Laos/P0106/07</td>
<td>&lt;</td>
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<tr>
<td>A/ck/Laos/P0127/07</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>A/ck/Laos/P0171/07</td>
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<td>&lt;</td>
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<tr>
<td>A/ck/Laos/37/08</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>A/dk/Laos/A0573/07†</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

*Values represent HI titres that are the reciprocal lowest dilutions of ferret antiserum that inhibited haemagglutination caused by 4 haemagglutination units of virus. Homologous titres are indicated in bold; < indicates a titre of <40.
†H3N8 subtype: hyperimmune goat serum to A/dk/Ukraine/1/63 (H3N8) influenza virus was generated by immunization with isolated HA protein.
Compared with other Lao H5N1 isolates, the pathogenicity of the reassortant viruses A/chicken/Laos/P0130/07 and A/duck/Laos/P0161/07 was maintained in chickens but attenuated in mice (Table 3).

DISCUSSION

The H5N1 outbreaks in poultry in Lao PDR in 2006–2008 were controlled, but the continuing emergence and reintroduction of H5N1 viruses emphasizes their threat to veterinary and human public health. Previous introductions of H5N1 viruses in Lao PDR in 2004 and early 2006 were stamped out in the local bird population, but in mid-2006 and early 2007, outbreaks of H5N1 viruses among domestic poultry generated mutants and reassortants. Phylogenetic analyses suggest that they persisted in the region for 6 months before causing outbreaks throughout Lao PDR in 2007. H5N1 clade 2.3.4 isolates and clade 2.3.2 viruses isolated in 2008 did not cluster with H5N1 viruses from 2007, demonstrating two new introductions. Only isolates from mid-2006 and 2007 clustered, suggesting that isolates from 2006 persisted in the region and resurfaced in 2007, causing outbreaks in avian species in five provinces over a 3-month period, which also resulted in the first two human fatalities in Lao PDR. The perpetuation of these H5N1 viruses may explain the phenotypic diversity of the genetically similar H5N1 viruses isolated in 2007.

Segmental reassortment among influenza viruses from different host species has caused the rapid evolution of influenza viruses and may aid the emergence of pandemic strains. We have isolated novel H5N1 reassortments in Lao PDR with genes from a circulating H5N1 virus and PB1 and PB2 genes from an unknown avian influenza virus subtype. Since its emergence, highly pathogenic H5N1 has continued to reassort with avian viruses from aquatic birds and undergo intra-subtype reassortment to generate novel genotypes in Southeast Asia (Guan et al., 2002a, b; Wan et al., 2008) and Africa (Owoade et al., 2008). In 2002,
Fig. 3. Phylogenetic relationships of the genes of the polymerase complex (PB1, PB2 and PA) and NP. Lao H5N1 isolates are highlighted in blue and the H3N8 isolate in green. Nucleotide sequences were analysed using the Bayesian approach. Clade credibilities (presented on the trees) were calculated using the parameters described in Methods. The phylogenetic trees were rooted to the PB1 and PB2 genes of A/swine/Henan/wy/04 (H5N1). bhg, Bar-headed goose; ck, chicken; ct, common teal; dk, duck; gs, goose; sck, silky chicken; mdk, Muscovy duck; ms, mute swan; tky, turkey; ts, tree swallow. Bars, 0.1 nucleotide substitution per site.
genotype Z became dominant in Asia (Duan et al., 2008; Li et al., 2004) and was eventually replaced by genotype V in 2006 (Duan et al., 2008; Smith et al., 2006). Variants of genotype V have been reported in Hong Kong (Smith et al., 2009); however, the PB1 and PB2 genes of the Lao reassortants are novel to H5N1 viruses. This new genotype P may have been transient because of successful control measures to eliminate circulating H5N1 viruses in Lao PDR in 2007; however, this must be confirmed by the complete sequence data of more recent H5N1 viruses isolated from Lao PDR and neighbouring countries.

Because amantadine-resistant H5N1 strains are present in many countries, the use of adamantanes is recommended only in countries where H5N1 is not resistant to the drug. Adamantanes would have been recommended before reintroduction of H5N1 viruses in Lao PDR in 2008, but the emergence of clade 2.3.4 viruses with the S31N mutation in the M2 protein would limit antiviral treatment to oseltamivir. To prepare for pandemics, stockpiles of oseltamivir and zanamivir have been manufactured. However, the emergence of H5N1 influenza viruses with mutations in the NA, which alter their sensitivity to NAIs

Table 2. Sensitivity of avian influenza viruses to NAIs

<table>
<thead>
<tr>
<th>Sensitivity to NA inhibitors</th>
<th>Influenza virus</th>
<th>NA mutations*</th>
<th>Oseltamivir</th>
<th>Zanamivir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>Laos 2006–2008 H5N1 isolates (n=23)</td>
<td>None‡</td>
<td>1.1 ± 0.7</td>
<td>1</td>
</tr>
<tr>
<td>Reduced sensitivity</td>
<td>A/ck/Laos/P0020/07 (H5N1)</td>
<td>K150N; I222L; S246N</td>
<td>84.3 ± 9.9</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>A/ck/Laos/P0169/07 (H5N1)</td>
<td>V116A</td>
<td>20.6 ± 2.4</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>A/ck/Laos/13/08 (H5N1)</td>
<td>S246N</td>
<td>27.2 ± 2.1</td>
<td>24</td>
</tr>
</tbody>
</table>

*Based on N2 numbering.
‡IC50 of NAI relative to a reaction mixture containing virus but no inhibitor. Values are from two independent experiments.
§No NA mutations were detected that conferred resistance to the NAIs (N2 NA numbering).

Fig. 4. Direct binding of viruses using sialyl glycopolymers. H5N1 viruses isolated in 2007 (a–c) or 2008 (d–f) were measured for their affinity to α2,3-linked (■) or α2,6-linked (●) sialic acids. Data are means ± SD of triplicate experiments.
of H5 viruses for α2,6 residues (Stevens et al., 2008). In the Lao H5N1 isolate A/chicken/Laos/17/08, the loss of glycosylation at position 154 reduced the receptor affinity for SAα2,3Gal and there was no switch to SAα2,6Gal. The additional HA mutation H179R may cause the reduced affinity for SAα2,3Gal. The H179 residue is conserved among influenza viruses and interacts with sialic acid residues and stabilizes the receptor-binding pocket (Iwata et al., 2008). The effect of the 179R mutation on affinity and specificity requires further investigation. Most importantly, we did not observe a switch to α2,6 linkages in individual HA mutations. Although previous studies have utilized this assay to characterize receptor binding (Auewarakul et al., 2007; Yamada et al., 2006), it utilizes only two glycans. Due to the multiple interactions of the HA molecules on the virus particle, a glycan array is necessary to further characterize receptor specificity and affinity and may give alternative binding patterns.

The pathogenicity of the studied viruses was high in chickens but heterogeneous in mice. The pattern of pathogenicity we observed in chickens correlates generally with known molecular determinants of H5N1 influenza virus pathogenicity (Horimoto & Kawaoka, 1994). No single mutation previously reported to increase pathogenicity of certain isolates in mice was found in Lao H5N1 viruses. Clade 2.3.4 viruses isolated in Lao PDR have amino acid substitutions in PB2 associated with mouse adaptation (Li et al., 2009) that may account for increased pathogenicity in mice. The attenuation of A/chicken/Laos/33/08 and A/chicken/Laos/37/08 in eggs and MDCK cells remains unclear, as these isolates retained pathogenicity in chickens and were pathogenic in mice. Interestingly, the PB1 and PB2 reassortant Lao H5N1 viruses replicated efficiently in eggs and MDCK cells and were highly pathogenic in chickens; however, they were not pathogenic in mice. Isolate A/Laos/P0050/07 clustered with the two reassortants for all genes except for the PB1 and PB2 genes and was highly pathogenic in mice. The reduced pathogenicity in mice with reassortant Lao H5N1 viruses supports previous reports that polymerase genes contribute to H5N1 virus pathogenicity in mice.

The highly pathogenic H5N1 avian influenza viruses have continued to circulate in epizootics in Eurasia for over a decade, resulting in the evolution of multiple clades and subclades. Because the transmission of H5N1 among humans is inefficient and limited, the public is becoming complacent about the pandemic potential of H5N1 viruses and their capacity to achieve human transmissibility. However, our study of H5N1 viruses in Lao PDR indicates that this notion is premature. Historically, it is not known how long it took for the Spanish, Asian and Hong Kong pandemics to acquire human transmissibility. Our results support the existence of a diverse population of H5N1 influenza viruses in Lao PDR – which might be true for all countries where H5N1 is endemic – that may mutate and reassort to cause a human pandemic.

### Table 3. Infectivity of H5N1 influenza viruses in different host systems

<table>
<thead>
<tr>
<th>Subtype/virus</th>
<th>Eggs*</th>
<th>MDCK cells†</th>
<th>MLD50‡</th>
<th>IVPI§</th>
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<tbody>
<tr>
<td>H5N1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A/dk/Laos/25/06</td>
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<td>7.1</td>
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<td>8.1</td>
<td>2.0</td>
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<td>H3N8</td>
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<td></td>
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<tr>
<td>A/dk/Laos/A0573/07</td>
<td>9.5</td>
<td>8.5</td>
<td>NPI</td>
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</tr>
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</table>

*Values are expressed as log10 EID50 ml⁻¹ from two independent determinations.
†Values are expressed as log10 TCID50 ml⁻¹ from two independent determinations.
‡MLD50 are expressed as log10 EID50 ml⁻¹ required to kill 50% of inoculated mice. Values are the means of two independent experiments.
§IVPI, Intravenous virus pathogenicity index in chickens. Chickens were examined for clinical signs of disease once a day for 10 days. Pathogenicity was scored as 0 (no signs of illness), 1 (signs of illness), 2 (signs of severe illness) or 3 (death within 24 h of inoculation). The IVPI was calculated as the mean score per bird per observation. NT, Not tested.
NPI, Non-pathogenic (MLD50 of <0.5 log10 EID50 ml⁻¹).
METHODS

Viruses. During outbreaks of highly pathogenic avian influenza in Lao PDR, avian influenza viruses were isolated from cloacal swabs and tissue samples were collected from sick, dead or apparently healthy birds (chickens, ducks and pigeons) from commercial farms, backyard farms and markets. Avian influenza A viruses were isolated by inoculating them into the allantoic cavities of 10-day-old embryonated chicken eggs and identified by haemagglutination and haemagglutination inhibition (HI) assays. We selected 29 H5N1 influenza isolates for analysis: four viruses isolated in 2006, 16 in 2007 and nine in 2008. A single H3N8 influenza isolated from an apparently healthy duck was also characterized. Experiments with highly pathogenic H5N1 viruses were conducted in a Biosafety Level 3+ containment facility according to the applicable laws and guidelines.

Growth of viruses. The 50% egg infectious dose (EID$_{50}$) titre was determined in duplicate by injecting 100 µl of 10-fold dilutions of virus into the allantoic cavities of 10-day-old eggs. Eggs were incubated at 37 °C for 48 h and HA activity was assayed. The 50% tissue culture infective dose (TCID$_{50}$) was determined by infecting MDCK cells with 10-fold dilutions of wild-type viruses, incubating at 37 °C for 1 h, and washing and overlaying cells with infection medium (minimal essential medium with 0.3 % BSA). HA activity was measured using 0.5% packed chicken red blood cells after incubation at 37 °C for 3 days, EID$_{50}$ and TCID$_{50}$ values were calculated by the method of Reed & Muench (1938).

Antisera and serological assay. Viruses were analysed antigenically by HI testing against a panel of ferret antisera. Sera were treated with a receptor-destroying enzyme, heat-inactivated at 56 °C for 30 min and tested using an HI assay with 0.5% packed chicken red blood cells.

Sequence analysis. Viral RNA was isolated from allantoic fluid using an RNeasy kit (Qiagen). Gene segments were amplified by RT-PCR using a universal primer set for influenza A viruses (Hoffmann et al., 2001). Viral cDNA and template cDNA were sequenced by the Hartwell Center for Biotechnology at St Jude Children’s Research Hospital. DNA sequences were analysed by the Lasergene sequence analysis software package (DNASTAR).

Phylogenetic analyses. Phylogenetic analyses were based on all eight full-length gene segments encoding viral surface antigens, except for the HA (nt 49–1020). Sequences were aligned using the CLUSTAL W algorithm in MEGALIGN version 7.2.1 (DNASTAR). All trees were calibrated using the CLUSTAL W method described by Kohler & Milstein (1976) and have been mapped on the three-dimensional HA structures (Kaverin et al., 2001), at a dilution of 1:2000. Horseradish peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin (Sigma-Aldrich) was used as the secondary antibody and developed with pre-mixed tetramethylbenzidine substrate (Sigma-Aldrich), and the absorbance was read at 450 nm.

Pathogenicity tests in chickens. The intravenous pathogenicity index (IVPI) was determined by intravenous injections of infective allantoic fluid diluted 1:10 in sterile PBS (0.1 ml) into each of ten 6-week-old, specific-pathogen-free chickens (Charles River Laboratories). Chickens were examined for clinical signs of disease once a day for 10 days.

Pathogenicity tests in mice. The 50% mouse lethal dose (MLD$_{50}$) of nine influenza H5N1 virus isolates and the H3N8 influenza isolate was determined in 6-week-old female BALB/c mice (Jackson Laboratories). Groups of five mice were anaesthetized with isoflurane and inoculated intranasally with 50 µl 10-fold serial dilutions of allantoic fluid in PBS. Mice were weighed and observed for mortality daily for 15 days. MLD$_{50}$ values were calculated by the method of Reed & Muench (1938). All studies were conducted under the applicable laws and guidelines and after approval from the St Jude Animal Care and Use Committee.

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