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Rapid Communication

Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam

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Abstract

Highly pathogenic avian influenza virus H5N1 is endemic in poultry in East and Southeast Asia with disease outbreaks recently spreading to parts of central Asia, Europe and Africa. Continued interspecies transmission to humans has been reported in Vietnam, Thailand, Cambodia, Indonesia and China, causing pandemic concern. Here, we genetically characterize 82 H5N1 viruses isolated from poultry throughout Indonesia and Vietnam and 11 human isolates from southern Vietnam together with sequence data available in public databases to address questions relevant to virus introduction, endemicity and evolution. Phylogenetic analysis shows that all viruses from Indonesia form a distinct sublineage of H5N1 genotype *Z* viruses suggesting this outbreak likely originated from a single introduction that spread throughout the country during the past two years. Continued virus activities in Indonesia were attributed to transmission via poultry movement within the country rather than through repeated introductions by bird migration. Within Indonesia and Vietnam, H5N1 viruses have evolved over time into geographically distinct groups within each country. Molecular analysis of the H5N1 genotype *Z* genome shows that only the M2 and PB1-F2 genes were under positive selection, suggesting that these genes might be involved in adaptation of this virus to new hosts following interspecies transmission. At the amino acid level 12 residues were under positive selection in those genotype *Z* viruses, in the HA and PB1-F2 proteins. Some of these residues were more frequently observed in human isolates than in avian isolates and

Abbreviations: BEB, Bayes Empirical Bayes; BH goose, bar-headed goose; Ck, chicken; CP heron, Chinese pond heron; Dk, duck; Env, environment; FJ, Fujian; GBH gull, great black-headed gull; GD, Guangdong; Gs, goose; GX, Guangxi; HA, hemagglutinin; HI, hemagglutination inhibition; HK, Hong Kong; HN, Hunan; IDN, Indonesia; JX, Jiangxi; KHM, Cambodia; MAb, monoclonal antibody; Mall, mallard; M, matrix protein; MDCK cell, Madin-Darby canine kidney cell; MDk, migratory duck; MYS, Malaysia; NA, neuraminidase; NP, nucleoprotein; NJ, neighbor joining; NS, non-structural; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2; Ph, pheasant; Qa, Quail; QH, Qinghai; RT-PCR, reverse transcription polymerase chain reaction; SCk, silky chicken; ST, Shantou; THA, Thailand; VNM, Vietnam; YN, Yunnan.

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are related to viral antigenicity and receptor binding. Our study provides insight into the ongoing evolution of H5N1 influenza viruses that are transmitting in diverse avian species and at the interface between avian and human hosts.

Keywords: Influenza A; Virus evolution; Molecular epidemiology; Positive selection

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) H5N1 virus were initially observed in southern China in 1996 and 1997 (Claas et al., 1998; Guan et al., 1999; Xu et al., 1999) and has caused major poultry outbreaks in Vietnam, Thailand, Indonesia and other East Asian countries since early 2004 (Li et al., 2004; Vieshakul et al., 2004; Tiensin et al., 2005; WHO, 2005a). The virus is now endemic in poultry in these countries and has caused repeated zoonotic transmission to humans (Chotpitayasunondh et al., 2005; Hien et al., 2004; Puthavathana et al., 2005). In Southeast Asia in 2004, the majority of human infections were recognized in Vietnam and Thailand (WHO, 2005b, 2005c). Since mid-2005, there has been a resurgence in the number of human H5N1 infections with cases recognized in Indonesia and also more recently in China and Turkey (WHO, 2005c; Yu et al., 2005). So far, a total of 184 people have been confirmed as having H5N1 disease, 103 of these cases having a fatal outcome (WHO, 2005c). The persistent introduction of H5N1 virus to humans raises the possibility of emerging as a human pandemic virus, either as a purely avian virus adapting to more efficient human transmission or through reassortment with current human influenza strains (Webster et al., 1992; Taubenberger et al., 2005). Although some cases of human H5N1 infection were of family clusters, human-to-human transmission is still very limited (Ungchusak et al., 2005). There is still no convincing evidence for sustained chains of transmission within humans. Thus, infection from affected poultry continues to be the major source of the human cases (WHO, 2005b).

Genetic analyses has shown that most H5N1 influenza viruses from poultry and from humans in these countries belong to a single dominant genotype *Z* that was first recognized in poultry from southern China in 2002 (Guan et al., 2004; Li et al., 2004; Puthavathana et al., 2005; WHO, 2005a). However, an additional genotype of H5N1 virus has been identified in Vietnam in 2005 and was first isolated from poultry in southern China in early 2005 (Chen et al., 2006). Recent findings show that long-term endemicity of H5N1 influenza in poultry has resulted in the establishment of multiple regional sublineages, even among viruses that belong to the same genotype (Chen et al., 2006; WHO, 2005a).

Genetic characterization of H5N1 viruses from Southeast Asia and southern China identified a number of amino acid residues in the HA that appear to be unique to virus clades from different regions, and that these changes are concentrated at antigenic and receptor-binding sites (WHO, 2005a). However, the limited number of H5N1 influenza isolates were genetically characterized hitherto has made comparison of isolates from different hosts difficult. In addition, there is currently no information available for genetic differences in the remaining 10 influenza gene products.

Here, we provide previously unpublished data on 11 human and 82 avian H5N1 influenza viruses from Indonesia and Vietnam, analyzed along with genetic data from 266 other genotype *Z* viruses available from public databases. The findings of the present study show that the H5N1 genotype *Z* viruses from Indonesia and Vietnam were originally derived from a single virus introduction. Those H5N1 viruses have subsequently developed into geographically distinct groups, reflecting what is known regarding the course of the disease outbreaks in each country. Molecular analysis revealed that the M2 and PB1-F2 genes were under positive selection, suggesting that these genes may be associated with adaptation of this virus to different hosts. Among all gene products, 12 amino acids were also under positive selection, within the HA and PB1-F2 proteins, that are related to antigenicity and receptor binding. These findings provide insight into the ongoing evolution of H5N1 genotype *Z* influenza virus at the interface between avian and human hosts.

Results

Phylogenetic analysis

Results of phylogenetic analysis indicated that all H5N1 viruses analyzed in this study had hemagglutinin (HA) genes derived from the Gs/GD/1/96-like (Gs/GD-like) lineage (Fig. 1A). Analysis of the remaining seven gene segments, represented by the NP gene, shows that all viruses from Indonesia, and all but one virus from Vietnam belonged to H5N1 genotype *Z* (Fig. 1B and data not shown). A single virus from Vietnam (Dk/VNM/568/05) belongs to genotype G, which differs from genotype *Z* viruses in the source of the PB2 gene. Viruses from both Indonesia and Vietnam consistently have a sister group relationship with viruses from southern China.

All 41 viruses isolated in Indonesia from geographically distant sites cluster together, which suggests a common introduction (Figs. 1 and 2). Similarly, apart from the genotype G virus, all those viruses sequenced from Vietnam form a single clade together with viruses from Cambodia and Thailand, also suggesting a single introduction into the Thailand/Vietnam region, but one clearly distinct from the virus introduced into Indonesia (Fig. 1).

Phylogenetic analysis of the viruses isolated within each of these countries revealed the emergence of groups of H5N1 viruses based on geographical regions within Indonesia and Vietnam (Figs. 2–4). In the Indonesian sublineage, there are three groups of viruses. Group A includes viruses from central and eastern Indonesia with isolates from Java, southern

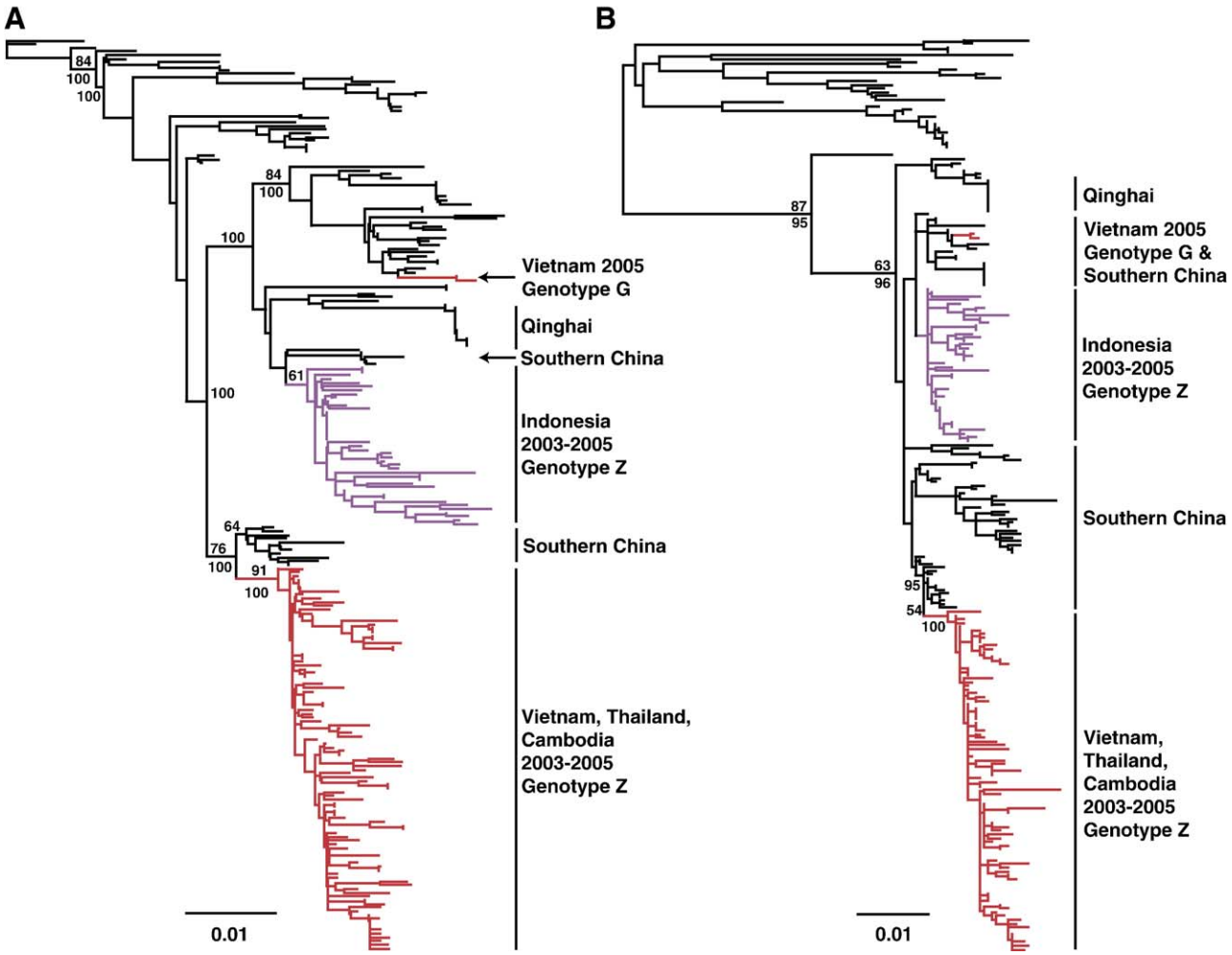

Fig. 1. Phylogenetic relationships of the (A) HA and (B) NP genes of influenza A viruses isolated in Indonesia and Vietnam. Numbers above and below branches indicate neighbor-joining bootstrap values and Bayesian posterior probabilities, respectively. Analysis was based on nucleotides 1–963 of the HA gene and 1–990 of the NP gene. The HA tree was rooted to Gs/GD/1/96 and the NP tree to Dk/HK/Y280/97. Scale bar, 0.01 substitutions per site.

Sulawesi and West Timor (Figs. 2 and 3; Table S1). Group B also contains viruses from central and eastern Indonesia but with isolates from Java, Bali, Flores Island and West Timor. In comparison, group C viruses are from central and western Indonesia being found throughout Java and Sumatra and also in Bangka Island. In all three groups, viruses from Java isolated in 2003 and 2004 are in a basal position to those viruses from other parts of the country (Fig. 3). Also, viruses from all groups are present in Java, whereas other affected areas have only viruses from a single group, with the exception of West Timor that has viruses from groups A and B. These relationships suggest an initial introduction of H5N1 virus into central Indonesia (Java) and its subsequent spread both east and west throughout the country, with single introductions to Flores Island, Sulawesi and Sumatra and two separate introductions to West Timor.

In the Vietnam sublineage, there are two groups of viruses. Group N includes those viruses from five provinces in the Red River Delta, northern Vietnam. These viruses are most closely related to H5N1 isolates from Thailand and Malaysia. Group S includes those viruses from 13 provinces in southern Vietnam, mostly in the Mekong River Delta (Figs. 2 and 4; Table S2). Viruses from Cambodia also fall in this group. All human isolates characterized in this study were from southern Vietnam and clustered into group S. For most human isolates, there were closely related avian viruses. Although some human isolates did cluster together, there was low statistical support for these relationships.

Molecular characterization

Most viruses from Indonesia and Vietnam characterized in this study maintained the motif of multiple basic amino acids at the HA cleavage site characteristic of HPAI (QRERRRKKR/G). Two viruses from Indonesia (Ck/Kulon Progo/BBVet-XII-1/04 and Ck/Kulon Progo/BBVet-XII-2/04) had a Lys deletion (QRERRRKR/G), while 11 avian viruses from Vietnam, including the genotype G virus Dk/VNM/568/05, had an Arg deletion (QRERRKKR/G) and another single virus (Ck/VNM/147/04) had an Arg to Ile substitution at position −5 from the cleavage site. The receptor-binding pocket of HA1 retains amino acid residues Gln 222 and Gly 224 (H5 numbering used throughout) that preferentially bind

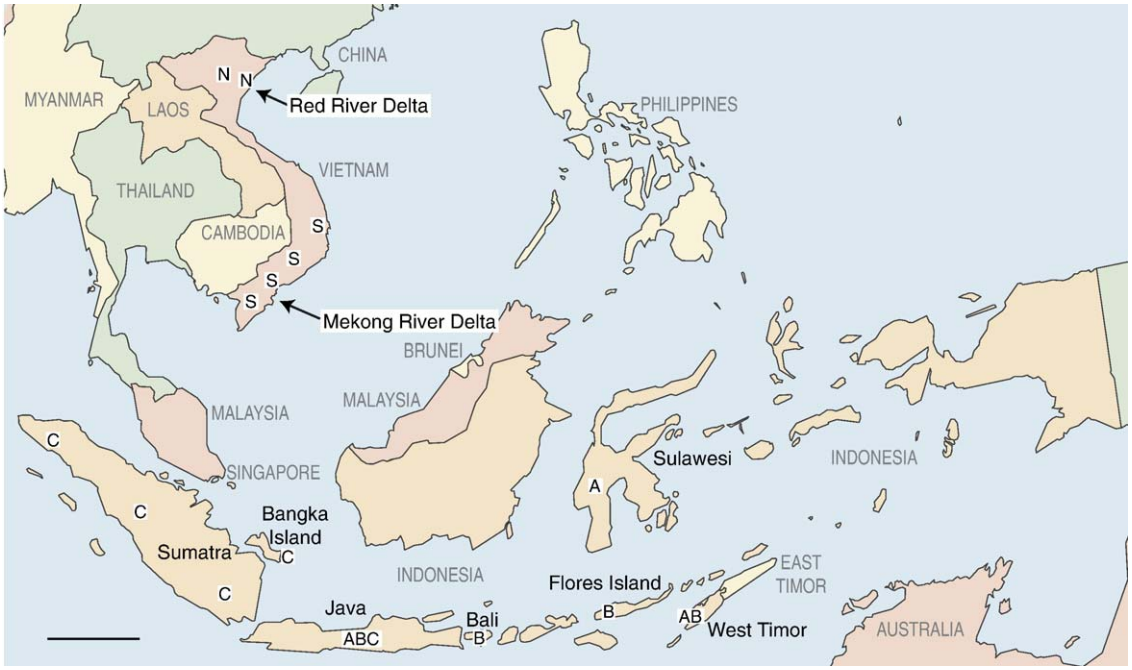

Fig. 2. Map of Southeast Asia. Groups identified in the phylogenetic analysis are marked onto the regions of Indonesia and Vietnam from where the viruses were isolated. Scale bar, 500 km.

to α-2,3-NeuAcGal receptors (Ha et al., 2001). Other amino acid residues relevant to receptor-binding sites were identical to those of HK/156/97 and Gs/GD-like viruses (Claas et al., 1998; Ha et al., 2001) in most isolates, but with some notable differences. The major change at a receptor-binding site was a Ser129Leu substitution that was present in all Vietnam isolates and also in five Indonesia isolates from a single clade in group C (Fig. 3). Eight avian viruses from Vietnam had a Ser133Ala mutation that was also seen in two human isolates (VNM/CL115/05 and VNM/CL2009/05) and that has been previously reported for avian and human viruses from Vietnam and Cambodia (WHO, 2005a). The viruses with this mutation all fall into a single clade in the HA phylogram as indicated in Fig. 4. The virus VNM/CL2009/05 also had a Tyr91Phe mutation, while another two human viruses had a single mutation each; Glu186Asp (VNM/CL01/04) and Ala134Val (VNM/CL105/05). The latter mutation was previously reported from a human H5N1 isolate in Vietnam (VNM/3046/04) (Li et al., 2004). A single avian virus (Mall VNM/16/03) had an Ile151Val mutation.

All avian and human viruses from Vietnam included in this study, excluding the single genotype G virus (Dk/VNM/568/05), had Leu26Ile and Ser31Asn mutations in the M2 protein that are known to confer resistance to amantadine (Scholtissek et al., 1998). Six avian viruses from the Indonesian group C viruses had the Ser31Asn mutation that was also previously reported for Ck/IDN/2A/04 (Fig. 3). Five of these viruses also had the HA Ser129Leu substitution (see above), and all five were isolated in central or northern Sumatra in 2005. Other Indonesian viruses did not have mutations known to confer amantadine resistance. As previously reported, the isolate VNM/CL2009/05 has a His274Tyr mutation in the NA protein associated with resistance to oseltamivir (de Jong et al., 2005b).

No avian isolates had the mutation Lys 627 in the PB2 protein while no avian or human isolates had the Glu 92 in the NS1 protein, both mutations that have been associated with increased virulence of influenza viruses (Hatta et al., 2001; Seo et al., 2002; Fouchier et al., 2004). However, five human viruses characterized in this study from Vietnam (VNM/CL20/04, VNM/CL26/04, VNM/CL36/04, VNM/CL105/05 and VNM/CL2009/05) did have the Lys 627 mutation in the PB2 protein. Furthermore, the Lys 627 substitution in the PB2 protein was the only consensus change that was identified in the deduced amino acid residues when comparing H5N1 genotype *Z* isolates from human, chicken and duck in the Vietnam sublineage. Of the sequences included in the consensus analysis, none of the chicken and duck sequences had Lys 627 in the PB2, while ten of 14 of human viruses did.

Detection of positive selection

To detect selection pressures that may be acting upon the H5N1 genotype *Z* genome, a comparison of synonymous and non-synonymous nucleotide substitutions was conducted on all available data. Results showed that, when averaged over all sites, only the M2 ($\omega = 1.23$) and PB1-F2 ($\omega = 3.01$) genes were under positive natural selection, while all other genes were under strong negative selection pressure ($\omega < 0.50$; Table 1 and data not shown). This was also the case for H5N1 genotype *Z* viruses from Indonesia, while in those viruses from Vietnam only, the PB1-F2 gene was under positive selection pressure (data not shown).

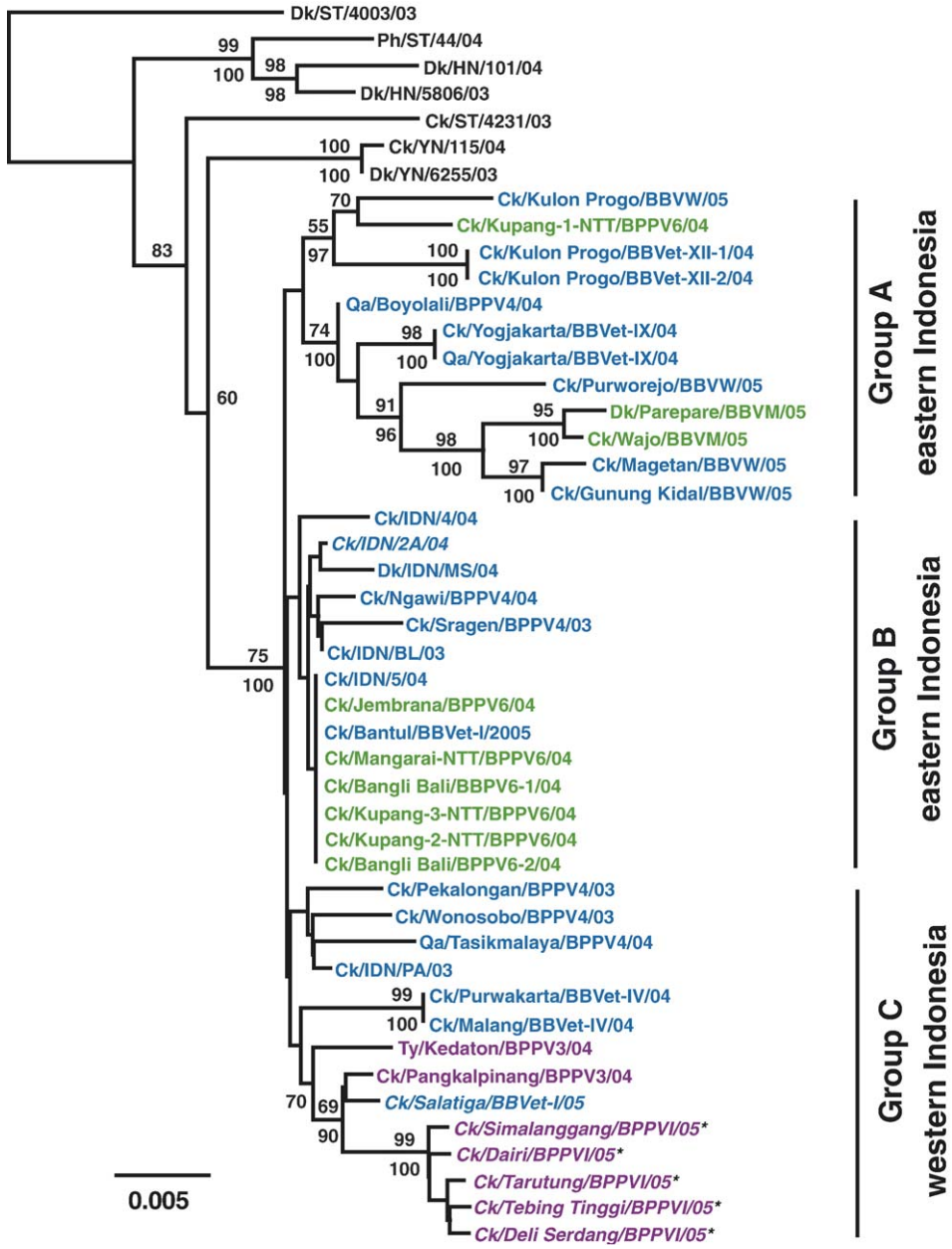

Fig. 3. Phylogenetic relationship of the HA gene of influenza A viruses isolated in Indonesia. Analysis was based on nucleotides 1–963 of the HA gene, and the tree was rooted to Gs/GD/1/96. Scale bar, 0.01 substitutions per site. Viruses with italicized names have the amantadine resistance mutation (Ser31Asn) in the M2 ion channel. Blue text indicates viruses isolated from Java; purple text indicates viruses isolated from Sumatra and Bangka Island; green text indicates viruses isolated from Bali, Flores, Sulawesi and West Timor. *Viruses with the Ser129Leu substitution in the HA.

Likelihood ratio tests revealed that the M7 model was rejected in favor of the M8 model for only the HA and PB1-F2 genes ($P < 0.01$). Detection of positive selection at the amino acid level indicated that eight residues in the HA and four residues in the PB1-F2 proteins were under positive selection pressure (Table 1). In the HA, these residues include five in antigenic sites A and E (positions 83, 86, 138, 140 and 141); two involved in receptor binding (positions 129 and 175); and position 156 that is a site for potential *N*-linked glycosylation that is near the receptor-binding site (Table 1 and Fig. 5). The biological function of the residues in the PB1-F2 protein is unknown.

To identify possible early adaptation of H5N1 genotype *Z* influenza viruses in humans, percentages of each residue that was under positive selection in those viruses from chicken, duck and human were calculated. Results revealed that three residues in the HA (Val 86, Ser 129, Thr 156) were more frequently observed in human isolates than in chicken and duck isolates (Table 2). Position 175 in the HA had two residues of almost equal frequency (54% Leu and 43% Met) in human isolates, while avian isolates had 86% Leu at the same position (Table 2). The remaining residues differ by less than 10% between hosts.

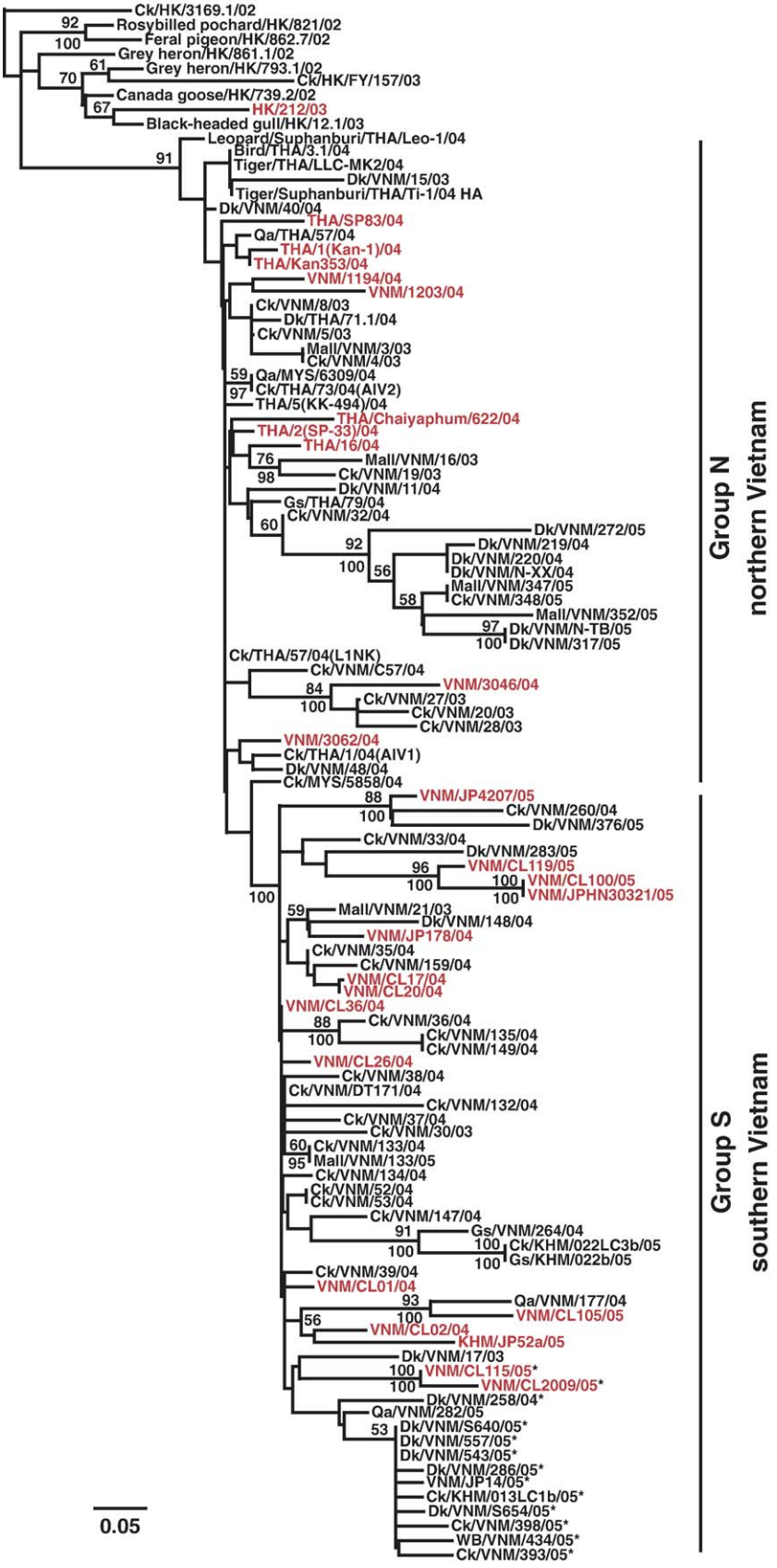

Fig. 4. Phylogenetic relationship of the HA gene of influenza A viruses isolated in Vietnam. Analysis was based on nucleotides 1-963 of the HA gene, and the tree was rooted to Gs/GD/1/96. Scale bar, 0.01 substitutions per site. Red text indicates viruses isolated from humans. *Viruses with a Ser133Ala substitution in the HA.

Table 1
Positively selected sites of H5N1 genotype Z influenza viruses from southern China and Southeast Asia 2002–2005

Gene	ω	Sites ($\omega > 1$)[a]	Residues[b]	Function	Site ω (±SE)
HA	0.198	**83**	A/D/T/V	Antigenic site E	2.77 ± 0.72
		86	A/I/T/V	Antigenic site E	2.77 ± 0.72
		129	L/S	Receptor binding	2.71 ± 0.81
		138	L/M/Q	Antigenic site A	2.85 ± 0.62
		140	E/K/N/Q/R/S/T	Antigenic site A	2.85 ± 0.60
		141	P/S	Antigenic site A	2.71 ± 0.80
		156	A/S/T	Glycosylation	2.85 ± 0.61
		175	L/M	Receptor binding?	2.74 ± 0.77
PB1-F2	3.012	**23**	N/S/V	–	5.39 ± 1.92
		33	L/P	–	5.32 ± 1.98
		79	Q/R	–	5.29 ± 1.99
		84	N/S	–	5.29 ± 2.00

[a] Sites were included if the posterior probability was ≥0.90 in the CODEML M8 model using the BEB method. Sites in bold had a posterior probability of ≥0.95. Sites are numbered from the start (Met) codon for each gene except the HA that is numbered from the beginning of the mature H5 HA1 protein.
[b] All amino acid residues, given in single letter code, present at that site in genotype Z viruses.

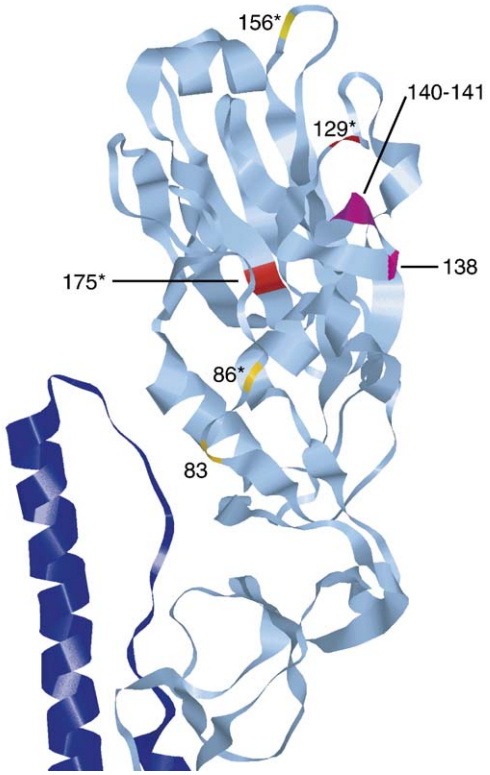

Fig. 5. Location of amino acid residues in the HA of H5N1 influenza viruses that are under positive selection. Those positively selected residues involved in receptor binding are highlighted in red; those in antigenic site A in pink and antigenic site E in orange; while the site of potential *N*-linked glycosylation is highlighted in yellow. *Indicates those residues that are more frequently observed in human isolates than in avian isolates (Table 2). Residue positions have been imposed upon the 3D structure of H5 hemagluttinin from the Protein Data Bank (1JSM). The light blue ribbons represent the HA1 chain and the dark blue represents the HA2 chain of the molecule. RasTop version 2.7 was used to visualize the molecule.

Antigenic analysis

Antigenic analyses of viruses from Vietnam have been reported previously (Chen et al., 2006; WHO, 2005a). Therefore, we have conducted antigenic analysis of H5N1 virus isolates from Indonesia with representative strains from Vietnam using a panel of mAbs and polyclonal antisera. The results show that viruses from Indonesia do not react to ferret antisera against VNM/1203/04, and that representative viruses from Vietnam do not react to ferret antisera against Indonesian viruses IDN/5/05 and Dk/IDN/MS/04. There is also antigenic diversity within the viruses from Indonesia with some viruses (e.g., Ck/Yogjakarta/BBVet-IX/04, Ck/Tarutung/BPPVI/05) reacting poorly to ferret anti-Dk/IDN/MS/04 (Table 3). In general, Indonesia group A viruses had lower reaction titers to MAbs 10HD2 and 3C8 than group B and C viruses (Table 3).

Discussion

Phylogenetic analysis of HPAI H5N1 influenza viruses from different regions in Asia has demonstrated that these viruses have become established in poultry and form regionally distinct sublineages (Chen et al., 2006; WHO, 2005a). It has also been shown that since 2001, there have been multiple transmissions of different H5N1 viruses from southern China to Vietnam (Chen et al., 2006). However, a lack of sufficient data has meant that little is known regarding the evolution of HPAI H5N1 viruses within those sublineages, particularly in Indonesia.

Results of the present study demonstrate that H5N1 genotype *Z* viruses have been endemic in poultry populations of Indonesia and Vietnam since 2003. Phylogenetic analysis showed that all H5N1 viruses, tested in this study, from Indonesia and Vietnam were both derived from a single introduction, likely to have come from domestic poultry in southern China (Li et al., 2004; WHO, 2005a; Chen et al., 2006). Furthermore, the continuing endemicity of those viruses has resulted in the establishment of geographically distinct groups within each of the Indonesian and Vietnamese sublineages. The phylogenetic differences between these

Table 2
Percentages of residues at positively selected sites of H5N1 genotype Z influenza viruses from southern China and Southeast Asia 2002–2005

	Residue[a]	Duck[b]	Chicken	Human
HA	86 (A/V)	96/0	30/70	3/97
	129 (L/S)	56/44	75/25	5/95
	156 (A/T)	40/58	11/87	0/100
	175 (L/M)	86/14	86/14	54/43

[a] Two most frequent amino acid residue in single letter code.
[b] Percentage of each residue found in isolates.

Table 3
Antigenic analysis of H5N1 influenza viruses from Indonesia by hemagglutination inhibition titer

Group	Virus	Region[a]	Ferret antisera			MAb 1203	MAb YU22	MAb YU22
			IDN/5/05	IDN/MS/04	VNM/1203/04	18H2	10HD2	3C8
A	Ck/Wajo/BBVM-12/05	Sulawesi	640	160	<	<	<	<
A	Ck/Kulon Progo/BBVet-XII2/04	Central Java	320	80	<	400	100	<
A	Ck/Yogjakarta/BBVet-IX/04	Central Java	40	<	<	200	<	<
A	Ck/Kulon Progo/BBVW/05	Central Java	320	80	<	400	<	<
A	Ck/Kupang-1-NTT/BPPV6/04	Timor	160	80	<	800	<	100
A	Qa/Boyolali/BPPV4/04	Central Java	160	40	<	800	100	<
A	Ck/Purworejo/BBVW/05	Central Java	640	160	<	1600	100	<
A	Ck/Magetan/BBVW/05	East Java	640	160	<	<	800	<
B	Ck/Bangli Bali/BBPV6-1/04	East Bali	320	160	<	1600	100	400
B	Dk/IDN/MS/04	East Java	640	**320**	<	800	400	1600
C	Ck/Malang/BBVet-IV/04	East Bali	640	320	<	12,800	12,800	1600
C	Ck/Simalangang/BPPVI/05	Central Sumatra	160	80	<	800	200	100
C	Ck/Tarutung/BPPV1-10/05	North Sumatra	80	<	<	400	800	<
	Dk/VNM/568/05		<	<	<	100	<	<
	VNM/1203/04		<	<	**640**	>**12,800**	>12,800	400
	Gs/HK/437.6/99		80	40	640	>12,800	>12,800	<
	Ck/HK/YU22		320	160	<	>12,800	**>12,800**	1600

< Indicates lowest dilution tested: 1:100 for monoclonal antibody titers, 1:40 for ferret anti-sera. MAb abbreviations: 1203/04, VNM/1203/04; YU22, Ck/HK/YU22/02.
[a] Regions are general areas and do not correspond to official Provinces of Indonesia.

sublineages are also reflected in significant differences in antigenic cross reactivity between these two groups of viruses. Ferret antisera against VNM/1203/04 (H5N1) virus react poorly or not at all to viruses isolated in Indonesia (Table 3).

It is remarkable that H5N1 viruses within Indonesia from geographically distant areas such as North Sumatra and West Timor, a distance of over 3000 km, and covering the time period from late 2003 to mid-2005, form a single sublineage without evidence for H5N1 viruses from any other phylogenetic lineage (Fig. 2). While the number of viruses studied is limited, the selection of viruses was made to represent H5N1 isolates from different geographic areas over the time period of the outbreak and also from diverse avian species.

This virus then probably spread across the Indonesian archipelago, possibly along routes of trade of poultry and poultry products, and has now evolved into regionally based groups within the sublineage. While Sumatra and West Java share viruses from one such group (i.e., group C), all three groups (A, B and C) are found in central and eastern Java. This is likely a reflection that these are the areas of most intensive poultry production and also of continued poultry movements across the Indonesian archipelago. Java is the hub of the poultry industry in Indonesia with approximately 35% of all poultry production being in East Java and approximately a further 25% in other parts of Java (TSP Naipospos, personal communication). Short range transmission by wild birds acquiring infection from poultry may well have amplified and contributed to such spread (Li et al., 2004; Guan et al., 2004). However, there is no evidence of repeated waves of introduction of H5N1 viruses via long range bird migration into Indonesia.

It is notable that the Ser31Asn mutation in the M2 protein, associated with amantadine resistance, appears to be largely associated with viruses from group C, predominantly in Sumatra (Fig. 3). Whether this is associated with use and abuse of the adamantanes for prevention or treatment of avian influenza or from other environmental factors remains to be established.

As with Indonesia, a single sublineage of H5N1 viruses was observed in Vietnam (Fig. 2), that also includes viruses isolated from Thailand and Cambodia, again indicating a single virus introduction that subsequently spread within this region. However, more recently, another H5N1 virus (genotype G) was recently detected in Vietnam. These latter viruses are similar to viruses that have been isolate from domestic poultry in Guangxi and Hunan, China (Chen et al., 2006). The H5N1 genotype *Z* viruses in Vietnam appear to have established geographically distinct groups in northern and southern Vietnam (Fig. 4). The group N viruses found in the Red River Delta in northern Vietnam are more closely related to viruses in Thailand (and Malaysia). The explanation for the similarity of viruses from these geographically separate areas remains obscure, although there is known to be legal and illegal animal trade between these regions via Laos. The group S viruses found in the Mekong River Delta are more closely related to those from Cambodia. Cambodia and southern Vietnam are both in the basin of the Mekong River and have a similar natural habitat. In addition, there is a well-established animal trade between southern Vietnam and Cambodia (TD Nguyen, personal communication). The human viruses from Vietnam reported here were isolated exclusively from the south of Vietnam and, as expected, cluster within group S viruses from the same region.

Molecular analyses of each of the gene products of H5N1 genotype *Z* influenza viruses show that only the M2 and PB1-F2 proteins were under positive selection pressure. The M2 ion channel, which is involved in hydrogen transport (Ciampor et al., 1992), may be under positive selection as the viruses repeatedly adapt between aquatic and terrestrial hosts that have different pH and cellular environments (Scholtissek, 1994). The PB1-F2 protein is thought to be pro-apoptotic and involved in downregulation of the host immune response to infection (Chen

et al., 2001; Yamada et al., 2004; Zamarin et al., 2005). Our results suggest that the biological role of the M2 and PB1-F2 proteins in interspecies transmission is worthy of further investigation.

While M2 and PB1-F2 were the only genes under positive selection pressure, further analysis revealed that 12 amino acid residues in the HA and PB1-F2 proteins were also being selected (Table 1). All eight of those residues in the HA were concentrated on the globular head of the protein. Five of these residues were at antigenic sites A and E, two were associated with receptor binding, and another is a site for potential *N*-linked glycosylation (Fig. 5 and Table 2) (WHO, 2005a). When the percentage of each amino acid residue at these positions in different species was calculated, it revealed that three specific residues are predominant in human isolates—one at antigenic site E, one involved in receptor binding and one at the glycosylation site, suggesting that these residues may be selected for in humans (Table 2).

Comparison of sequences of H5N1 influenza viruses from avian and human hosts in Vietnam identified only a single consensus amino acid substitution, Lys 627 in the PB2. This mutation was previously associated with increased virulence of H5N1 viruses in mammals (Hatta et al., 2001) and has also been associated with increased replication competence at 33 °C, the naturally occurring temperature in the human nasopharynx (Massin et al., 2001). Ten of 14 human H5N1 genotype *Z* isolates carry the PB2 Lys 627 substitution that may indicate a selective advantage for viruses with this residue in a mammalian system. This may be of some concern given that this substitution was found in all isolates from the Lake Qinghai outbreak (Chen et al., 2005; Liu et al., 2005). However, for the human isolates, there was no apparent correlation between the presence of this mutation and disease outcome (data not shown).

However, there is no convincing evidence that these viruses have adapted to humans or are capable of efficient human-to-human transmission. Phylogenetic and genetic analyses indicate that most human cases of H5N1 infection were directly introduced from an avian source. The distribution of the amino acid residues in the HA indicates that the major source for human infection is from chicken (Table 2).

As the virus is broadly endemic in poultry in these countries, it is imperative that systematic surveillance of poultry is implemented to identify the earliest indications of a pandemic influenza. This study provides a focus for future experimental work that may lead to a better understanding of the factors that are involved in the adaptation of H5N1 and other influenza viruses to different hosts.

Materials and methods

Virus isolation and characterization

Virus isolates were collected from outbreaks of poultry disease throughout Indonesia (n = 34) and Vietnam (n = 94) from October 2003 to June 2005 and August 2003 to May 2005, respectively, from chicken, duck, geese, mallard, quail and turkey. The avian viruses were selected to represent geographically different parts of each country. Additional 11 viruses were isolated from human patients in southern Vietnam between January 2004 and January 2005. Avian viruses were grown in embryonated eggs and isolates identified and subtyped using reference antisera as previously described (Guan et al., 2000). Initial diagnoses of human isolates were made on pharyngeal swabs by RT-PCR as described (Hien et al., 2004); virus isolation was done on MDCK cells, and viruses were identified by RT-PCR and HI (de Jong et al., 2005a). All virus isolation was conducted in bio-safety level 3 facilities.

Phylogenetic analysis

To understand the evolutionary history of H5N1 viruses isolated in this study, 34 (100%) avian isolates from Indonesia; 53 (56%) avian isolates from Vietnam; and 11 (100%) human isolates from Vietnam were sequenced. All eight gene segments of these viruses were characterized and phylogenetically analyzed together with virus sequence data available in GenBank of 266 H5N1 genotype *Z* viruses.

VRNA extraction, cDNA synthesis and PCR of avian isolates and sequencing of all isolates were carried out as described previously (Guan et al., 2002; Butt et al., 2005). RNA extraction of human isolates was conducted by the Boom-method (Boom et al., 1990, 1999) and cDNA synthesis and PCR as previously described (Hien et al., 2004; de Jong et al., 2005a) with the exception that uni-12 primers were used instead of random hexamer primers. All sequences were assembled and edited with Lasergene version 6.0 (DNASTAR, Madison, WI); BioEdit version 7 was used for alignment and residue analysis (Hall, 1999).

The program MrModeltest version 2.2 (Nylander, 2004) was used to determine the appropriate DNA substitution model and gamma rate heterogeneity. The generated model was used in all subsequent analyses with gaps treated as missing data. Neighbor-joining (NJ) and maximum-likelihood trees were constructed by using PAUP* version 4.0 (Swofford, 2001). Bayesian analysis was conducted with MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) using two replicates of 1 million generations. Estimates of the phylogenies were calculated by performing 1000 NJ bootstrap replicates, and Bayesian posterior probabilities were calculated from the consensus of 16,000 trees after excluding the first 2000 trees as burn-in.

Molecular characterization

In an attempt to identify amino acid residues involved in the interspecies transmission of H5N1 genotype *Z* viruses, consensus sequences were generated from full-length or almost full-length sequences for all 11 gene products (PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1 and NS2). As substantial numbers of human isolates are only available from Thailand and Vietnam, viruses from Indonesia were excluded from the consensus analysis to avoid the affects of any regional differences in the virus sequence data. Therefore, only viruses from the Cambodia, Malaysia, Thailand and Vietnam clade (WHO, 2005a; Chen et al., 2006) were included in this analysis.

The consensus alignments were compared for chicken, duck and human to identify any amino acid differences. Genotype *Z* viruses from this study and those available in GenBank were used. Majority-rule consensus sequences were generated using Se-Al version 2 (Rambaut, 1996).

Detection of positive selection

Selection pressure at sites of the H5N1 genotype *Z* genome was investigated using codon substitution models as implemented in PAML version 3.14b (Yang, 1997). Comparison of non-synonymous and synonymous nucleotide substitution rate ratios ($\omega = d_N/d_S$) under different models (M7 and M8) was used to test for individual codons under positive natural selection ($\omega > 1$) (Nielsen and Yang, 1998; Yang et al., 2000). Likelihood ratio tests (LRTs) were used to determine whether model M7 (sites restricted to $0 < \omega < 1$) or M8 (allows sites with $\omega > 1$) was a statistically better fit to the data (Yang et al., 2000; Anisimova et al., 2003; Sainudiin et al., 2005). If M7 was rejected in preference for M8 (i.e., $P < 0.01$ in the LRT), then the Bayes Empirical Bayes (BEB) method was used for a posteriori estimation of individual codons under positive selection (Yang et al., 2005). A HA sequence alignment consisting of 343 genotype *Z* viruses, isolated from 2002 to 2005 across different regions in Southeast Asia, was generated. Sequences with 100% homology to other sequences in the alignment, and sequences that were not full-length, were removed from the alignment and standardized to a final alignment of 50 sequences. This alignment was used as the basis for the other gene products. The percentages of each amino acid at the positively selected sites in genotype *Z* were then calculated using alignments of our data and all available data from GenBank, with isolates from chicken, duck and human (HA, $n = 298$; PB1-F2, $n = 202$).

Antigenic analysis

The antigenic characteristics of the H5N1 influenza viruses from different regions were compared by hemagglutination inhibition (HI) assay with mAbs and polyclonal antisera to H5 subtype viruses as previously described (Guan et al., 2004). The mAbs 3C8 and 10H4D2 to the HA of Ck/HK/YU22/02 were produced in our laboratories, and mAb 18H2 to the HA of VNM/1203/04 was produced by the Department of Infectious Diseases at St. Jude Children's Research Hospital.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been deposited in the GenBank database (accession numbers DQ492818–DQ493428 and DQ497642–DQ497729).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.03.048.

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