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Two-dimensional antigenic dendrogram and phylogenetic tree of avian influenza virus H5N1

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Abstract

The hemagglutination-inhibition (HI) titers of a panel of twenty-five mouse monoclonal antibodies (MAbs) against forty-four isolates of highly pathogenic avian influenza virus H5N1 were determined. A two-dimensional antigenic dendrogram was constructed by hierarchical clustering of HI titers. Viruses with similar reactivity patterns were clustered horizontally, whereas MAbs were clustered vertically. In this 2-D dendrogram, with 40% similarity as a cut-off, four virus clusters and four MAbs clusters were identified. A phylogenetic tree based on the deduced amino acid sequence of the hemagglutinin (HA) gene of tested viruses was constructed and its topology was compared to the antigenic dendrogram. Interestingly, viruses with high genetic homology in the phylogenetic tree also had high similarity in their reactivity patterns, as indicated by their relatedness in the tree and close clustering in the dendrogram, respectively. However, the reverse and the converse were also true. Of the five pairs of viruses in the dendrogram with bootstrap values higher than 75, four pairs were in concordance with their genetic relatedness. However, one pair contained viruses belong to two distinct genetic clades. These results were discussed in the context of antigenic variation, genetic polymorphism, and the potential application of MAbs in antigenic analysis.

Keywords

avian influenza virus H5N1; antigenic cluster; phylogenetic tree; monoclonal antibody; antigenic dendrogram; hierarchical clustering

1. Introduction

In 1997, an epizootic of highly pathogenic avian influenza (HPAI) H5N1 virus in poultry occurred in Hong Kong. Significantly, this virus also infected humans, and resulted in six fatalities (Claas, *et al.*, 1998, Shortridge, *et al.*, 1998, Shortridge, *et al.*, 2000). Avian influenza virus H5N1 circulates in waterfowl and domesticated avian species, and has evolved into multiple genotypes, sublineages and clades (Guan, *et al.*, 2002, Smith, *et al.*, 2006, 2009). Furthermore, this virus has now become enzootic in many countries across Asia, part of Africa, and several Middle Eastern countries (Chen, *et al.*, 2005, Balish, *et al.*, 2010). Interestingly, distinctive sublineages or genetic clades circulate in different geography locations, suggesting the evolution of H5N1 virus is influenced by multiple factors. In addition to high mortality in poultry and other land-based avian species, this virus occasionally infects humans with high case-fatality rates. Therefore, this virus poses a significant public health threat (Peiris, *et al.*, 2007).

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Elucidating the mechanism of diversity and evolution of H5N1 virus remains a major knowledge gap. The role of immune selection and the relationship between genetic polymorphism and antigenic variation remain to be elucidated. Whereas genetic analysis of the virus is relatively straight forward by sequencing, antigenic characterization is problematic and reagent dependent. Using multiple mouse monoclonal antibodies (MAbs) against H5N1 viruses, we have reported extensive antigenic profiling (Wu*, et al.*, 2008) of forty-one post-2002 H5N1 viruses from major genetic clades isolated from various geographic locations (2005). Four antigenic groups were identified. Furthermore, it was found that genetic polymorphism was associated with antigenic variation for some viruses, but not for others.

To further delineate the relationship between genetic polymorphism and antigenic variation, in this report, by using a dataset of twenty-five MAbs and forty-four H5N1 viruses, a two-dimensional (2-D) antigenic dendrogram was constructed by hierarchical clustering of hemagglutination-inhibition (HI) titration data. This dendrogram was then compared to the topology of phylogenetic tree generated based on the deduced amino acid sequence of the hemagglutinin (HA) gene of the viruses in this dataset.

2. Materials and methods

2.1 Virus strains

The accession number of the hemagglutinin (HA) gene of forty-four H5N1 viruses and their corresponding taxonomy were shown in Table 1. Viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. Virus titration was conducted in MDCK cells. Hemagglutination-inhibition titration were performed using 05% suspension of turkey red blood cells (Webster, 2002). Experiments using live viruses were conducted in BSL-3 facility at The University of Hong Kong.

2.2 Monoclonal antibodies

Twenty-five MAbs (20A11, 16F13, 13D4, 16G3, 14E5, 20H2, 8H5, 10F7, 4D1, 2F2, 3C8, 9H12B, 3G4, 7C3, 7H8, 16A12, 14D4, 1G2, 3F1B8, 16F8, 4GE1, 1D8, 6CF3, 10DD2, and13E1) were raised in Balb/c mice by immunization with five representative H5N1 strains, Chicken/Hong Kong/YU22/2002 (Ck/HK/YU22/2002; clade 8), Duck/VNM/S654/2005 (Dk/VNM/S654/2005; clade 1), Dk/Indonesia/MS/2004 (Dk/IDN/MS/2004; clade 2.1), Bar-headed goose/Qinghai/15C/2005 (BH goose/QH/15C/2005; clade 2.2), and Dk/VNM/568/2005 (clade 2.3). Standard hybridoma production protocol was used. MAbs were purified from ascitic fluid by ammonium sulfate precipitation, followed by DE-52 ion exchange chromatography. Hemagglutination-inhibition assays were conducted with starting dilution at 1:100 and 0.5% suspension of turkey red blood cells.

2.3 Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura*, et al.*, 2007). Deduced amino acid sequences of the HA gene of viruses listed in Table 1 were aligned by ClustalW, and the phylogenetic tree was generated by Neighbor-Joining with a bootstrap set at 1000 replicates. The result of bootstrap analysis was displayed as the percentage of 1000 replicates.

2.4 Hierarchical antigenic clustering

The HI titers of the viruses using the listed MAbs were analyzed by hierarchical cluster explorer package (HCE version 3.5, University of Maryland Human-Computer Interaction Laboratory). The titers ranged from <100 to >12800. As the program handles positive numbers only, <100 titer was set as 100, and >12800 was set as 12800. Similarities were

computed by Euclidean matrix, and hierarchical clustering was performed two-dimensionally both for the rows and for the columns, using the complete linkage method. The combined heat map (color-coded: green for low similarity and red for high similarity) and the dendrogram shown in Fig. 1 were set at a similarity of 0.40 (40%) both for the rows and for the columns. The node distance was shown in scale according to the percentage of similarity in HI titers, with horizontal lines for the viruses and vertical lines for the MAbs, respectively. Bootstrap values were computed by Cluster Calculator (University of Alberta, Canada; http://www.biology.ualberta.ca/jbrzusto/cluster.php) with bootstrap set at 1000 replicates. The results were displayed as percentages (with the decimal rounded up) of bootstrap values.

3. Results

3.1 Antigenic clustering

Using the HI titers and computation with HCE 3.5, a two-dimensional antigenic dendrogram was generated as shown in Fig. 1. The bootstrap values, scale of similarity, and “heat map” (green=low HI titer; red=high HI titer) were also shown. At a minimum similarity of 0.40 (40%), this panel of tested viruses was classified into four clusters (A through D). The same setting was applied to the panel of MAbs, and coincidentally, four groups were also identified (a through d).

As shown in the heat map, viruses in cluster A were the most reactive to these MAbs, with very high HI titers (red), followed by B, C, and D clusters. Among these forty-four viruses, the most reactive virus was A/Ck/Malang/BBVet-IV/2004 (mostly red), and the least reactive was A/C.Mp/HK/645/2006 (mostly green).

On the other hand, the reactivity patterns among the MAbs groups was in the order of b>a>d>c. For visual effect and for simplicity, the clustering of MAbs was not color-coded.

No two MAbs patterns were exactly alike (vertical spectrum), suggesting these MAbs were distinctive regarding the epitopes they recognized, and their binding affinities and avidities were different. Similarly, no two viruses had the exact reactivity pattern (horizontal spectrum). Although grouping of viruses could be altered by adjusting the level of similarity (horizontal line), the robustness of the clustering was affirmed by the bootstrap values.

3.2 Comparison of antigenic clustering to phylogeny

The phylogenic tree generated from the deduced amino acid sequence of the HA gene by Neighbor-Joining was shown side by side to the 2-D antigenic dendrogram (Fig. 1). The color-coding of the viruses was according to the antigenic clustering (A=blue, B=red, C=green, D=black), and their corresponding taxonomy (clade or sub-clade classification) were also shown. The topology of trees generated by other algorithm such as Maximum Parsimony was similar (data not shown).

Almost all of the virus clades shown in Fig. 1 had member viruses in different antigenic clusters. For example, in clade 2.3.4, it had four viruses (A/Dk/FJ/1734/2005, A/W.Eg/HK737/2007, A/O.Mp/HK/366/2006, and A/SZ/1/2006) clustered in antigenic cluster B while the other two viruses (A/C.Mp/HK/645/2006 and A/Ck/HK/282/2006) were clustered in antigenic cluster D. In clade 1, three viruses (A/HK/213/2003, A/R.pochard/HK/821/2002, A/Dk/VNM/283/2005) were clustered in antigenic cluster A, four viruses (A/VNM/1203/2004, A/Dk/VNM/N-XX/2004, A/Ck/MYS/5858/2004 and A/Dk/VNM/S654/2005) were clustered in antigenic cluster D, while A/VNM/1194/2004 was clustered in antigenic cluster C.

Conversely, in the antigenic dendrogram, viruses with high antigenic similarity could be from highly genetically related viruses, or could be from genetically divergent viruses. For example, for a pair of viruses in antigenic cluster D, A/Qa/GX/575/2005 and A/Dk/VNM/568/2005, they had more than 80% in similarity. Both of them were clade 2.3.2 viruses. Therefore, genetic relatedness was associated with antigenic similarity. However, for another pair of viruses in antigenic cluster A, A/Ck/HK/YU22/2002 and A/Ck/Malang/BBVet-IV/2004, although they had more than 75% similarity, these viruses were genetically diverged, as they were classified in clade 8 and clade 2.1.1, respectively. Therefore, antigenic similarity was associated with genetic polymorphism.

3.3 Detailed comparison of five pairs of viruses with significant bootstrap values

Since the dendrogram was constructed by setting the similarity value at 0.4 (40%), to test the validity of this dendrogram, five pairs of viruses [(i) to (v)] with a bootstrap value >75 were chosen for further comparison. The characteristics were summarized in Table 2. For pair (i), both A/Qa/GX/575/2005 and A/Dk/VNM/568/2005 were clade 2.3.2 viruses, and their bootstrap value at the nearest node in phylogenetic tree was 27. In the antigenic dendrogram, they were clustered in Cluster D, with a similarity of 92% and bootstrap value of 84. Therefore, the antigenic dendrogram was in concordance with the phylogenetic tree for this pair. Similarly, for pair (ii) A/C.Mp/HK/645/2006 and A/Ck/HK/282/2006, (iii) A/Dk/GX/951/2005 and A/Dk/HN/1265/2005, and (v) A/Dk/VNM/283/2005 and A/R.pochard/HK/821/2002, the clustering of these pairs in the dendrogram was in concordance with the phylogenetic tree. However, for pair (iv), A/Ck/HK/YU22/2002 was a clade 8 virus while A/Ck/Malang/BB-Vet4-IV/2004 was a clade 2.1.1 virus, even though they shared 83% similarity in reactivity pattern and a bootstrap value of 76 in the antigenic dendrogram. This pair was obviously in discordance with their genetic relatedness. These results are summarized in Table 2. It should be noted that the bootstrap values in the dendrogram were independent of the similarity patterns (% similarity) toward the MAbs.

3.4 Antigenic relationship for human isolates

One of the impetuses to conduct a comprehensive antigenic analysis of avian influenza virus H5N1 virus was to identify vaccine candidate virus. To evaluate if this antigenic dendrogram could be applied to identify antigenic-matched viruses, human isolates were included in this study. In cluster D, A/IND/5/2005 shared the highest similarity to A/Dk/IDN/MS/2004 (>80% similarity). A/IND/5/2005 was a clade 2.1.3 virus whereas A/Dk/IND/MS/2004 a clade 2.1.1 virus. Similarly, in antigenic cluster B, A/SZ/1/2006 and A/W.Eg/HK/737/2007 were the most similar, with over 80% in similarity. Both viruses were clade 2.3.4 viruses. Therefore, genetic relatedness was associated with antigenic similarity for these viruses.

If the antigenic similarity was set at less than 70%, genetically divergent viruses could be grouped into the same antigenic cluster. For example, in antigenic cluster C, A/VMN/1194/2004 (clade 1) was closely clustered with A/Gs/GY/337/2006 (clade 4). Similarly, in antigenic cluster D, A/VMN/1203/2004 (clade 1) was clustered with A/M.Dk/JX/1653/2005 (clade 9) and A/Dk/FJ/897/2005 (clade 9). Therefore, genetically divergent viruses were grouped in same antigenic clusters if the similarity was set at less stringent conditions.

However, no definitive conclusion could be drawn from these observations by their relatively low bootstrap values (46, 60, 38, and 23, respectively).

Interestingly, earlier viruses of A/HK/483/1997 and A/HK/486/1997 were grouped into separate antigenic clusters C and A, respectively.

4. Discussion

The basis for the rapid evolution and genetic divergence of H5N1 remains to be elucidated. Immune selection (Ferguson, *et al.*, 2003) appears to be a significant driving force for the evolution of human influenza virus H3N2 and H1N1, as the long term evolutionary pattern is represented by a single dominant lineage (Fitch, *et al.*, 1997). For H3N2, there are codon biases at putative antigenic sites (Bush, *et al.*, 1999). In addition, using "antigenic cartography", Smith et al. has shown that antigenic evolution of human influenza virus H3N2 is "punctuated" (Smith, *et al.*, 2004). Whether this step-wise antigenic evolution is a result of accumulated herd immunity or by other unknown mechanism remains to be determined. Another possible mechanism for influenza virus to evade host immunity is by alternate circulation of distinct genetic and antigenic viral lineages (Lai, *et al.*, 2004). Therefore, the antigenic properties of influenza virus play a major role in viral evolution.

A major gap in elucidating the mechanism of H5N1 evolution is a lack of complete antigenic characterization of the virus. In contrast to genetic analysis, antigenic analysis is dependent on the availability of specific antibodies. Ideally, antigenic studies are conducted using polyclonal antiserum from homologous host species. However, ferret polyclonal antiserum is currently used and is regarded as the gold standard, particularly in vaccine strain selection (Webster, 2002). Polyclonal antiserum is a composite of a large spectrum of individual antibodies. For specific antiserum, it is generally assumed that as a result of clonal expansion, the quantity and quality of individual antibodies towards the antigenic sites are higher. We have developed over 400 MAbs reactive to a broad spectrum of H5N1 viruses. There is a high probability that some of these MAbs are specific to the antigenic sites, although these sites are yet to be unequivocally defined. The dataset described in this paper was chosen because the complete genetic sequences of the tested viruses are known and their reactivity towards this panel of MAbs has been well characterized. It should be noted that the epitope and the affinity and avidity of these MAbs have not been completely determined. This dataset is intended as an experimental model to test the usefulness of this approach in determining the relationship between genetic polymorphism and antigenic variation of H5N1 viruses. The usefulness of MAbs in mapping antigenic changes in influenza virus has been demonstrated previously (Gerhard, *et al.*, 1981, Underwood, 1982, Kaverin, *et al.*, 2004, Kaverin, *et al.*, 2007), and for H3N2, the results of these analyses concur with the results of structural determination of antigenic sites (Wiley, *et al.*, 1981). Furthermore, de Jong et al. (Weijers, *et al.*, 1985) has demonstrated the usefulness of MAbs in clustering related influenza viruses albeit with a small sample size.

Interestingly, A/HK/483/1997 and A/HK/486/1997 were isolated from a fatal case and a mild case, respectively (Zitzow, *et al.*, 2002). As shown in Fig. 1, both viruses are classified as clade 0 virus. However, A/HK/483/1997 and A/HK/486/1997 viruses are clustered in separate antigenic groups, C and A, respectively in the antigenic dendrogram. There is a difference of seven amino acids in the HA protein between these two viruses, five of which are in the HA1 portion of the hemagglutinin (125N, 156R, 172A, 202M, 336T). It is highly likely that at least one of these residues is located at the antigenic sites. Of note, ferret antiserum did not distinguish the two viruses (Rowe, *et al.*, 1999). The validity of this two-dimensional antigenic dendrogram is further illustrated by its ability to distinguish A/HK/213/2003 (clade 1) and A/VMN/1203/2004 (clade 1), as immunization with either of these viruses failed to cross-protect reciprocally in a ferret model (Forrest, *et al.*, 2009). A/HK/213/2003 and A/VMN/1203/2004 are clustered in group A and D, respectively in the dendrogram. Conversely, virus-like particles made based on A/IND/5/2005 (clade 2.1.3) cross-protected against A/VMN/1203/2004 (clade 1) (Mahmood, *et al.*, 2008), as both viruses are clustered in antigenic group D. These observations support the usefulness of this antigenic dendrogram in identifying antigenically matched viruses.

Clustering is an assignment of multiple objects with similar properties into groups or clusters. Hierarchical algorithms find successive clusters using previously established clusters. Members within a cluster share a high degree of similarities, in this case, reactivity towards this panel of MAbs. Hierarchical clustering had been shown to reliably classify MAbs based on reactivity patterns (Pratt*, et al.*, 2009). Of note, a phylogenetic tree is based on the homology of genetic sequence with an assumption of common ancestry. Antigenic clustering, on the other hand, is dependent on the phenotype. Unlike genetic phylogeny, the root of the dendrogram consists of a single cluster containing all observations. It should be noted that this analysis is not for elucidating epitopes recognized by individual MAb, as other reliable methods such as construction of site-specific mutants are available. These studies are in progress.

Four out of five pairs of viruses in this antigenic dendrogram are in concordance with the genetic relatedness, making this antigenic analysis a potential method for rapid identification of antigenic matched viruses. By mix and match of a panel of MAbs, it is possible to "mimic" a specific polyclonal antiserum, provided that critical parameters such as the epitopes and the binding affinities of these MAbs have been characterized. Furthermore, for the pair of viruses that are in discordance, it is interesting to elucidate the basis of this discordance and may shed some light on the mechanism of antigenic variation.

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Abbreviations

MAbs	monoclonal antibodies
HA	hemagglutinin
HI	hemagglutination-inhibition
HCE	hierarchical cluster explorer
HPAI	highly pathogenic avian influenza

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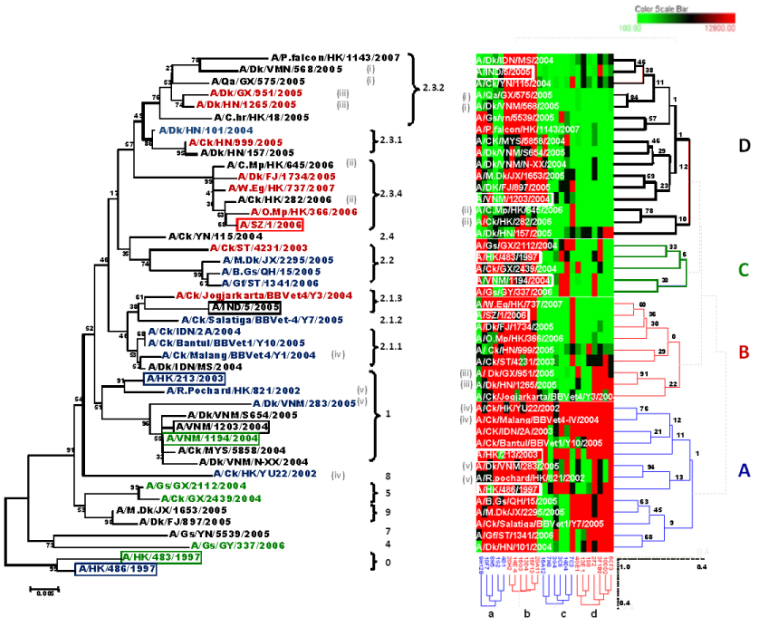

Fig. 1.

Two-dimensional antigenic dendrogram and phylogenetic tree. *Left:* Phylogenetic tree generated based on the deduced amino acid sequence of the HA gene of viruses in the dataset. Genetic clade designation is indicated adjacent to the virus name on the right. Branch lengths are in scale to the scale bar shown at lower left corner. Color-coding is in accordance to the antigenic clustering in the dendrogram on the right. *Right:* Two-dimensional antigenic dendrogram with heat map constructed by hierarchical clustering. Scale of heat map(green = 100; red=12800) is according to the color scale bar shown at upper right corner. Virus clustering is shown horizontally; MAbs clustering is shown vertically. Both horizontal and vertical dendrogram are in scale to the scale bars shown in lower right corner (from 0.4 to 1.0, representing 40% to 100% similarity, respectively), that is, the similarity is indicated by the position of the node relative to the scale bar. For detailed nomenclature of viruses, refer to Table 1. Boxed viruses are human isolates. Note that virus A/C.hr/HK/18/2005 and MAb 16F8 are not shown in the dendrogram due to being outliners for virus cluster C and D and MAb cluster c and d, respectively. Five pairs of viruses [(i) through (v)] chosen for detailed comparison are indicated in both the phylogenetic tree and the dendrogram. Bootstrap values shown at the nodes of both phylogenetic tree and dendrogram are percentages of 1000 replicates, with the decimal rounded up to the next whole number in the dendrogram.

Table 1

Accession number and taxonomy of avian influenza virus H5N1 used in this study

H5N1 viruses	Accession No.	Clade
A/Ck/HK/YU22/2002	AY651349	8
A/Ck/IDN/2A/2004	AY651323	2.1.1
A/Dk/IDN/MS/2004	AY651322	2.1.1
A/Ck/Malang/BBVet-IV/2004	DQ497642	2.1
A/Ck/Salatiga/BBVet1/Y7/2005	DQ320928	2.1.2
A/HK/213/2003	AB212054	1
A/Ck/Bantul/BBVet1/Y10/2005	DQ320932	2.1.1
A/Ck/YN/115/2004	AY651372	2.4
A/Gs/GX/2112/2004	DQ320889	5
A/Ck/Jogjarkarta/BBVet4/Y3/2004	DQ320930	2.1.3
A/Dk/VNM/283/2005	DQ497708	1
A/R.pochard/HK/821/2002	AY575874	1
A/CK/MYS/5858/2004	DQ320934	1
A/VNM/1194/2004	AY651333	1
A/VNM/1203/2004	AY818135	1
A/Dk/VNM/N-XX/2004	DQ497686	1
A/Dk/VNM/S654/2005	DQ320936	1
A/M.Dk/JX/1653/2005	DQ320916	9
A/DK/FJ/897/2005	DQ320875	9
A/Gs/GY/337/2006	DQ992765	4
A/Gs/YN/5539/2005	DQ992805	7
A/IND/5/2005	EF541394	2.1.3
A/B.Gs/QH/15/2005	DQ095621	2.2
A/Gf/ST/1341/2006	DQ992780	2.2
A/M.Dk/JX/2295/2005	DQ320920	2.2
A/ Ck/HN/99920/05	DQ320910	2.3.1
A/Dk/HN/157/2005	DQ320906	2.3.1
A/Ck/ST/4231/2003	AY651368	2.2
A/ Dk/GX/951/2005	DQ320900	2.3.2
A/Dk/HN/1265/2005	DQ320911	2.3.2
A/Ck/GX/2439/2004	DQ320893	5
A/Qa/GX/575/2005	DQ320897	2.3.2
A/Ck/VNM/568/2005	DQ320939	2.3.2
A/C.Mp/HK/645/2006	DQ992839	2.3.4
A/O.Mp/HK/366/2006	DQ992837	2.3.4
A/Ck/HK/282/2006	DQ992836	2.3.4
A/W.Eg/HK/737/2007	CY036077	2.3.4
A/P.falcon/HK/1143/2007	CY036094	2.3.2
A/SZ/1/2006	EF137706	2.3.4

H5N1 viruses	Accession No.	Clade
A/Dk/FJ/1734/2005	DQ095629	2.3.4
A/Dk/HN/101/2004	AY651365	2.3.1
A/C. Hr/HK/18/2005	DQ320925	2.3.2
A/HK/483/1997	AF046097	0
A/HK/486/1997	AF084281	0

*In bold=human isolates. Virus names are according to standard nomenclature: Type/species/location/laboratory-assigned number/year. Abbreviations: Ck=chicken; Dk=duck; Gs=goose; Qa=quail; hr=heron; Eg=egress; Mp=magpie; HK=Hong Kong; IND=Indonesia; VMN=Vietnam; MYS=Malaysia. YN, GX, JX, FJ, GY, ST, HN, SZ are abbreviation for Yunnan, Guangxi, Jiangxi, Fujian, Guiyang, Shantou, Hunan, and Shenzhen, respectively, representing various provinces or cities in Peoples' Republic of China.

Table 2

Comparison of virus pairs from phylogenetic tree and the dendrogram

	Virus pairs	Clade	*Bootstrap value (NJ Tree)	Cluster Group	Similarity	Bootstrap value	Concordance
(i)	A/Qa/GX/575/2005 A/Dk/VNM/568/2005	2.3.2 2.3.2	27	D	0.92	84	Yes
(ii)	A/C.Mp/HK/645/2006 A/Ck/HK/282/2006	2.3.4 2.3.4	99	D	0.80	78	Yes
(iii)	A/Dk/GX/951/2005 A/Dk/HN/1265/2005	2.3.2 2.3.2	74	B	0.83	91	Yes
(iv)	A/Ck/HK/YU22/2002 A/Ck/Malang/BB-Vet4-IV/2004	8 2.1.1	NA	A	0.83	76	No
(v)	A/Dk/VNM/283/2005 A/R.pochard/HK/821/2002	1 1	51	A	0.78	94	Yes

*Bootstrap values are from the nearest node in the phylogenetic tree, if they are in the same clade.