Identification of Precursors of Indonesia and Vietnam Avian Influenza A (H5N1) Viruses From Southern China

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The transmission of highly pathogenic avian influenza (HPAI) H5N1 virus to Southeast Asian countries triggered the first outbreak wave of this virus in late 2003. Subsequently H5N1 influenza virus has become endemic in poultry in this region, which has lead to the sustained transmission of those viruses and repeated outbreaks in poultry and human infection cases. This situation has raised global concern of a coming influenza pandemic sometime in the near future. Although surveillance work in market poultry had been strengthened following this initial outbreak in Southeast Asian countries, the lack of influenza surveillance prior to the outbreaks made it difficult to identify the precursors and transmission pathways of those H5N1 viruses. To determine the possible source of those H5N1 viruses responsible for this first transmission wave we recently conducted further sequencing of samples collected in live-poultry markets from Guangdong, Hunan and Yunnan Provinces in southern China from 2001 to 2003. Phylogenetic analysis of the HA gene of 50 H5N1 isolates from this period indicated that eight viruses, exclusively from Yunnan, fell as the direct progenitor to viruses isolated from Vietnam. A further two viruses isolated from Hunan were the direct precursor to those viruses from Indonesia. In general, phylogenetic analysis revealed similar relationships for the NA gene and each of the 6 internal genes, indicating that these viruses also belonged to the same H5N1 genotype Z that is predominant throughout Southeast Asia. These results clearly show a transmission of H5N1 viruses from Yunnan to Vietnam and from Hunan to Indonesia. Trade of poultry may be the major route of virus transmission between Yunnan and Vietnam, while the transmission route from Hunan to Indonesia remains unclear and could be either via migratory birds or poultry movement.

Introduction

It has been ten years since the first avian-to-human transmission of influenza virus was confirmed in Hong Kong. In the past decade, the "bird flu" caused by highly pathogenic influenza (HPAI) H5N1 virus has developed from an endemic disease in China to an epidemic disease affecting 60 countries in Eurasia and Africa. There have been 3 transmission waves of H5N1. Wave 1 involved transmission to Vietnam and Indonesia resulting in outbreaks in late 2003/early 2004 [1]. Wave 2 was initiated following the Qinghai Lake outbreak in early 2005 with subsequent spread west through Eurasia and Africa, while Wave 3 was initiated in late 2006 with spread to Laos and Malaysia [2,3]. Although surveillance in market poultry was strengthened following the Wave 1 transmission, the source of the Southeast Asian outbreak and how the virus spread from country to country remained obscure due to a lack of data prior to this period. Here we studied 50 sequences of H5N1 influenza viruses isolated from 2001 to 2003 in southern China to investigate the relationships between these viruses and those detected in Thailand, Laos, Vietnam, Cambodia, Malaysia and Indonesia during the period of the first wave of H5N1 outbreaks.

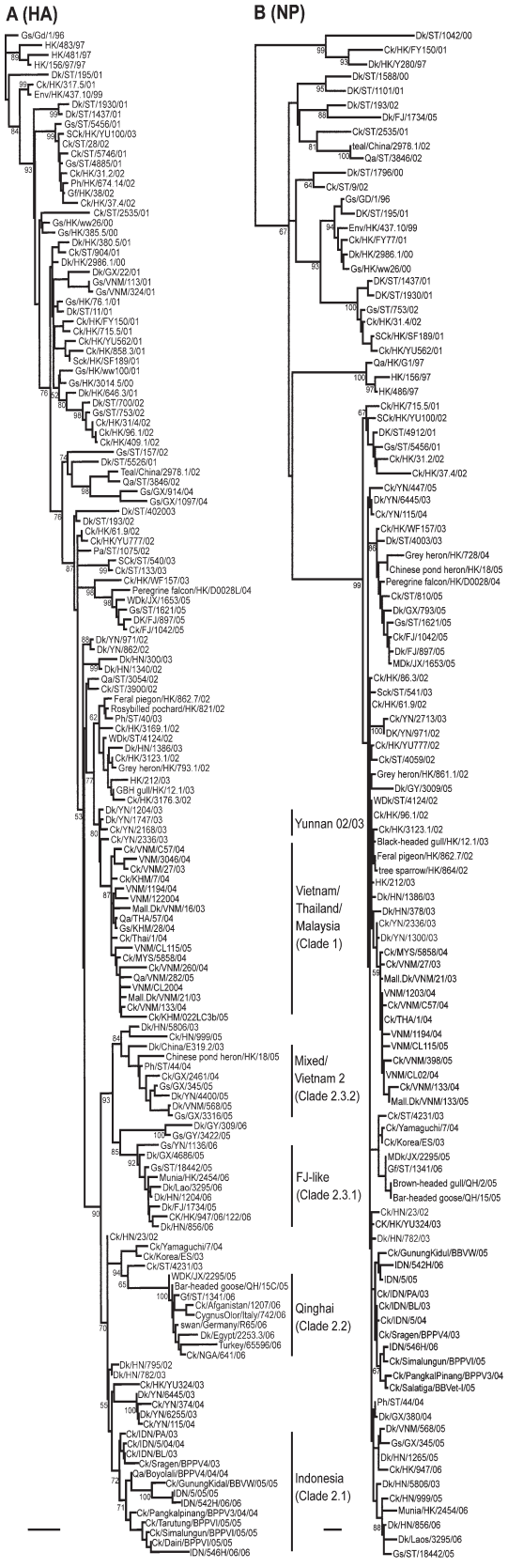
Methods

Viruses were isolated in 9- to 11-day-old embryonated chicken eggs as previously described [4]. Virus isolates were subtyped by standard hemagglutination inhibition (HI) tests using a panel of the World Health Organization reference antisera. Antigenic analysis was performed using five World Health Organization H5N1 reference antisera, monoclonal antibodies to Ck/Pennsylvania/1/83 and four monoclonal antibodies to Ck/HK/YU22/02. To visualize similarity between the antigenic reaction patterns of different viruses, numerical analysis of HI titers was conducted using PRIMER version 5.2.9 (PRIMER-E, Plymouth, United Kingdom). The data were standardized and square-root transformed, and the Bray-Curtis coefficient was used to construct a similarity matrix. Hierarchical agglomerative clustering with group-average linking was conducted and a dendrogram produced. Nonmetric multidimensional scaling was also used to produce two- and three-dimensional ordinations over 100 iterations. The two-dimensional configuration with lowest overall stress is presented. RNA extraction, cDNA synthesis, PCR and sequencing were carried out as described previously [4]. All sequences were assembled and edited with Lasergene 6.0 (DNASTAR, Madison, WI); BioEdit 7 was used for alignment and residue analysis. The program MrModeltest 2.2 was used to determine the appropriate DNA substitution model and rate heterogeneity. The generated model was used in all subsequent analyses. Neighbor-joining and maximum likelihood trees were constructed by using PAUP* 4.0. Estimates of the phylogenies were calculated by performing 1,000 neighbor-joining bootstrap replicates. All eight genes were sequenced for each virus isolate.

Results

Phylogenetic analysis. The HA gene of the 50 H5N1 isolates characterized in this study indicated that eight viruses exclusively from Yunnan (represented by Ck/YN/2336/03) fell as the direct progenitor to Clade 1 viruses isolated from Vietnam, Thailand, Malaysia and Cambodia (Fig. 1A). A further two viruses isolated from Hunan (represented by Dk/HN/795/02) were the direct precursor to those Clade 2.1 viruses from Indonesia. Phylogenetic analysis of those 10 precursor viruses revealed similar relationships for the NA gene and each of the 6 internal genes (represented by the NP gene), indicating

Figure 1. Phylogenetic relationships of the (A) HA and (B) NP genes of influenza A viruses in Eurasia. Numbers below branches indicate neighbor-joining bootstrap values. 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 Equine/Prague/1/56. Scale bar, 0.01 substitutions/site.

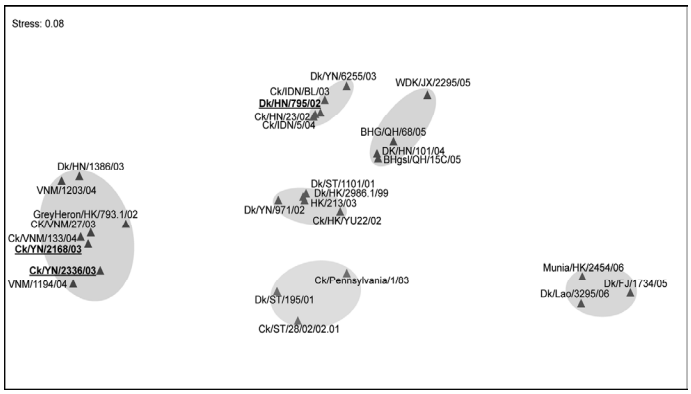


that these viruses also belonged to the same H5N1 genotype Z that is predominant throughout Southeast Asia (Figure 1B). However, analysis of the remaining 40 viruses isolated from southern China in that period revealed the presence of multiple genotypes of H5N1 virus as previously described [1].

Molecular characterization. All of the 50 viruses characterized were highly pathogenic with variations of the multi-basic cleavage site (QRERRRKKR/G) in the HA molecule. The receptor-binding pocket of HA1 retains amino acid residues Gln 222 and Gly 224 (H5 numbering used throughout) that preferentially bind to α-2,3-NeuAcGal receptors [5]. Other amino acid residues relevant to receptor-binding sites were identical to those of HK/156/97 and Gs/GD-like viruses [5,7] in most isolates, but with some notable differences. Most of the Yunnan viruses characterized had a Ser129Leu substitution (LGVSS), which had been observed previously in viruses from Vietnam [7,8]. While most of the Hunan and Indonesia isolates had SGVSS at positions 129-133. In the NA amino acid sequences, all isolates characterized had 274Y, indicating resistance to oseltamivir. In the M2 protein, all eight Yunnan viruses that are precursors to Clade 1 had both the Leu26Ile and Ser31Asn mutations. These mutations may confer resistance to the amantadines and both are present in all Clade 1 viruses characterized to date [7]. This provides further evidence that these amantadine resistance mutations were present in the viruses that were introduced into Vietnam to form Clade 1. No amantadine resistance mutations were observed in the two Hunan viruses that are precursors to Indonesian isolates.

Antigenic analysis. The hemagglutinin inhibition reaction patterns of viruses from Yunnan and Hunan characterized in this study were similar to those of viruses from Vietnam and Indonesia, respectively (Figure 2). These reaction patterns correspond to their phylogenetic relationships and further suggest that these viruses are the precursors to those Wave 1 viruses.

Figure 2. Numerical analysis of HI titers using nonmetric multidimensional scaling.



Discussion

The outbreaks of H5N1 bird flu in Southeast Asia in late 2003 onset suddenly and spread quickly. However, the source of the virus and the route of introduction into each country were unclear. In our study, the phylogenetic trees showed a close relationship between Hunan and Yunnan with Indonesia and Vietnam, respectively. The antigenic analysis correlated strongly to their phylogenetic relationships. Molecular characterization at the amino acid level also revealed a similar pattern, especially with amantadine resistance mutations in the M2 protein of Vietnam (Clade 1) viruses. These results clearly show a transmission of H5N1 viruses from Yunnan to Vietnam and from Hunan to Indonesia. Trade of poultry may be the major route of virus transmission between Yunnan and Vietnam, while the transmission route from Hunan to Indonesia remains unclear and could be either via migratory birds or poultry movement.

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