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<b>Author(s)</b>	<b>Dhanasekaran, V; Smith, GJ; Bahl, J; Wang, J; Webster, RG; Guan, Y</b>
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## Dating the Emergence of Influenza A (H5N1) Virus

Dhanasekaran Vijaykrishna<sup>1</sup>, Gavin JD Smith<sup>1</sup>, Justin Bahl<sup>1</sup>, Jia Wang<sup>1</sup>, Robert G Webster,<sup>2</sup> Yi Guan<sup>1</sup>

<sup>1</sup>State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China; <sup>2</sup>Virology Division, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

Since the first detection of highly pathogenic avian influenza (H5N1) virus in geese in Guangdong, China, H5N1 viruses have transmitted to poultry throughout southern China. In late 2003 the first transmission wave spread the virus to multiple Southeast Asian countries. In May 2005, the second transmission wave of H5N1 virus westwards to Europe and Africa was initiated following a major outbreak in migratory birds at Qinghai Lake, China, while a third transmission wave has been initiated since mid-2005. Those viruses are now endemic in poultry populations in some affected regions and cause repeated outbreaks in poultry and increasing human infection cases, creating persistent pandemic concerns. Genetic data from systematic surveillance of H5N1 for the past seven years in marketing poultry, along with sequence data from outbreaks throughout the region, provide us with a unique opportunity to estimate the most recent common ancestor (MRCA) and postulate the dates of introduction of H5N1 variants into different affected countries. In this study, we estimated the time of emergence of those three transmission waves, based on their hemagglutinin genes, and compared these MRCA estimates with the date of detection of either human or poultry disease. These analyses indicated that the time interval between the first detection of H5N1 disease and the MRCA for the wave 1 outbreaks, in both Vietnam and Indonesia, was 5 months. For the Qinghai outbreak the MRCA was estimated at three months prior to their first detection among migratory birds. Remarkably, the mean time of the MRCA for the third transmission wave was estimated as exactly the same day as the first virus (Dk/FJ/1734/05) from this lineage was isolated. The early detection of H5N1 viruses in the second and third transmission waves, as compared to the first wave outbreaks in Vietnam and Indonesia, highlights the importance of systematic influenza surveillance in apparently healthy market poultry.

### Introduction

The first outbreak of highly pathogenic avian influenza (H5N1) occurred in goose populations in southern China in 1996. Subsequently H5N1 viruses had transmitted to poultry throughout southern China, and have since become endemic in this region. In late 2003 the first transmission (Wave 1) was detected that had originated in southern China and spread to multiple Southeast Asian countries [1,2]. In May 2005, a second transmission (Wave 2) westwards to Europe and Africa was initiated following an H5N1 disease outbreak in migratory

birds at Qinghai Lake, China [3], and a third transmission (Wave 3) was initiated in mid-2005 [4]. Those H5N1 viruses are now endemic in poultry populations in affected regions and cause repeated outbreaks in poultry and subsequent human infection cases with high mortality, increasing pandemic concern. Genetic data from systematic surveillance of H5N1 for the past eight years in apparently healthy market poultry, along with sequence data from outbreaks throughout the region, provide us with a unique opportunity to estimate the most recent common ancestor (MRCA) and therefore postulate the dates of introduction of H5N1.

### Materials and Methods

To estimate the time of emergence of the three major transmission waves of H5N1 viruses, we analyzed the hemagglutinin (HA) gene of representative viruses belonging to the major sub-lineages of the A/goose/Guangdong (Gs/GD)-like viruses that also included the recently sequenced viruses isolated from southern China from 2000-2003 [2]. We used the uncorrelated relaxed clock method in BEAST v1.4 using sampling dates and skyline population coalescent priors [5,6]. MCMC chains were run thrice for 20 million generations sampling every 1,000 generations under the codon based SRD06 model [7]. The times of divergence were estimated with a discarded burn-in of approximately 10% using the program Tracer v1.3 [8], and utilizing optimized operator tuning values to increase the efficiency of sampling for subsequent runs.

### Results

The most recent common ancestors (MRCA) for the three transmission waves were calculated for the HA gene using the uncorrelated relaxed clock method (Figure 1). The MRCA for the Vietnam, Thailand and Malaysia (VTM) lineage (Clade 1) was estimated at Mar/2003 (Highest Posterior Density {HPDs}, Dec/2002, Oct/2003), while the MRCA for the Indonesia lineage (Clade 2.1) was estimated at Apr/2003. (HPDs, Jan/2003, Aug/2003). These results indicate that Wave 1 transmission of H5N1 viruses from southern China to Vietnam and Indonesia was initiated at the same time in early 2003. The emergence of Wave 2 (Qinghai-like, Clade 2.2) and Wave 3 (Fujian-like, Clade 2.3.4) were estimated at March/2005 (HPDs, Jan/2005, May/2005) and Mar/2005 (HPDs, Oct/2004, Aug/2005), respectively, suggesting that MRCAs of the virus that caused an outbreak among migratory waterfowl in Qinghai Lake, China and the emergence of a new H5N1 variant (Fujian-like) in southern China occurred during the same time period in early 2005.

### Discussion

In this study, we estimated the time of emergence of the three transmission waves, based on the hemagglutinin (HA) gene. We then compared these MRCA estimates with the date of detection of either human or poultry disease and postulated an establishment time for the virus in the absence of control measures. These analyses indicated that the time intervals

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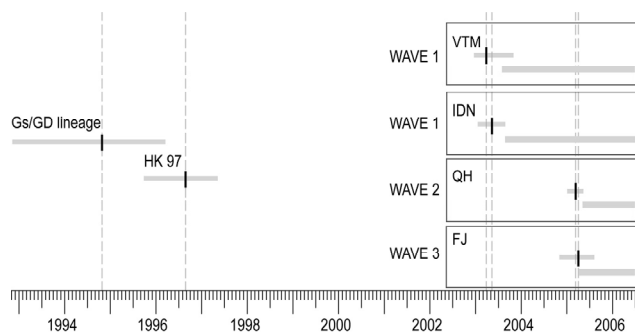
**Figure 1.** Phylogenetic tree of the hemagglutinin (HA) gene of representative viruses belonging to the Gs/GD lineage. The analysis was based on 963 nucleotides of the HA1 gene. The divergence times correspond to the mean posterior estimates. Bars indicate 95% confidence intervals for the divergence estimates. Tip dates correspond to the date of isolation.



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between the first detection of H5N1 disease and the MRCA for the Wave 1 outbreaks in both Vietnam and Indonesia, that were initiated from Yunnan and Hunan provinces of southern China, was 5 months (Figure 2). For the Qinghai outbreak (Wave 2) the MRCA was estimated at 3 months prior to their first detection among migratory birds. Remarkably, the mean time of the MRCA for Wave 3 was the same day that the first virus (Dk/FJ/1734/05) from this lineage was isolated (Figure 2). The early detection of H5N1 viruses in the second and third transmission waves, as compared to the first wave outbreaks in Vietnam and Indonesia, highlights the importance of systematic influenza surveillance in apparently healthy market poultry in the early detection, and potential for control, of the virus.

**Figure 2.** Time line of emergence of major H5N1 lineages and disease outbreaks in southeast Asia. Mean divergence times are shown in black; bars indicate 95% highest posterior density (HPD). The bars adjacent to the divergence estimates indicate outbreak/occurrence events.



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