# Establishment of Influenza A Virus (H6N1) in Minor Poultry in Southern China

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An H6N1 virus, A/teal/Hong Kong/W312/97 (W312), was isolated during the 'bird flu' incident of Hong Kong in 1997. Genetic analysis suggested that this virus might be the progenitor of the A/Hong Kong/156/97 (HK/97) H5N1 virus as seven of eight gene segments of those viruses had a common source. Continuing surveillance in Hong Kong showed that a W312-like virus was prevalent in quail and pheasant in 1999; however, the further development of H6N1 viruses has not been investigated since 2001. Here we report influenza surveillance data from 2000 to 2005 in southern China that shows H6N1 viruses have become established and endemic in minor poultry, and mainly replicated in the respiratory tract. Phylogenetic analysis indicates that all H6N1 isolates had W312-like hemagglutinin and neuraminidase genes. However, reassortment of internal genes between different subtype virus lineages, including H5N1, H9N2 and other avian viruses, generated multiple novel H6N1 genotypes in different types of poultry. These novel viruses are double, triple or even quadruple reassortants. Molecular analyses suggest that W312-like viruses may not be a precursor of HK/97 virus but a reassortant from HK/97-like virus and another unidentified H6 subtype virus. These results provide further evidence of the pivotal role of the live-poultry market system of southern China in generating increased genetic diversity of influenza viruses in this region.

## Introduction

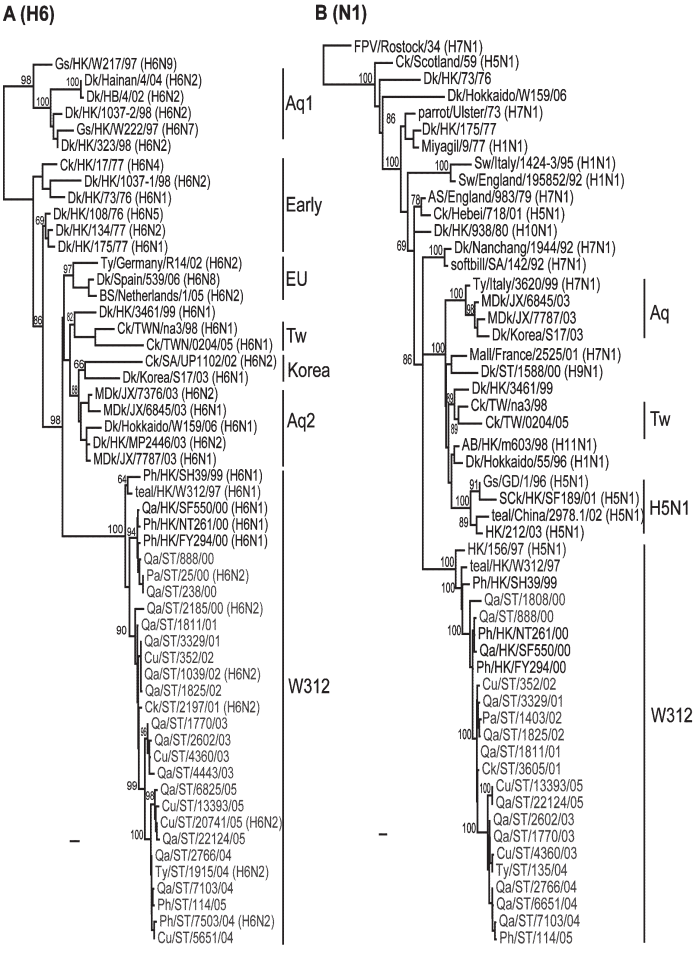
H6 influenza viruses are one of the most commonly recognized subtypes in domestic duck in southern China (4). During the Hong Kong H5N1 'bird flu' incident in 1997 an H6N1 avian influenza virus, A/teal/Hong Kong/W312/97 (W312), was isolated from live-poultry market. Genetic characterization of this virus revealed that, except for the hemagglutinin (HA) gene, its remaining seven gene segments were closely related to highly pathogenic avian influenza (HPAI) H5N1 viruses found in both poultry and humans (3). These findings suggested that a W312-like H6N1 virus might have been involved in the generation of the Hong Kong H5N1 virus (A/Hong Kong/156/97, HK/97) (2, 3). However, it is still unknown how the HK/97-like virus was generated as an H9N2 virus lineage, represented by A/quail/Hong Kong/G1/97 (G1), also shared the same internal gene complex as that of W312-like and HK/97-like viruses. As those three different subtypes of influenza viruses (H5N1, H6N1 and H9N2) were initially detected simultaneously during the Hong Kong 'bird flu' incident (2), the direction of gene flow among those viruses could not be determined.

## Results

Systematic surveillance of minor poultry including quail, chukkar, Guinea fowl, partridge and pheasants in live-poultry markets from 2000 and 2005 resulted in 414 H6 subtype viruses out of a total of 11,415 samples collected (overall isolation rate, 3.6%). Most of these isolates were H6N1 subtype. Those viruses were prevalent year-round but with a higher isolation rate during the winter since 2004. However, the isolation rate for each year varied markedly from 1.7% to 6.4%. Of the minor poultry, chukkar and quail provided the main body of H6N1 isolates and had remarkably high isolation rates of 8.9% and 4.2%, respectively. In comparison, only a single H6N1 virus was isolated from 8,788 chicken and 2,530 silkie chicken specimens collected during the same period and from the same markets. A total of 402 of 414 (97%) H6 subtype viruses were isolated from tracheal swabs, and only eight from cloacal swabs and four from fecal material. These findings suggest that H6N1 viruses mainly replicated in the respiratory tract of those birds. Phylogenetic analysis of the surface genes: To understand the evolution and genesis of the H6N1, 77 of 414 (18.6%) virus isolates were sequenced and phylogenetically analyzed. Phylogenetic analysis of the H6 HA gene revealed that all viruses separated into the American and Eurasian gene pool. Within the Eurasian gene pool, four major lineages could be recognized, including Contemporary 1 and 2, Early, Aquatic. Contemporary 1 only contained viruses isolated from 1997 to 2004 in domestic ducks and geese in southern China (Fig. 1A). The second lineage (Early) mainly contained viruses isolated from 1976 to 1977, however, one virus isolated in 1998 in Hong Kong (Dk/HK/1037-1/98) also joined this lineage. The third lineage (Aquatic) consists of multiple different clades from the Eurasian influenza gene pool. Within this lineage H6 subtype influenza viruses were introduced into terrestrial poultry in several regions, e.g. Taiwan and South Africa. The fourth lineage (Contemporary 2) contains all H6 viruses that are prevalent in terrestrial poultry in southern China. These viruses were closely related to and derived from that of A/teal/HK/W312/97 and hence are referred to as "W312-like". The H6 HA gene tree clearly demonstrates that the phylogenetic position of the viruses tested corresponds to the different time points of their evolutionary pathway, and has formed a stable lineage (Fig 1A). This evolutionary pattern is different from that of the H5N1 and H9N2 viruses, which appear to have multiple evolutionary pathways with diverse co-circulating sublineages [4]. Phylogenetic analysis of N1 NA genes also shows that all H6N1 viruses isolated from minor poultry in southern China were closely related to and derived from that of A/teal/Hong Kong/W312/97 virus (Fig 1B). In concordance with the relationship of the Contemporary 1 lineage of the HA gene, the W312-like lineage appears to be derived from the Eurasian gene pool. All H6N1 viruses isolated from chicken in Taiwan also clustered with the same virus, Dk/HK/3461/99, which suggests the establishment of that virus lineage in Taiwan poultry. Phylogenetic analysis of the internal genes: In general, four different sources for the internal genes of the H6 viruses

tested were recognized, indicating that multiple reassortment events had occurred. Two of the internal gene sources were from the W312- or G1-like and Ck/Bei-like H9N2 virus lineages, while the remaining two were either from aquatic birds or from an unidentified source that was shared with current H5N1, H6N1 and H9N2 variants. For example, in the PB2 gene most of the H6N1 viruses clustered with G1-like or W312-like H9N2 viruses, while a few viruses clustered with recently identified H9N2 Ck/Bei-like variants. However, in the PA gene most H6N1 viruses were closely related to PA genes from aquatic bird isolates or novel H9N2 and H5N1 variants (data not shown). These results suggest that further reassortment between H9N2 and H6N1 viruses occurred after the Ck/Bei-like H9N2 variants were generated. It is noteworthy that the matrix (M) and non-structural (NS) genes of the H6N1 viruses showed much less genetic diversity than the other genes and belonged to either the G1-like or Ck/Bei-like H9N2 lineages.

**Figure 1.** Phylogenetic relationships of the H6 hemagglutinin (HA) (A) and N1 neuraminidase (NA) (B) genes of representative influenza A viruses. Trees were generated by the neighbor-joining method in the PAUP* program. Numbers above and below branches indicate neighbor- joining bootstrap values and Bayesian posterior probabilities, respectively. Analysis was based on nucleotides 49-1032, 1-1353 and 1-1229 of the H6 HA and N1 NA gene segments respectively. The H6 HA trees are rooted to A/turkey/Canada/63 (H6N2) and the N1 NA tree is rooted to A/Wisconsin/1/33 (H1N1). Virus subtypes are indicated in parenthesis, while those viruses with no subtype designations are of H6N1 subtype.




Molecular evolutionary analysis: Analysis of nonsynonymous substitutions (dN) of H6N1 influenza viruses from Hong Kong and Shantou in different years showed that the dN rate was higher in the N1 gene in comparison to all other genes, including the HA gene, but with the exception of the NS1 gene (Table 1). The dN rates of both the HA and NA genes gradually reduced over time since 1999. These findings suggest that the N1 gene may have been incorporated into the H6N1 viral particle later than the other genes.

**Table 1.** Rates of nonsynonymous subtitutions (Dn) of H6N1 influenza virus in Southern China from 1999 to 2003.

| Gene | Virus groups | | | | | |
|---|---|---|---|---|---|---|
| | HK/99 (HK) | HK/00 | ST/00 | ST/01 | ST/02 | ST/03 |
| **HA** | 4.4 ± 1.4 | 2.8 ± 0.6 | 2.9 ± 0.3 | 3.4 ± 0.3 | 2.5 ± 0.0 | 3.1 ± 0.4 |
| **N1** | 5.7 ± 1.0 | 4.9 ± 0.4 | 5.0 ± 0.3 | 4.4 ± 0.5 | 3.6 ± 0.4 | 4.4 ± 0.1 |
| **PB2** | 0.7 ± 0.6 | 0.7 ± 0.5 | 1.4 ± 0.4 | 1.1 ± 0.2 | 1.1 ± 0.1 | 1.3 ± 0.2 |
| **PB1** | 1.7 ± 0.3 | 0.9 ± 0.5 | 1.5 ± 0.9 | 0.8 ± 0.4 | 1.0 ± 0.0 | 1.3 ± 0.1 |
| **PA** | 2.0 ± 0.8 | 1.1 ± 0.2 | 1.5 ± 0.5 | –[a] | – | – |
| **NP** | 1.1 ± 1.0 | 1.6 ± 2.7 | 1.7 ± 0.7 | – | – | – |
| **M1** | 0 | 0 | 0 | 0 | 0 | 0.1 ± 0.2 |
| **NS1** | 6.7 ± 4.9 | 5.0 ± 4.9 | 6.1 ± 1.2 | – | – | – |

[a]Not calculated as novel gene segments were incorporated from other virus lineages.

## Discussion

Here we have provided the first comprehensive surveillance data for H6N1 viruses from 2000 to 2005 in poultry in this region. The findings of the present study revealed that H6N1 influenza viruses derived from W312-like viruses have become established in minor terrestrial poultry in southern China since 2000. Genetic analyses demonstrated that this virus lineage underwent broad reassortment with other influenza viruses of multiple origins, as also observed in H5N1 and H9N2 viruses, including directional gene exchange with those H9N2 and H5N1 viruses in poultry. Epidemiological data and phylogenetic analyses revealed that the H6N1 viruses have become established in terrestrial minor poultry, mainly in chukkar and quail. Those viruses have already adapted in this host, as their main replication site is the respiratory tract, similar to the adaptation of Ck/Bei-like H9N2 viruses in chickens and G1-like viruses in quail (5). The H6 HA gene tree clearly demonstrates that the phylogenetic position of all those viruses tested corresponds to the different time points of their evolutionary pathway. This evolutionary pattern is different from that of the H5N1 and H9N2 viruses, which appear to have multiple evolutionary pathways with diverse co-circulating sublineages (1, 5). The mechanism in the ecosystem for those differences remains to be explored. Phylogenetic analyses of the NA and internal genes revealed that those reassortant H6N1 viruses might have acquired their novel gene segments from the established H9N2 virus lineages and their reassortants, or vice versa. Like H9N2 Ck/Bei-like variants, some novel segments of the H6N1 virus genotypes have also been incorporated from the aquatic bird influenza gene pool. Currently, we have difficulty in identifying the sources of those G1-like or W312-like internal gene segments as their genetic origins are the same. Molecular analysis suggested that the N1 NA gene of W312-like virus had a higher dN substitution rate than the HA gene, which reduced

gradually from 1999 to 2003. The higher dN rate of the N1 NA gene and its dynamic change indicates that this gene segment may have been incorporated latest in the virus particle, to which it has gradually adapted. Therefore, H6N1 W312-like virus may not be the precursor of the H5N1/97-like virus, but rather a derived strain which resulted from reassortment between H5N1/97-like and an unknown H6 subtype virus. Although we have systematically analyzed H5N1, H9N2 and H6N1 viruses from 2000 to 2005, the sources of some viral genes that were repeatedly detected in different subtypes, and obviously with a common origin (e.g. the NP gene), remains to be identified. This situation demonstrates a common genesis pathway for the emergence of variants of these three subtypes of influenza virus. Future studies are therefore needed that elucidate more clearly gene precursors to fully understand the ecology and evolution of influenza in southern China.

**Acknowledgements**

This study was supported by the Li Ka Shing Foundation, the National Institutes of Health (NIAID contract HHSN266200700005C), and the Research Fund for Control of Infectious Diseases and the Research Grants Council of the Hong Kong SAR Government.

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