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Genetic characterization of H5N1 avian influenza viruses isolated in southern China during the 2003–04 avian influenza outbreaks

Brief Report

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Summary. The recent H5N1 avian influenza outbreaks in Asia spread over more than 8 countries. It has caused enormous economic loss and grand challenges for the public health. During these breakouts we isolated three strains of H5N1 Avian Influenza Virus (AIV) from chickens and one from duck in different farms of Southern China. We completely sequenced these four AIVs. Molecular characterization demonstrated that these strains retain the reported H5N1 AIV sequence properties relevant to virus virulence and host adaptation. Phylogeny results demonstrated that three of these isolates (except A/Chicken/Guangdong/174/04) were closely linked to other H5N1 AIVs isolated from the recent H5N1 outbreaks in Asia. Six of 8 segments (except PA and M) of A/Chicken/Guangdong/174/04 also shares a close linkage to other H5N1 AIVs isolated from the recent H5N1 outbreaks. However, the PA gene of A/Chicken/Guangdong/174/04 and another H5N1 strain forms a distinct subgroup along with an H6N1 AIV, and the M gene of A/Chicken/Guangdong/174/04 shows a close linkage to some H5N1 AIVs from aquatic species in China. Our findings suggest that a new genotype of AIV (in addition to previous reported ones) was present during the 2003–04 Asian bird flu outbreaks and that continuing virus surveillance of AIVs be conducted to monitor the evolutionary paths of the A/Chicken/Guangdong/174/04-like AIVs.

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The bird flu is caused by the avian influenza virus (AIV), which has 8 genomic segments (HA, NA, PA, PB1, PB2, NP, NS, and M). To date, 15 HA (haemagglutinin) and 9 NA (neuraminidase) subtypes have been reported [15]. The genetic shift and genetic drift lead to a rapid emergence of novel genotypes of the avian influenza viruses during their evolution. The Southern China has been shown to be the avian influenza virus pool for flu outbreaks in history, including H2N2 (1957), H3N2 (1968), H5N1 (1997 & 2003), and H9N2 (1999) [18]. After the 1997 H5N1 outbreak, several small outbreaks were reported in Hong Kong until the recent H5N1 outbreaks [6–8, 14, 19, 26]. The scientists have isolated many H5N1 AIV strains from different avian species in Hong Kong. Multiple genotypes of H5N1 AIVs have been demonstrated to coexist within a single epidemic in Hong Kong [6–8, 11, 13].

The recent avian influenza outbreaks, especially the H5N1 outbreaks in Asia, have caused enormous economic loss and grand challenges for the public health. From November 2003 to March 2004, the H5N1 avian influenza cases were reported in more than 8 countries, including Cambodia, China, Indonesia, Japan, Laos, South Korea, Thailand, and Vietnam (http://www.cdc.gov; http://www.who.it/). Approximately 80 million birds died of the disease or were slaughtered. To date, 32 out of 44 confirmed H5N1 patients died. In Mainland China, 49 areas across China were reported with the H5N1 avian influenza based on the reports from Chinese Minister of Agriculture. Through 53 samples (tracheal and cloacal swaps, trachea, lung, spleen, pancreas, kidney, spleen, and brain) collected from Southern China (Guangdong Province), we isolated 12 strains of H5N1 AIVs. The virus isolation and related experiments are followed the biosafety level 3 (BSL-3) Ag containment procedures [1].

We completely sequenced all 8 gene segments of four strains isolated from different territorial areas: A/Duck/Guangdong/173/04 (H5N1) (Dk/GD/173/04 in brief, Central Guangdong), A/Chicken/Guangdong/174/04 (H5N1) (Ck/GD/174/04 in brief, Western Guangdong), A/Chicken/Guangdong/178/04 (H5N1) (Ck/GD/178/04 in brief, Eastern Guangdong), and A/Chicken/Guangdong/191/04 (H5N1) (Ck/GD/191/04 in brief, Northern Guangdong). The sequence data have been deposited into GenBank with accession numbers AY609309 to AY609316, and AY737285 to AY737308. Here we reported the results from genetic characterization of these four AIVs.

The alignment of the derived amino acid sequences of these four AIVs shows that Ck/GD/174/04 has the same motif of basic amino acids (RRRKKR) as most of other public H5N1 2003–04 outbreak strains between the two domains of HA (HA1 and HA2) [12]. However, Ck/Gd/178/04 and Ck/GD/191/04 have a deletion of nucleotides in this bridge, which results in a loss of an amino acid K at the fifth position within the motif RRRKKR, as in A/duck/China/E319.2/03. In contrast, Dk/Gd/173/04 lost the R residue at the first position within this motif, and its motif is RRKKR. Based on the manual of standards for diagnostic tests and vaccines (OIE, 2000; http://www.oie.int), these basic amino acid motifs indicate that all the 2003–04 outbreak AIVs contain features of Highly Pathogenic AIVs (HPAIVs).

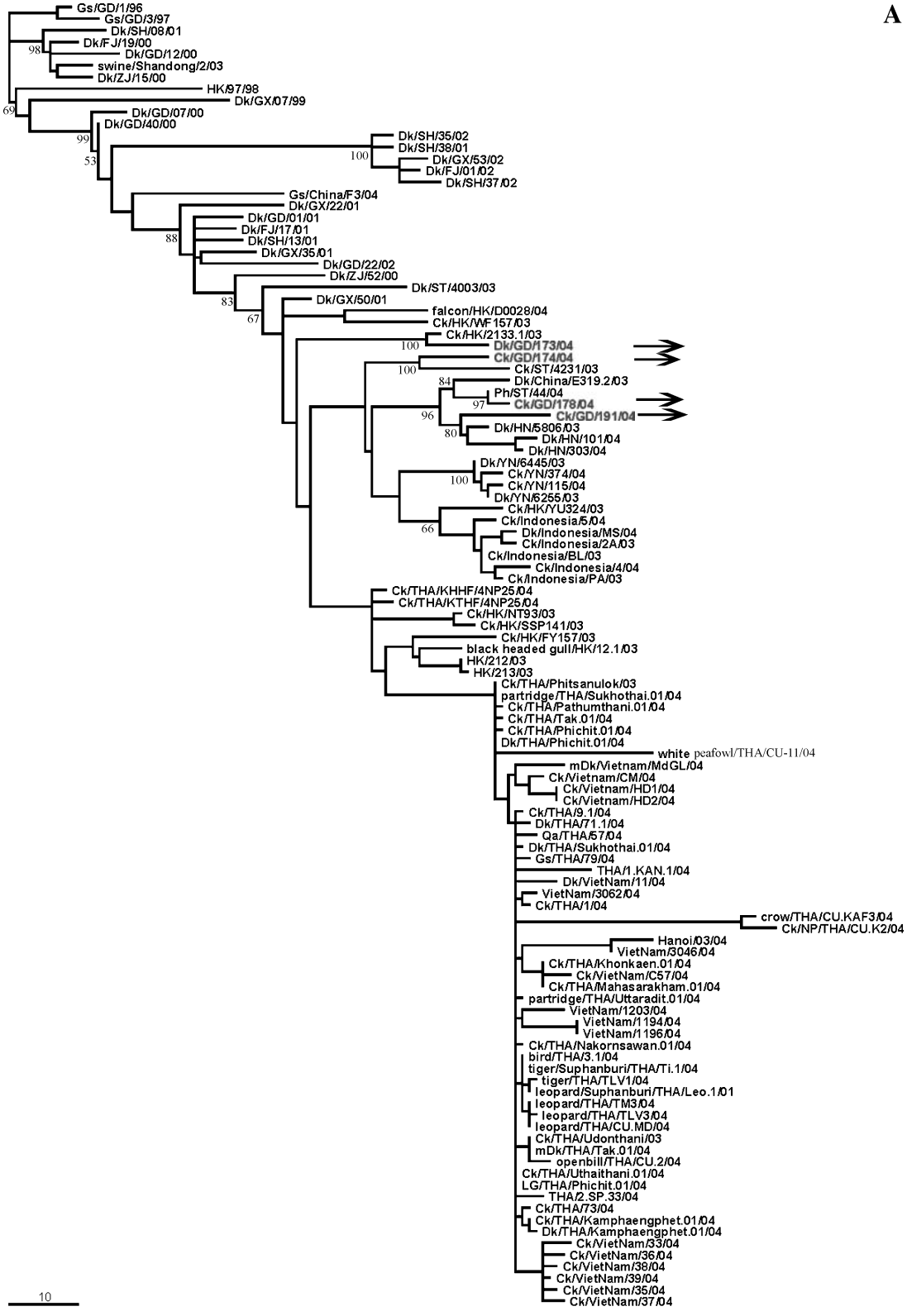
Position of Gln226 to Gly228 of HA was demonstrated to retain affinity for avian cell-surface receptor [9, 10]. All four strains retain this molecular feature as well as other 2003–04 outbreak strains [12]. The other amino acid residues for amino acid binding (residue Tyr98, Trp149, Ile151, His179, Glu186, Leu190) are identical to other 2003–04 outbreak isolates as well as A/HK/156/97. Within the 130 loop, Dk/Gd/173/04, Ck/GD/174/04 and Ck/GD/191/04 have Ser129-Gly130-Val130-Ser131-Ser132, Trp149, which is identical to most 2003–04 outbreak strains (except from Vietnam and Thailand), A/HK/156/97, and A/Goose/GD/1/96. However, Ck/Gd/178/04 has a mutation at position 129 from Ser to Leu (Leu129-Gly130-Val130-Ser131-Ser132), which is identical to some H5N1 isolates from Vietnam and Thailand. It is unknown whether this change will affect HA binding property.

Compared to the strains isolated before 2001, the NS1 genes from all four strains have a conserved deletion (position 80–84) in the middle of peptide, which is similar to other 2003–04 outbreak isolates [7, 12]. The NA genes of these four strains have a similar 20 amino acid deletion in the stalk region (positions 49 to 68) as the other isolates during the 2003–04 outbreaks as well as the viruses isolated from ducks since 2000 in Southern China [2]. This deletion may contribute to the adaptation of AIVs from wild birds to land-based poultry [7].

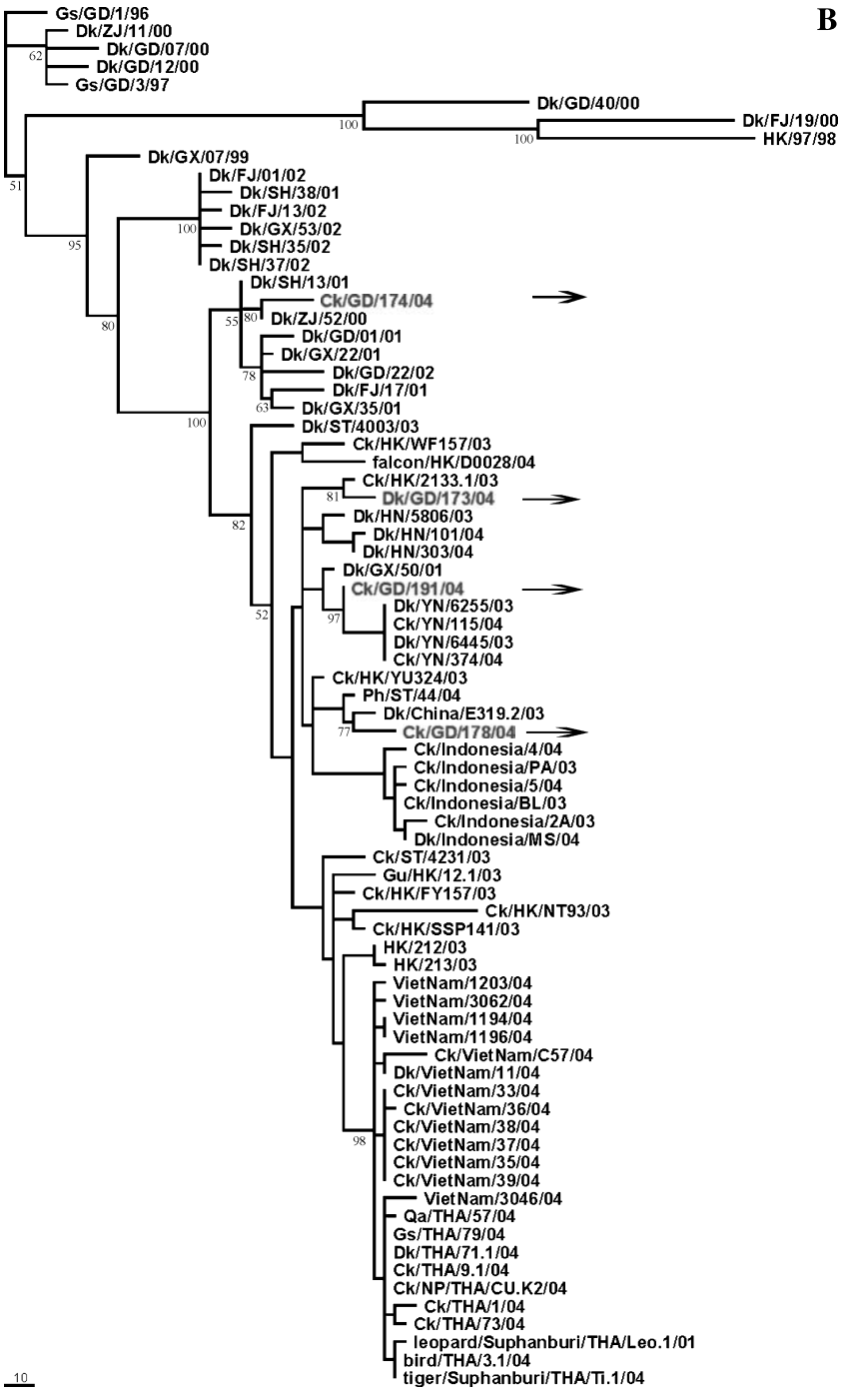
Previous research demonstrated that the point mutation Lys (position 627) of polymerase (PB2) increased the replicate efficiency in mice, which limited the virus replication only in respiratory organs [17]. The position 627 of PB2 in all these four strains are Glu627, which is the same as the avirulent strains of Hong Kong H5N1 on mice and the recent outbreak isolates from chickens. However, some H5N1 viruses isolated from Human in Vietnam were shown with Lys [12]. The Glu92 of NS1 was demonstrated to increase the virulence of the viruses in pigs [16]. All the 4 isolates have Asp at the 92 position of NS1, which are similar to recent outbreak isolates [12].

We performed phylogenetic analyses using nucleotide sequences for 8 genome segments of Ck/GD/174/04 along with other published AIV sequence data in the influenza sequence database (http://www.flu.lanl.gov). The HA genes included all H5 nucleotide sequences and H9N2 entries, NA genes include all of N1 nucleotide sequences and H9N2 entires. PA, NS, PB1, PB2, M, NP include all of nucleotide sequences for serotypes H5N1, H9N2, and H6N1 in the influenza sequence database. The sequence alignments were performed using ClustalW [21], and the phylogenetic analyses were based on PAUP with the tree bisection reconnection branch-swapping option for heuristic search of the maximum parsimony [20]. Phylogenetic analyses were based on different nucleotide sequence lengths of 8 genomic segments varied from 221 to 2,342 bps: PB1 (221 to 2,342 bps), PB2 (280 to 2,341 bps), PA (273 to 2,233 bps), HA (287 to 1,776 bps), NP (238 to 1,565 bps), NA (391 to 1,458 bps), M (231 to 1,027 bps), and NS (266 to 890 bps).

Our phylogeny results demonstrated that all 8 gene segments of Dk/Gd/173/04, Ck/GD/178/04, and Ck/GD/191/04 have a close linkage to other 2003–04 outbreak isolates (Fig. 1) [12]. Six of eight gene segments (HA, NA, NS, PB1, PB2, and NP) of Ck/GD/174/04 are closely linked to other 2003–04 outbreak



Figs. 1A, B (*continued*)



Figs. 1A, B. Phylogenetic analyses of the H5N1 AIVs isolated from Southern China during 2003–04 H5N1 outbreaks. **A** The HA genes of all isolates have a close linkage to other 2003–04 Asian outbreak H5N1 AIVs. **B** The M of Ck/GD174/04 is closer to the H5N1 AIVs isolated from ducks during 2000–2002 in China

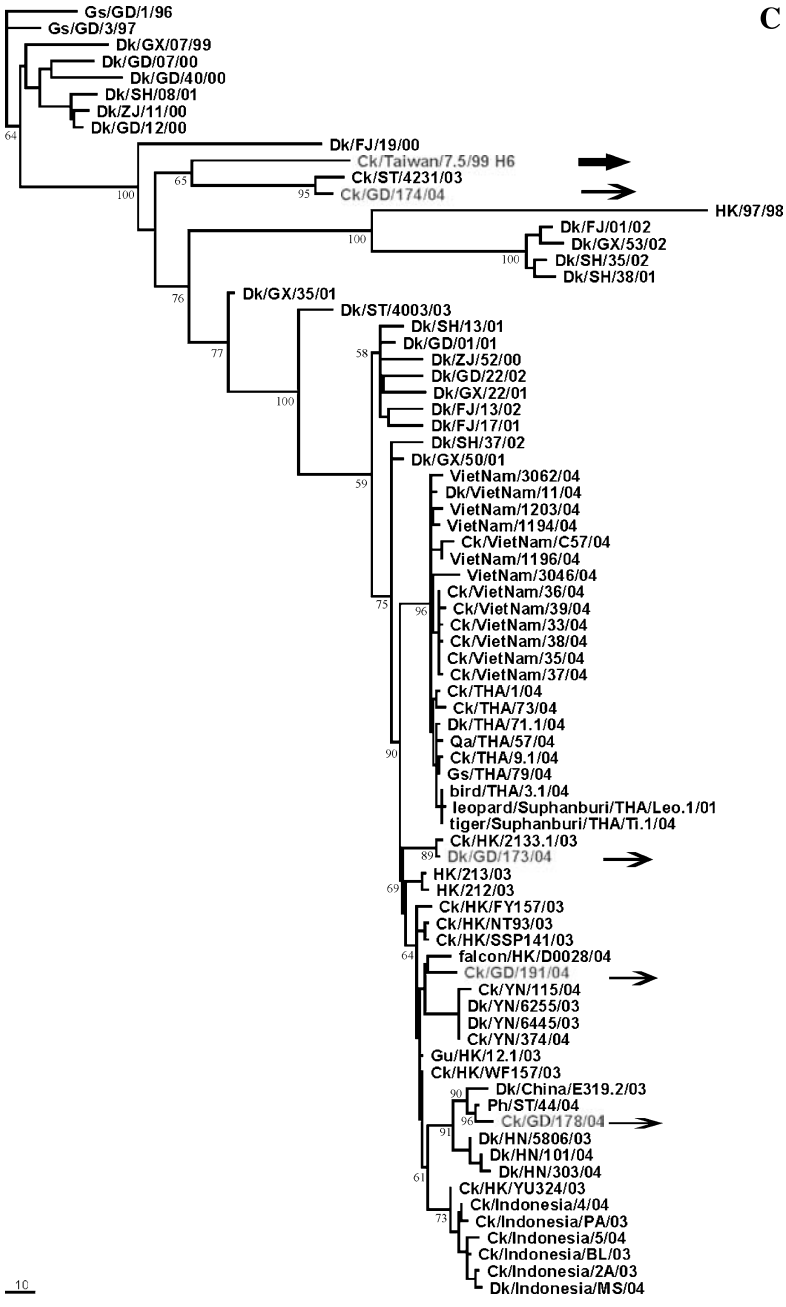


Fig. 1C. The PAs of Ck/GD174/04 and another strain (Ck/ST/4231/03) isolated in 2003 from Guangdong Province form a distinct subgroup, which has a close linage to an H6N1 AIV (Ck/Taiwan/7.5/99). The four isolated H5N1 strains were pointed by narrow arrows, and the H6N1 AIV (Ck/Taiwan/7.5/99) was pointed by a wide arrow. Numbers below branches indicate bootstrap values from 100 replicates. Only bootstrap values for the selected important groups have been included due to space constrains. All the trees were rooted with Gs/GD/1/96. The results of PB2, PB1, NP, NA, and NS are available at http://digbio.missouri.edu/~wanx/flu/. Phylogenetic analyses were based on different nucleotide sequence lengths of 8 genomic segments varying from 221 to 2,342 bps: PB1 (221 to 2,342 bps), PB2 (280 to 2,341 bps), PA (273 to 2,233 bps), HA (287 to 1,776 bps), NP (238 to 1,565 bps), NA (391 to 1,458 bps), M (231 to 1,027 bps), and NS (266 to 890 bps). FJ, Fujian; GD, Guangdong; GX, Guangxi; HK, Hong Kong; HN, Hunan; SH, Shanghai; ST, Shantou; THA, Thailand; TW, Taiwan; YN, Yunnan; and ZJ, Zhejiang; Ck, Chicken; Dk, Duck; Gs, Goose

isolates, so called genotype Z [12], especially those that isolated from duck in Hunan Province, China [12] (Fig. 1A). However, the internal segment M of Ck/GD/174/04 is closer to the H5N1 AIVs isolated from ducks during 2000–2002 in China (Fig. 1B). The internal segment PAs of Ck/GD/174/04 and another strain (Ck/ST/4231/03) isolated in 2003 from Guangdong Province form a distinct subgroup (genotype V), which has a close linage to an H6N1 AIV (Ck/Taiwan/7.5/99) (Fig. 1C). This subgroup is closer to Gs/GD/96-like virus as well. Our phylogenetic analyses also demonstrated that PA of Ck/Taiwan/7.5/99 is far away from other nine H6N1 AIVs in the flu database, which were close to H9N2 linkages isolated from Southern China (data not shown). The results of PB2, PB1, NP, NA, and NS are available at http://digbio.missouri.edu/∼wanx/flu/. These results suggest a new genotype is present within the 2003–04 outbreaks in addition to the genotypes previously reported [12].

The genetic reassortment, which was demonstrated to cause the genomic segment exchanges within the virus subgroups, posed a grand challenge for avian influenza prevention and control [23, 24]. Since 1997, small outbreaks occurred

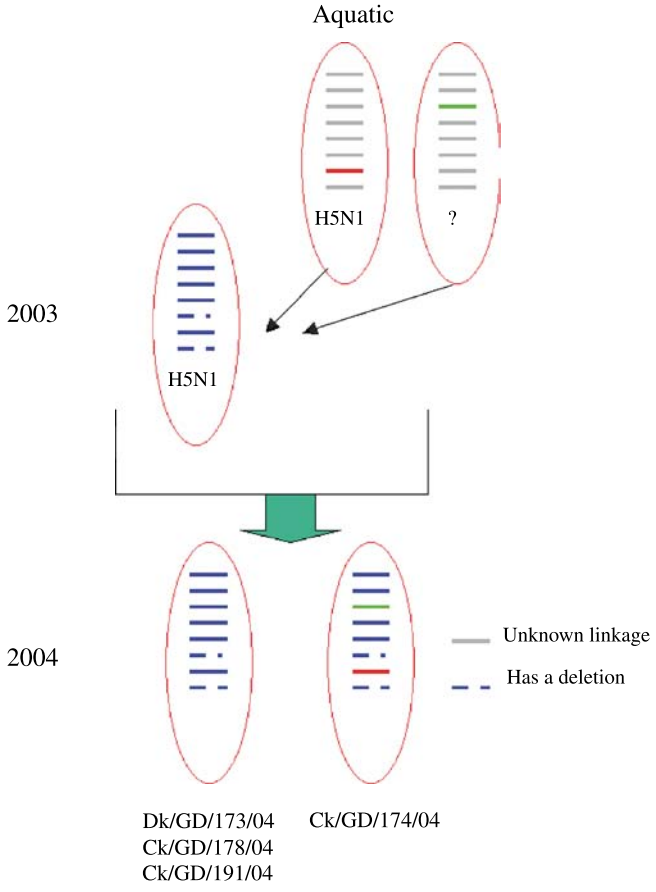


Fig. 2. The evolution model for the four isolates from Southern China during the 2003–04 H5N1 outbreaks. Different lines from top to bottom in each virus particle represent PB2, PB1, PA, HA, NP, NA, M, and NS. The quote denotes as a likely ancestor

almost every winter season in Hong Kong [6–8, 11, 13]. However, the 1997-linkage H5N1 AIVs seem to have disappeared after the slaughtering and clearing program in 1997 [7]. Meanwhile, many new types of H5N1 AIVs were isolated in the past several years [6–8, 22]. The majority of 2003–04 outbreak isolates have been originated from one subgroup from reassortment between territorial AIVs such as H9N2 and H5N1. Previous reports demonstrated that, besides H9N2 [5], H6N1 AIVs have also been isolated very frequently in southeastern China [3]. Our results suggest that a possible active reassortment occurred between H5N1 and H6N1 AIVs and generated novel H5N1 AIVs, which could be potential seeds for future flu pandemics. Figure 2 shows the possible reassortment model for this new subgroup. How prevalence of the viruses closely linked to Ck/GD/174/04 and Ck/ST/4231/03 in birds is still unknown. The further surveillance of AIVs in this area may shed some lights on this question.

In summary, we here genetically characterized four AIVs isolated from Southern China during the 2003–04 bird flu outbreaks. Our results demonstrated that these four strains retain the reported most of H5N1 AIV sequence properties relevant to virus virulence and host adaptation. Phylogenetic analyses demonstrated a new genotype (Ck/GD/174/04-like) was present in the recent outbreaks in addition to the genotypes reported before [12]. As a newly emerged AIV, Ck/GD/174/04-like AIV, could be a potential factor causing future potential flu pandemic. Future surveillance of AIVs should pay an attention to the evolutionary paths of this distinct subgroup.

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