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


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An H6N1 virus, A/teal/Hong Kong/W312/97 (W312), was isolated during the “bird flu” incident in 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 pheasants in 1999; however, the further development of H6N1 viruses has not been investigated since 2001. Here we report influenza virus surveillance data collected in southern China from 2000 to 2005 that show that H6N1 viruses have become established and endemic in minor poultry species and replicate mainly in the respiratory tract. Phylogenetic analysis indicated 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 H6N1/N2 viruses are double, triple, or even quadruple reassortants. Reassortment between a W312-like H6N1 virus and an A/quail/Hong Kong/G1/97 (HK/97)-like H9N2 virus simultaneously generated novel H6N2 subtype viruses that were persistent in poultry. Molecular analyses suggest that W312-like viruses may not be the precursors of HK/97 virus but reassortants from an 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 in influenza viruses in this region.

H6 influenza viruses make up one of the most commonly recognized subtypes in domestic ducks in southern China (24). During the Hong Kong H5N1 “bird flu” incident in 1997, an H6N1 avian influenza virus, teal/Hong Kong/W312/97 (W312), was isolated from a 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 those of highly pathogenic avian influenza H5N1 viruses found in both poultry and humans (15). These findings suggested that a W312-like H6N1 virus might have been involved in the generation of the Hong Kong H5N1 virus (Hong Kong/156/97 [HK/97]) (4, 15). However, it is still unknown how the HK/97-like virus was generated, as an H9N2 virus lineage, represented by quail/Hong Kong/G1/97 (G1), also shared the same internal gene complex as that of W312-like and HK/97-like viruses. Because these three different subtypes of influenza viruses (H5N1, H6N1, and H9N2) were initially detected simultaneously during the Hong Kong bird flu incident (4), the direction of gene flow among these viruses could not be determined.

Subsequent influenza virus surveillance in live poultry markets in Hong Kong from 1998 to 2000 revealed that a W312-like H6N1 virus was still prevalent in minor terrestrial poultry species, such as quail, pheasant, and chukar (4). In the meantime, the primary precursor of the HK/97 virus, goose/Guangdong/1/96 (Gs/GD) H5N1 virus, was also detected in geese imported from mainland China (11). Even though the HK/97-like virus has not been detected by surveillance since the mass culling of poultry in Hong Kong, the continued cocirculation of Gs/GD-like and W312-like viruses in poultry raises the possibility of the reemergence of an HK/97-like virus.

Accumulated information suggests that the influenza virus ecology in southern China, a hypothetical influenza epicenter (25), has increased in complexity in the last 10 years (19, 20). Previous studies demonstrated that G1-like H9N2 viruses had become established in quail (9, 10, 32). A recent investigation also showed that the G1-like virus underwent reassortment, as a donor or receiver, with viruses from multiple virus lineages and that the “pure” G1-like virus has not been detected since 2002 (32). Another H9N2 influenza virus lineage, represented by chicken/Beijing/1/94 (Ck/Bei), has developed into more than 30 different genotypes in southern China. It is well known that novel highly pathogenic avian influenza H5N1 virus reassortants with human infectious potential have continued to emerge in this region and have been transmitted to as many as 60 different countries in Eurasia and Africa (31). All of the novel H5N1 variants have a Gs/GD-like HA but have acquired multiple internal genes from different origins, including both H9N2 virus lineages (19, 32). The cocirculation of H5N1 and G1 and Ck/Bei H9N2 viruses has therefore contributed to increased viral genetic diversity in southern China (9, 10, 19, 32). Since H5N1, H9N2, and H6N1 influenza viruses are all 10402
en endemic and prevalent in poultry species in this region, it is necessary to determine whether H6N1 influenza viruses in terrestrial poultry have been involved in the generation of the recent H5N1 and H9N2 variants.

In the present study, the results of our systematic influenza virus surveillance program in southern China from 2000 to 2005 indicate that H6N1 and H6N2 influenza viruses are prevalent year-round in terrestrial minor poultry species and that H6 influenza viruses are still among the most frequently detected subtypes from domestic ducks (unpublished data). Genetic and antigenic analyses of representative influenza viruses revealed that all H6N1 viruses had W312-like virus HA and neuraminidase (NA) genes and that reassortment between a W312-like H6N1 and a G1-like H9N2 virus also generated the novel H6N2 subtype virus. Reassortment events involving these H6N1 and H6N2 viruses have generated different novel H6N1 and H6N2 genotypes with internal genes from multiple sources, including H5N1 and H9N2 viruses that were cocirculating in poultry in this region. Further molecular analyses also suggest that W312-like H6N1 viruses may not be the precursors of the HK/97-like viruses but were derived by reassortment between an HK/97-like virus and another unidentified H6 subtype virus. These results demonstrate that the continued cocirculation of H5N1, H6N1/N2, and H9N2 influenza viruses in southern China led to frequent reassortment in minor poultry species that greatly increased the genetic diversity in influenza viruses in this region, thereby increasing the chance for development of a human influenza pandemic.

MATERIALS AND METHODS

Sampling and virus isolation. A total of 11,415 swabs were collected by weekly sampling from apparently healthy poultry, including chukar, guinea fowl, partridges, pheasants, and common quail, in live poultry markets in Shantou, Guangdong Province, China, from 2000 to 2005. Minor poultry species in the markets were from local farms or were introduced from neighboring provinces, such as Hunan, Jiangxi, and Zhejiang. Of those swabs, 5,658 were paired swabs of tracheal and cloacal specimens, while for the remaining samples only cloacal or tracheal swabs were taken. In the meantime, our surveillance also included major poultry, such as chickens, geese, and domestic ducks, as well as migratory birds. Virus isolation was conducted using 9- to 11-day-old embryonated chicken eggs as previously described (19, 20).

Antigenic analysis. Virus isolates were subtyped by using standard hemagglutination inhibition (HI) and NA inhibition tests, using a panel of World Health Organization reference antisera (http://www.who.int/cs/resources/publications/en/influenza). Antigenic analysis was conducted using postinfection chicken antisera raised against chicken/Hong Kong/17/77, laughing gull/Delaware/490, teal/Hong Kong/W312/97, quail/Hong Kong/1721-20/99, and quail/Hong Kong/YU1564/00 that were generated in our laboratory. Numerical analysis of HI titers was conducted as previously described, using PRIMER 21, version 5.2.9 (PRIMER-E, Plymouth, United Kingdom) (28). The data were standardized, and the Bray-Curtis coefficient was used to construct a similarity matrix. Hierarchical agglomerative clustering with group-average linking and nonmetric multidimensional scaling were also used to produce two- and three-dimensional ordinations over 100 iterations (28).

Phylogenetic and molecular analyses. One or two virus isolates from each positive sampling occasion were randomly selected for sequence analyses. RNA extraction, cDNA synthesis, and PCR were carried out as previously described (32). Sequencing was performed by using a BigDye Terminator v3.1 cycle sequencing kit on an ABI 3700 genetic analyzer (Applied Biosystems) following the manufacturer’s instructions. DNA sequences were compiled and edited by using Lasergene 6.0 (DNASTAR, Madison, WI) and were aligned with ClustalW software in BioEdit 7.1. Potential N-glycosylation residues were identified using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). All eight gene segments were characterized and phylogenetically analyzed together with virus sequences available in GenBank. The program MrModeltest 2.2 (21) was used to determine the appropriate DNA substitution model and γ-rate heterogeneity, and the generated models were used in all subsequent analyses. Neighbor-joining (NJ) and maximum likelihood trees were constructed by using PAUP* 4.0 (29). Bayesian analysis was performed with MrBayes 3.1 (16) by using two replicates of 1 million generations with six chains, sampled every 100 generations. Phylogenetic supports were calculated by performing 1,000 NJ bootstrap replicates, and Bayesian posterior probabilities were calculated from the consensus of 18,000 trees after excluding the first 2,000 trees as burn-in.

Rates of nonsynonymous substitutions (dN) were determined using the Pamilo-Bianchi-Li method in MEGA3 software (17). The virus groups recruited include HK/99 (Ph/HK/SF39/99, Qa/HK/1721-20/99, and Qa/HK/1721-30/99), HK/00 (Ph/HK/NT261/00, Qa/HK/SF58/00, Qa/HK/SF59/00, Cu/HK/FY295/00, and Ph/HK/FY294/00), ST/00 (Qa/ST/238/00, Qa/ST/388/00, Qa/ST/1808/00, and Qa/ST/1821/00), ST/01 (Qa/ST/1811/01, Qa/ST/2370/01, Cu/ST/3605/01, and Qa/ST/3234/01), ST/02 (Cu/ST/3522/02, Cu/ST/476/02, Cu/ST/823/02, Pa/ST/1403/02, Qa/ST/4680/02), and ST/03 (Qa/ST/1052/03, Qa/ST/1770/03, Qa/ST/3692/ 03, Cu/ST/3978/03, and Qa/ST/4443/03) (see Table 4). Tel/HK/W312/97 was used as the representative virus to compare with virus groups isolated from 1999 to 2003. In some instances, it was not possible to calculate the dN rate for the PA, NP, and NS genes due to reassortment between different lineages.

Nucleotide sequence accession numbers. The nucleotide sequences obtained from this study are available from GenBank under accession numbers EU049892 to EU050635.

RESULTS

Epidemiology. Systematic surveillance of minor poultry species, including quail, chukar, guinea fowl, partridges, and pheasants, in live poultry markets from 2000 to 2005 resulted in 414 subtype H6 viruses out of a total of 11,415 samples

TABLE 1. Isolation of H6 influenza viruses from terrestrial poultry in southern China during 2000 to 2005a

<table>
<thead>
<tr>
<th>Yr of isolation</th>
<th>No. (%) of H6-positive isolates</th>
<th>Total sample no.</th>
<th>No. of viruses sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>14 (3.5)</td>
<td>400</td>
<td>7</td>
</tr>
<tr>
<td>2001</td>
<td>48 (4.3)</td>
<td>1,124</td>
<td>7</td>
</tr>
<tr>
<td>2002</td>
<td>24 (2.1)</td>
<td>1,140</td>
<td>9</td>
</tr>
<tr>
<td>2003</td>
<td>38 (2.6)</td>
<td>1,917</td>
<td>12</td>
</tr>
<tr>
<td>2004</td>
<td>238 (6.4)</td>
<td>3,705</td>
<td>43</td>
</tr>
<tr>
<td>2005</td>
<td>5 (1.7)</td>
<td>3,129</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>414 (3.6)</td>
<td>11,415</td>
<td>93</td>
</tr>
</tbody>
</table>

a Based on types of poultry, the overall isolation rates over 6 years were as follows: chukar, 150/1,692 samples (8.9%); guinea fowl, 16/511 samples (3.1%); partridge, 31/1,100 samples (2.8%); pheasant, 18/1,436 samples (1.3%); quail, 195/4,601 samples (4.3%); and turkey, 4/108 samples (3.7%).
collected (overall isolation rate, 3.6%). Most of these isolates were of subtype H6N1, and 26 were H6N2 viruses. Those viruses were prevalent year-round, but with a higher isolation rate during the winter since 2004 (Fig. 1). However, the isolation rate for each year varied markedly, from 1.7% to 6.4%. Of the minor poultry species, chukar and quail provided the main body of H6N1 isolates and had remarkably high isolation rates, of 8.9% and 4.2%, respectively (Table 1). In contrast, only a single H6N1 virus was isolated from 8,788 chicken and 2,530 silky chicken specimens collected during the same period and from the same markets. A total of 402 of 414 (97%) subtype H6 viruses were isolated from tracheal swabs, and only 8 were...
isolated from cloacal swabs and 4 were isolated from fecal material. These findings suggest that H6N1 viruses replicated mainly in the respiratory tracts of those birds.

Antigenic analysis. Antigenic analyses using different postinfection chicken antisera revealed three distinct antigenic groups (Fig. 2). The viruses isolated from domestic and migratory ducks in southern China from the 1970s to 2003 reacted against teal/HK/W312/97 and Qa/HK/1721-20/99 (Table 2). The fourth lineage (contemporary 2) contains all H6 viruses that are prevalent in terrestrial poultry in several regions, e.g., Taiwan and South Africa. The second lineage (early) contains viruses isolated from 1997 to 2004 from domestic ducks and geese in southern China (Fig. 3A). The second lineage (early) contains mainly viruses isolated from 1976 to 1977, but 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 virus gene pool. Within this lineage, subtype H6 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 teal/HK/W312/97 and hence are referred to as “W312-like.”

Phylogenetic analysis of the H6 HA gene revealed that all viruses were separated into the American and Eurasian gene pool. Within the Eurasian gene pool, four major lineages could be recognized, including the contemporary 1 and 2, early, and aquatic lineages. The contemporary 1 lineage contains only viruses isolated from 1997 to 2004 from domestic ducks and geese in southern China (Fig. 3A). The second lineage (early) contains mainly viruses isolated from 1976 to 1977, but 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 virus gene pool. Within this lineage, subtype H6 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 teal/HK/W312/97 and hence are referred to as “W312-like.”

Phylogenetic analysis of N1 NA genes also showed that all H6N1 viruses isolated from minor poultry species in southern China were closely related to and derived from the teal/Hong Kong/W312/97 virus (Fig. 3B). 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 chickens in Taiwan also clustered with the same virus, Dk/HK/3461/99, which suggests the establishment of that virus lineage in Taiwanese poultry.

In the NA gene tree, all N2 genes of H6N2 viruses tested, except for Qa/ST/892/00, were derived from chicken/Beijing/1/94-like (Ck/Bei-like) viruses that have circulated in this region since the mid-1990s (Fig. 3C). The N2 gene of Qa/ST/892/00 was derived from the quail/Hong Kong/G1/97-like virus (G1-

### TABLE 2. HI titers from antigenic analysis of influenza A H6 viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>Ck/HK/17/77</th>
<th>Laughing gull/DE/4/90</th>
<th>Teal/HK/W312/97</th>
<th>Qa/HK/1721-20/99</th>
<th>Qa/HK/YU1564/00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dk/HK/73/76</td>
<td>H6N1</td>
<td>320</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>MDk/JX/7787/03</td>
<td>H6N1</td>
<td>320</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Dk/JX/227/03</td>
<td>H6N2</td>
<td>160</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Dk/HK/1037-2/98</td>
<td>H6N2</td>
<td>80</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Dk/HK/323/98</td>
<td>H6N2</td>
<td>40</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Dk/HK/1037-1/98</td>
<td>H6N2</td>
<td>160</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>MDk/JX/6845/03</td>
<td>H6N1</td>
<td>320</td>
<td>&lt;</td>
<td>160</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>Teal/HK/W312/97</td>
<td>H6N1</td>
<td>320</td>
<td>&lt;</td>
<td>1,280</td>
<td>640</td>
<td>1,280</td>
</tr>
<tr>
<td>Qa/ST/238/00</td>
<td>H6N1</td>
<td>640</td>
<td>&lt;</td>
<td>1,280</td>
<td>640</td>
<td>1,280</td>
</tr>
<tr>
<td>Ph/HK/NT261/00</td>
<td>H6N2</td>
<td>320</td>
<td>&lt;</td>
<td>160</td>
<td>160</td>
<td>640</td>
</tr>
<tr>
<td>Qa/HK/YU1564/00</td>
<td>H6N2</td>
<td>320</td>
<td>40</td>
<td>320</td>
<td>1,280</td>
<td>2,560</td>
</tr>
<tr>
<td>Qa/ST/1039/02</td>
<td>H6N2</td>
<td>640</td>
<td>80</td>
<td>320</td>
<td>640</td>
<td>1,280</td>
</tr>
<tr>
<td>Qa/ST/1770/03</td>
<td>H6N1</td>
<td>1,280</td>
<td>40</td>
<td>320</td>
<td>640</td>
<td>1,280</td>
</tr>
<tr>
<td>Cu/ST/4360/03</td>
<td>H6N1</td>
<td>1,280</td>
<td>40</td>
<td>320</td>
<td>640</td>
<td>1,280</td>
</tr>
<tr>
<td>Qa/ST/4443/03</td>
<td>H6N1</td>
<td>640</td>
<td>40</td>
<td>160</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>Qa/ST/1811/01</td>
<td>H6N1</td>
<td>1,280</td>
<td>40</td>
<td>320</td>
<td>1,280</td>
<td>&lt;</td>
</tr>
<tr>
<td>Qa/ST/2766/04</td>
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<td>1,280</td>
<td>40</td>
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<td>320</td>
<td>&lt;</td>
</tr>
<tr>
<td>Cu/ST/5651/04</td>
<td>H6N1</td>
<td>1,280</td>
<td>40</td>
<td>160</td>
<td>320</td>
<td>&lt;</td>
</tr>
<tr>
<td>Cu/ST/5344/04</td>
<td>H6N1</td>
<td>640</td>
<td>&lt;</td>
<td>80</td>
<td>160</td>
<td>&lt;</td>
</tr>
<tr>
<td>Qa/ST/7103/04</td>
<td>H6N1</td>
<td>1,280</td>
<td>&lt;</td>
<td>160</td>
<td>80</td>
<td>&lt;</td>
</tr>
<tr>
<td>Ph/ST/7503/04</td>
<td>H6N1</td>
<td>640</td>
<td>&lt;</td>
<td>160</td>
<td>80</td>
<td>&lt;</td>
</tr>
<tr>
<td>Qa/ST/22124/05</td>
<td>H6N1</td>
<td>640</td>
<td>&lt;</td>
<td>160</td>
<td>80</td>
<td>&lt;</td>
</tr>
<tr>
<td>Ph/ST/114/05</td>
<td>H6N1</td>
<td>640</td>
<td>&lt;</td>
<td>160</td>
<td>80</td>
<td>&lt;</td>
</tr>
<tr>
<td>Qa/ST/6825/05</td>
<td>H6N2</td>
<td>640</td>
<td>&lt;</td>
<td>80</td>
<td>40</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

* HI titer of <40 (serum dilution of 1:40). Virus abbreviations are listed in the legend to Fig. 3.
like) lineage (9). These findings suggest that reassortment has already occurred between H6N1 and H9N2 virus lineages.

**Phylogenetic analysis of internal genes.** Phylogenetic analysis of the PB2 gene demonstrated that most of the H6N1/N2 viruses clustered with the G1-like or W312-like lineage, while a few viruses clustered with recently identified H9N2 Ck/Beijing/1/94-like variants (Fig. 4A) (32). None of the PB2 genes from the H6 viruses tested were derived from Ck/Beijing-like or H5N1 goose/Guangdong/1/96 (Gs/GD)-like viruses (Fig. 4A). These results suggest that further reassortment between H9N2 and H6N1 viruses occurred after the Ck/Beijing-like H9N2 variants were generated. It is noteworthy that the Taiwanese H6N1 viruses also clustered into different sublineages but that each of them was clearly derived from the viruses resident in domestic ducks from southern China (Fig. 4A). A similar phylogenetic relationship was observed in the PA phylogeny. Most of the H6N1/N2 viruses tested had a G1-like PA gene. The PA genes of all other H6 viruses isolated from 2000 to 2005 clustered with cocirculating subtype H5 and H9 viruses, indicating that the PA genes of the H6 viruses were derived from resident.

**FIG. 3.** Phylogenetic relationships of the H6 HA (A), N1 NA (B), and N2 NA (C) genes of representative influenza A viruses. Trees were generated by the NJ method in the PAUP* program. Numbers above and below branches indicate NJ bootstrap values and Bayesian posterior probabilities, respectively. Analysis was based on nucleotides 49 to 1032, 1 to 1353, and 1 to 1229 of the H6 HA, N1 NA, and N2 NA gene segments, respectively. The H6 HA and N2 NA trees are rooted to turkey/Canada/63 (H6N2), and the N1 NA tree is rooted to Wisconsin/1/33 (H1N1). Virus subtypes are indicated in parentheses, and viruses with no subtype designation are of the H6N1 subtype. Viruses in blue are viruses characterized in this study, and viruses in red are prototype viruses. Genotypes for viruses characterized in this study are indicated in parentheses along with their clade designations. Abbreviations: Aq, aquatic; AB, aquatic bird; AS, African starling; BS, Bewick's swan; Bei, Beijing; Ck, chicken; Ck/Be, chicken/Beijing/1/94-like; Cu, chukar; Dk, duck; EU, Europe; FJ, Fujian; FPV, fowl plague virus; G1, Qa/HK/G1/97-like; GD, Guangdong; Gf, guinea fowl; Gs, goose; Gs/GD, goose/Guangdong/1/96-like; GX, Guangxi; GY, Guiyang; HK, Hong Kong; HLJ, Heilongjiang; HN, Hunan; JX, Jiangxi; MDk, migratory duck; Pa, partridge; Ph, pheasant; Qa, quail; SA, South Africa; SCK, silky chicken; ST, Shantou; Sw, swine; Tw, Taiwan; Ty, turkey; VNM, Vietnam; WDk, wild duck; W312, teal/HK/W312-like. Bar, 0.01 substitution per site.
viruses in the gene pool in this region. It is noted that the H6N1 viruses from Taiwan also had multiple sources for their PA genes (Fig. 4C). Taken together, these findings suggest that the PA genes of the currently cocirculating H6N1/N2 viruses were derived from the aquatic gene pool via H9N2 viruses.

Phylogenetic analysis also revealed that the H6N1/N2 viruses had NP genes of multiple origins. In addition to the G1-like and Ck/Bei-like H9N2 NP genes, some of the NP genes of the viruses were closely related to H9N2 and H5N1 reassortants recognized from 1998 to 2005 (Fig. 5A). These findings provide updated evidence of the interaction between the three major subtypes (H5N1, H9N2, and H6N1) of influenza viruses found in poultry in southern China.

Like recent observations with H9N2 reassortant viruses (20, 32), the matrix (M) and nonstructural (NS) protein genes of the H6N1/N2 viruses showed much less genetic diversity than the other genes and belonged to either the G1-like or Ck/Bei-like H9N2 lineage (Fig. 5B and C). It has been noted that H6N1/N2 viruses isolated from 1999 to 2000 had mainly G1-like or W312-like NS genes, which have been replaced by a Ck/Bei-like NS gene since 2001. Phylogenetic analysis of the M and NS genes also revealed that the H6N1 viruses from Taiwan...
had multiple sources and belonged to different sublineages from the influenza virus gene pool (Fig. 5B and C).

**Genotyping.** As multiple reassortants of H6N1/N2 viruses are identified, it is necessary to have a systematic nomenclature for the recognition of viruses with different gene constellations. Here we propose the assignment of H6N1/N2 virus genotypes as follows. All H6N1 viruses with W312-like HA and NA genes will be designated the genotype A series, while the H6N2 viruses that have a W312-like HA but a Ck/Bei-like or G1-like N2 NA gene will be designated the genotype B series (Fig. 6).

Pure W312-like viruses have not been detected since 2001, while a group of new variants that incorporated gene segments from aquatic and Ck/Bei-like H9N2 viruses and their variants (genotypes A2 and A3) were generated the same year. The A2 genotype continued to be persistent in terrestrial minor poultry species during our surveillance (Fig. 6). However, every year, several novel genotypes were generated and the genetic diversity of H6N1 viruses increased.

It is interesting that an H6N2 virus that shared seven gene segments with a G1-like virus (genotype B1) was detected during 2000 and that the reassortant H6N2 viruses incorporated Ck/Bei-like virus gene segments as early as 2000 (Table 3 and Fig. 6). The number of Ck/Bei-like gene segments within H6N2 viruses increased over the course of our surveillance. Several H6N2 genotypes (B2, B3, B6, and B7) with gene segments from aquatic sources were also recognized from 2000 to 10408 CHEUNG ET AL. J. VIROL.

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FIG. 5. Phylogenetic relationships of nucleoprotein (NP) (A), matrix (M) (B), and nonstructural (NS) protein (C) genes of representative influenza A viruses isolated in Asia. Trees were generated by the NJ method in PAUP*. Numbers above and below branches indicate NJ bootstrap values and Bayesian posterior probabilities, respectively. Analysis was based on the following nucleotides: NP, 22 to 915; M, 70 to 835; and NS, 1 to 815. The NP, M, and NS trees are rooted to equine/Prague/1/56 (H7N7). Viruses characterized in this study are highlighted in blue. Viruses in red represent prototype viruses. Genotypes for viruses characterized in this study are indicated in parentheses along with their clade designations. “Mix” indicates co-circulating H5N1, H6N1/N2, and H9N2 subtype viruses. Abbreviations are listed in the legends to Fig. 3 and 4.

Bar, 0.01 substitution per site.
2004, but they were transient and not persistent in poultry in this region (Fig. 6).

Genetic reassortment between H5N1, H9N2, and H6N1/2 viruses occurred as early as 1996–1997 (Fig. 6). Interestingly, since 2001, the “pure” H5N1 Gs/GD-like, H9N2 G1-like, and H6N1 W312-like viruses were replaced by novel reassortants (8, 19, 32). The novel H5N1 variants reassorted with H9N2 viruses and further reassorted with the H6N1/N2 viruses, as revealed in this study. These genotypic designations will help to trace the dynamic generation and evolution of the different subtype virus lineages (Fig. 6) (6, 27, 28).

Molecular characterization. Most of the viruses characterized in this study maintained the previously identified subtype H6 amino acid motif at the HA cleavage site (PQIETR↓G). Two viruses isolated from chukar during 2003 (Cu/ST/3809/03 and Cu/ST/3978/03) had a Thr-to-Ala substitution at position 11002 from the cleavage site (PQIEAR↓G), while one isolate (Ph/ST/1297/04) had a Gln-to-Arg substitution at position 11005 from the cleavage site (PRIETR↓G). The receptor binding pocket of HA1 retains the amino acid residues Gln 224 and Gly 226 (H6 numbering of the mature protein is used throughout), as also seen in H5 HA, which preferentially binds to α-2,3-NeuAcGal receptors (12), except for the HAs of two viruses isolated in 2001 (Ck/ST/2197/01 and Qa/ST/4770/01) that had a Gly226Ala substitution. An Asp insertion at position 157 of HA, which is present in all subtype H6 isolates from terrestrial poultry (4), was retained in all but two viruses (Qa/ST/2185/00 and Cu/ST/3322/04) that had an Asp157Gly substitution. Most of the viruses characterized had six potential glycosylation sites (positions 12 or 13, 25, 169, 293, 298, and 485), as previously recognized (4), although an additional potential glycosylation site at position 165 was also detected in 2004.
the majority of viruses isolated since 2004 due to a Lys167Asn mutation.

The NAs of all H6N1 isolates had a 19-amino-acid deletion in the stalk region, similar to those of HK/97-like viruses, while the majority of viruses isolated since 2004 due to a Lys167Asn mutation.

Only one virus (Ty/ST/752/04) had an Asn 31 substitution in the M2 protein that is associated with amantadine resistance. No viruses tested had the Lys 627 mutation in the PB2 protein, while nine viruses isolated in 2000–2001 had the NS1 mutation; both mutations are associated with increased virulence of influenza viruses (7, 14, 23).

Analysis of \( d_N \) rates of H6N1 influenza viruses from Hong Kong and Shantou in different years showed that the \( d_N \) rate was higher for the N1 gene than for all other genes, including the HA gene, with the exception of the NS1 gene (Table 4). The \( d_N \) rates for both the HA and NA genes have gradually reduced over time since 1999 (Table 4). These findings suggest that the N1 gene may have been incorporated into the H6N1 viral particle later than the other genes. The high \( d_N \) rate for the NS1 gene was also observed in a previous study and may reflect host adaptation (4, 23). Thus, the W312-like H6N1 virus might be derived from reassortment between the HK/97 H5N1 virus and another unknown H6 virus rather than being a precursor to the HK/97 virus (15).

### DISCUSSION

As the world is preparing for a possible influenza pandemic, in comparison to H5N1 and H9N2 influenza viruses, subtype H6 influenza viruses have not been investigated systematically since the Hong Kong “bird flu” incident. Here we have provided the first comprehensive surveillance data for H6N1/N2 subtype viruses in poultry in this region from 2000 to 2005. The findings of the present study revealed that H6N1/N2 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 the same host species, resulting in the emergence of novel virus lineages.
multiple origins, as also observed for H5N1 and H9N2 viruses, including directional gene exchange with H9N2 and H5N1 viruses in poultry, which generated novel reassortant viruses every year along with novel subtype H6N2 viruses.

The findings from the epidemiological data and phylogenetic analyses revealed that H6N1/N2 viruses have become established in terrestrial minor poultry species, mainly in chukar and quail. The viruses have already adapted in these hosts, 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 (32). The H6 HA gene tree clearly demonstrates that the phylogenetic positions of all viruses tested correspond to the different time points of their evolutionary pathway (Fig. 3A). This evolutionary pattern is different from that of the H5N1 and H9N2 viruses, which appear to have multiple evolutionary pathways with diverse cocirculating sublineages (3, 28, 32). The mechanism in the ecosystem for this difference remains to be explored.

Phylogenetic analyses of the NA and internal genes revealed that the reassortant H6N1/N2 viruses might have acquired their novel gene segments from the established H9N2 virus lineages and their reassortants, or vice versa (Fig. 3 to 5). However, it remains unknown why the same virus gene segments are incorporated in these three influenza virus subtypes. Like H9N2 Ck/Bei-like variants, some novel segments of the H6N1/N2 virus genotypes have also been incorporated from the aquatic bird influenza virus gene pool. The cocirculation of multiple distinct lineages of H5, H6, and H9 subtype viruses has greatly magnified the genetic diversity and complexity of influenza virus ecology in this region. Currently, we have difficulty in identifying the sources of the 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 viruses had a higher $d_N$ substitution rate than that of the HA gene, which reduced gradually from 1999 to 2003. The higher $d_N$ rate of the N1 NA gene and its dynamic change indicate 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 subtype H6 virus. The detection of an H6N2 virus (Qx/ST/892/00; genotype B1) that also shares seven of eight gene segments with the G1-like H9N2 virus provides additional evidence for the interaction between G1-like and W312-like viruses during this time (Fig. 6).

Although we have systematically analyzed H5N1, H9N2, and H6N1 viruses isolated from 2000 to 2005, the sources of some viral genes that were repeatedly detected in different subtypes, obviously with a common origin (e.g., the NP gene), remain to be identified (Fig. 5A). This situation demonstrates a common genesis pathway for the emergence of variants of these three subtypes of influenza virus. Future studies are therefore needed to elucidate gene precursors more clearly to fully understand the ecology and evolution of influenza virus in southern China.

Interspecies transmissions of subtype H6 viruses from aquatic birds to terrestrial poultry are usually associated with low-mortality disease outbreaks in the new hosts (1, 18, 30). The present study revealed that interspecies transmission events are not rare (18) and that these events increase the genetic diversity of influenza viruses in terrestrial poultry. These novel introductions provide new sources for further reassortment between H6 and other virus lineages that are already established in terrestrial poultry. These novel reassortants may reversely transmit back to aquatic birds, as demonstrated for H9N2 viruses (20). The index isolate of the H6N1/N2 lineage, teal/Hong Kong/W312/97, was originally isolated from a teal, a typical aquatic bird.

As yet, we still do not have any reports of human infection cases with subtype H6 viruses. One possibility is that the H6 subtype is not able to infect humans or that this subtype causes no or few disease symptoms, making detection unlikely. However, the present study demonstrated that the replication site of current H6N1/N2 viruses is the respiratory tract of terrestrial poultry, and genetic analysis revealed several residue substitutions related to tissue tropism in the HA protein.

Our influenza virus surveillance in southern China during the past 10 years has provided insight into the ecology and evolution of H5N1 and H9N2 viruses in southern China (2, 5–8, 10, 19, 26, 32). The present study demonstrates the cocirculation of different subtypes of virus in southern China and the frequent introduction of gene segments from aquatic birds into terrestrial poultry. However, the sources of some gene segments that continually reassorted among H5N1, H6N1/N2, and H9N2 viruses remain to be identified. This study further highlights the direct benefit of systematic surveillance of influenza viruses in both animal and human populations in preparing for a possible pandemic.

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