Characterization of H9N2 influenza viruses isolated from vaccinated flocks in an integrated broiler chicken operation in eastern China during a 5 year period (1998–2002)

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In the current study, we characterized H9N2 influenza viruses isolated from vaccinated flocks in an integrated broiler chicken operation during a 5 year period (1998–2002). Phylogenetic analysis of the 8 genes of 11 representative viruses showed that they all shared high similarity to that of the first isolate, A/Chicken/Shanghai/F/1998 (Ck/SH/F/98), and clustered to the same lineages. Furthermore, all 11 viruses had a 9 nt deletion between positions 206 and 214 of the neuraminidase gene. These genetic characteristics strongly suggest that these viruses are descendants of the first isolate. In addition, our study also showed that the H9N2 viruses circulating in the operation during this 5 year period were evolving, as shown by antigenic variations between viruses manifested by reactivity with polyclonal antisera and monoclonal antibodies, by haemagglutination with erythrocytes from different animals, by amino acid differences in haemagglutinin and neuraminidase proteins, and by variation in their ability to replicate in the respiratory and intestinal tract and to be transmitted by aerosol. Phylogenetic analysis revealed that the internal genes from some H5N1 viruses of duck origin clustered together with those from H9N2 virus and that the RNP genes of these H5N1 viruses isolated after 2001 are more closely related to the genes of the Ck/SH/F/98-like H9N2 viruses, indicating more recent reassortment events between these two subtypes of viruses. Continuous surveillance of influenza virus in poultry and waterfowl is critical for monitoring the genesis and emergence of potentially pandemic strains in this region.

INTRODUCTION

Wild birds, including waterfowls, gulls and shorebirds, are the natural reservoirs for influenza A viruses, in which these viruses are thought to be in evolutionary stasis (Alexander, 2000; Webster et al., 1992). However, when avian influenza viruses are transmitted to new hosts, such as terrestrial poultry or mammals, they evolve rapidly and may cause occasional severe systemic infection with high morbidity (Ludwig et al., 1995; Suarez, 2000). Despite the fact that avian influenza virus infection occurs commonly in chickens, it is unable to persist for a long period of time due to control efforts and/or a failure of the virus to adapt to new hosts (Suarez, 2000). However, increasing numbers of outbreaks in poultry have occurred in the past 20 years, suggesting that avian influenza virus can infect and spread in aberrant hosts for an extended period of time (Liu et al., 2003; Li et al., 2005; Webby et al., 2003; Choi et al., 2004; Lee et al., 2006). In Hong Kong and mainland China, the H9N2 subtype of influenza virus has been isolated from pigs and humans with influenza-like illness during the past decade (Peiris et al., 1999, 2001; Saito et al., 2001; Xu et al., 2004). These findings confirm that the H9N2 subtype virus is ubiquitous in China and poses a great threat to both economics and public health.

Infections with the H9N2 subtype of the avian influenza virus (AIV) in domestic poultry, especially in chickens,
have been reported frequently in China and other Asian countries since the late 1990s (Naeem et al., 1999; Guo et al., 2000; Liu et al., 2003; Choi et al., 2004; Perk et al., 2006; Lee et al., 2006; Xu et al., 2007). In 1994, for the first time, H9N2 virus was isolated from diseased chickens in Guangdong province, China. Since then, this subtype of influenza virus has been found in domestic poultry in other provinces in China (Chen et al., 1994; Guo et al., 2000; Liu et al., 2003; Choi et al., 2004; Li et al., 2005; Xu et al., 2007). In late October 1998, a severe outbreak of H9N2 avian influenza occurred in 3–5-week-old broiler chickens in a large integrated broiler chicken operation in eastern China; a virus designated A/chicken/Shanghai/F/98 (Ck/SH/F/98) was isolated and characterized (Liu et al., 2003; Lu et al., 2005). To control infection and transmission of H9N2 viruses in the operation, an intensive vaccination program was implemented in the following years with a killed oil-emulsion vaccine based on Ck/SH/F/98. Despite the vaccination effort and the strict biosecurity measures employed, H9N2 viruses were still isolated from chickens during the next 5 years.

In this study, our goal was to determine whether the circulating H9N2 viruses isolated from the operation during a 5 year period were from a single introduction or from repeated reintroduction, by using genetic analysis, and to explore the extent of antigenic variation among these viruses.

**Table 1.** H9N2 influenza A viruses isolated from an integrated chicken operation during a 5 year period

All virus strains were isolated from sick chickens. ND, Not determined.

<table>
<thead>
<tr>
<th>Virus Abbreviation</th>
<th>Date of isolation</th>
<th>Titre (log_{10} EID_{50} per 0.2 ml)</th>
<th>Pathogenicity*</th>
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<tr>
<td>A/chicken/Shanghai/2/1999 Ck/SH/2/99</td>
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<td>Low</td>
</tr>
<tr>
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<td>7.50</td>
<td>Low</td>
</tr>
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<td>ND</td>
<td>ND</td>
</tr>
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<td>6.50</td>
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</tr>
<tr>
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<td>Mar 2001</td>
<td>ND</td>
<td>ND</td>
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</tr>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>Apr 2001</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>Apr 2001</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>A/chicken/Shanghai/12/2001 Ck/SH/12/01</td>
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</tr>
<tr>
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<td>ND</td>
</tr>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
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<td>Apr 2002</td>
<td>8.50</td>
<td>Low</td>
</tr>
</tbody>
</table>

*Pathogenicity for 6-week-old SPF chickens.
Monoclonal antibodies (mAbs). A panel of mAbs to the HA of two H9N2 strains were used: two mAbs (2A1 and 5D3) to Ck/SH/F/98 and four mAbs (2B12, 5B1, 4A2 and 8E6) to A/Chicken/Shanghai/14/2001 (Ck/SH/14/01) which were prepared as described by Kaverin et al. (2004).

Transmission experiments in chickens. For the transmission experiments, 10 or 11 5-week-old SPF white Leghorn chickens for each virus were divided into: (i) infected group (three chickens), (ii) physical contact group (three to four chickens) and (iii) aerosol contact group (four chickens) (Table 3). The physical contact group was raised in the same cage as chickens from the infected group; they were put in the cage about 30 min after inoculation of the infected group. The aerosol contact group was placed in a cage directly adjacent to the infected group with a distance of 100 cm between cages. The infected group was inoculated orally, intraanally or intratracheally with 107 EID50 ml−1 virus (about 100 chicken infectious doses) (Reed & Muench, 1938). Tracheal and cloacal swabs were collected 3, 5 and 7 days post-inoculation, respectively, and AIVs were titrated for infectivity in embryonated chicken eggs. Meanwhile, birds were observed daily for signs of disease within 21 days.

RT-PCR and sequence analyses. Viral RNA was extracted directly from the allantoic fluid of the first passage virus with the Trizol LS kit (Invitrogen) to reduce the effects of passage history in embryonated chicken eggs on additional mutations. Reverse transcription was conducted with Uni-12 primer and PCR was performed with primers specific for each of the eight RNA segments (primer sequences are available on request). PCR products were purified with a gel DNA purification kit (TakaRa) and sequenced using an ABI Prism dye terminator cycle sequencing kit on an ABI 377 DNA Sequencer (Perkin-Elmer).

The nucleotide sequences were analysed with the Seqman module and the nucleotide and deduced amino acid sequences were aligned and analysed using the MEGALIGN module of the Lasergene sequence analysis package (DNASTAR) and MEGA version 3.1 (Kumar et al., 2004). Evolutionary trees were constructed using the neighbour-joining method (MEGA version 3.1) on the basis of the following gene sequences: nt 88–1048 (960 bp) of HA1; 88–1369 or 1378 (1282 or 1291 bp) of NA; 589–1785 (1197 bp) of PB2; 736–1888 (1153 bp) of PB1; 784–2016 (1233 bp) of PA; 76–1483 (1408 bp) of NP; 53–984 (932 bp) of M; and 66–838 (773 bp) of NS. Estimates of the phylogenies were calculated by performing 1000 neighbour-joining bootstrap replicates. The nucleotide sequence data obtained in this study are available from GenBank with accession numbers EU753271–EU753350.

RESULTS

Background information

In 1998, a disease outbreak was reported in chicken flocks in a large integrated broiler chicken operation in eastern China (60 million broiler chickens each year), resulting in the isolation of an H9N2 influenza virus, Ck/SH/F/98 (Liu et al., 2003). The disease outbreak was associated with coughing and respiratory distress in 80 % of the birds, with a 30–50 % mortality rate within 2 weeks of the first appearance of clinical signs. Microbial assay showed that Escherichia coli was the most common bacterial pathogen, suggesting that it contributed to the severity of the disease. The outbreak started in a 5-week-old broiler flock and spread quickly to many flocks in different locations of the operation, including breeder flocks. To control the disease in the operation, an intensive vaccination programme was implemented with the inactivated whole virus vaccines prepared from Ck/SH/F/98; all chickens were immunized. Additionally, biosecurity measures around the broiler operation were heightened. The antibody titres induced by this vaccine in the chickens vaccinated in this operation were approximately 2–3. In order to assess the protective efficacy of the inactivated vaccine, tracheal and cloacal swabs were collected from flocks in the operation during a 5 year surveillance period (1998–2002). A total of 22 H9N2 influenza viruses were isolated (Table 1).

Phylogenetic analysis of HA, NA, matrix protein (M) and non-structural protein (NS) genes

To determine the genetic characterization of these viruses, all eight gene segments of three viral isolates from 1998, 1999 and 2000, six viral isolates from 2001 and two viral isolates from 2002 in six different chicken flocks were partially sequenced. The phylogenetic relationship of the HA, NA, M and NS genes are shown in Fig. 1. The HA and NA genes of these 11 viruses shared 94.6–96.9 % and 94.7–96.6 % nucleotide identity, respectively, with those of the A/Chicken/Beijing/1/1994 (Ck/Bj/1/94) H9N2 virus, an earlier H9N2 isolate from southern China (Fig. 1a and b). These results indicated that the HA and NA genes of these viruses belonged to the Ck/Bj/1/94-like lineage of H9N2 viruses. Furthermore, the NA genes of all 11 viral isolates had a deletion of 9 nt between positions 206 and 214, suggesting that they were of the same origin.

Similar to analysis of the HA and NA genes, phylogenetic analysis of the M and NS genes revealed that they also belonged to the Ck/Bj/1/94-like lineage (Fig. 1c and d), sharing 96.6–100 and 96.7–97.9 % nucleotide identity, respectively, with those of the Ck/Bj/1/94 H9N2 virus. The M and NS genes of these viruses were different from the Gs/GD/1/96-like H5N1 viruses, indicating that they belonged to different lineage. However, it is notable that the M gene of H5N1 virus Dk/GD/01/01 and M and NS genes of H5N1 virus Dk/Fj/19/00, which were isolated from domestic duck in 2001 and 2000, respectively, were clustered together with those of the H9N2 viruses included in this study, suggesting that reassortment events might have occurred between these two virus subtypes.

Taken together, our findings clearly indicated that the HA, NA, M and NS genes of these H9N2 variants isolated from this integrated operation belonged to Ck/Bj/1/94-like lineage and that they are different from those of Qa/HK/G1/97-like H9N2 viruses. However, phylogenetic analysis of the M and NS genes showed some of the duck H5N1 isolates were closely related to these H9N2 variants, suggesting that a reassortment event may have occurred in this region. Our findings further supported the view that interspecies transmission between different types of poultry exists in southern China.
Phylogenetic analysis of the PB2 and PB1, PA and NP genes

The results of a parallel phylogenetic analysis of the PB2, PB1, PA and NP genes which code for the proteins of the RNP complex are very similar (Fig. 2a–d). The four RNP complex genes of the H9N2 viruses shared more than 96.4% nucleotide identity. The topologies of the phylogenetic trees of the four RNP complex genes were also very similar. The four RNP genes of the 11 H9N2 viral isolates had evolved in a sequential fashion from 1998 to 2002 and

Fig. 1. Phylogenetic trees for the HA1 (a), NA (b), M (c) and NS (d) genes of the H9N2 influenza A viruses that were analysed. The HA1 and M phylogenetic trees are rooted to A/Duck/Alberta/60/1976 (Dk/Alberta/60/76) (H12N5) (GenBank accession no. AB288334) and A/Equine/Prague/1/1956 (Eq/Prague/1/56) (H7N7) (GenBank accession nos M73519 and CY005803), respectively. The length of the horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing and labelling. The viruses obtained in this study are underlined and their names can be found in Table 1. Abbreviations: Qa, quail; Gs, Geese; G1, A/quail/HongKong/G1/97; Ck, chicken; Dk, duck; Ty, Turkey; CA, California; WI, Wisconsin; HK, Hong Kong; Kor, Korea; BJ, Beijing; SD, Shandong; GD, Guangdong; SH, Shanghai; NC, Nanchang; YN, Yunnan; HB, Hubei; HN, Hunan; FJ, Fujian; ZJ, Zhejiang.

Phylogenetic analysis of the PB2 and PB1, PA and NP genes

The results of a parallel phylogenetic analysis of the PB2, PB1, PA and NP genes which code for the proteins of the RNP complex are very similar (Fig. 2a–d). The four RNP complex genes of the H9N2 viruses shared more than 96.4% nucleotide identity. The topologies of the phylogenetic trees of the four RNP complex genes were also very similar. The four RNP genes of the 11 H9N2 viral isolates had evolved in a sequential fashion from 1998 to 2002 and
formed a unique lineage, a Ck/SH/F/98-like lineage (Li et al., 2005), which was distinguishable from the Ck/BJ/1/94-like (H9N2) lineage and Gs/GD/1/96-like (H5N1) lineage. We noted that the RNP complex genes of a few H5N1 viruses derived from domestic duck also clustered into the Ck/SH/F/98-like (H9N2) lineages, indicating that reassortment events had occurred between these two subtypes of viruses. For example, the PB2 gene of H5N1 viruses Dk/FJ/19/00 and Dk/SH/35/02 (Fig. 2a), the PB1 gene of Dk/SH/35/02 (Fig. 2b), the PA gene of H5N1 viruses WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211/05, Dk/SH/38/01 and Dk/SH/35/02 (Fig. 2c) and the NP gene of eight H5N1 viruses, including WDk/HN/211.

**Fig. 2.** Phylogenetic trees for the PB2 (a), PB1 (b), PA (c) and NP (d) genes of the H9N2 influenza A viruses that were analysed. The phylogenetic trees of PB2, PB1, PA and NP are rooted to Eq/Prague/1/56 (H7N7) (GenBank accession nos M73519 and CY005803) and A/Equine/London/1416/1973 (Eq/London/16/73) (H7N7) (GenBank accession nos M25928 and M26087), respectively. The length of the horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing and labelling. The viruses obtained in this study are underlined, and the name of the viruses and abbreviations are as described in Fig. 1.
05 and Dk/SH/35/02 (Fig. 2d), all clustered into a corresponding Ck/SH/F/98-like lineage. It is interesting to note that many H5N1 viruses isolated from domestic duck since 2001 in mainland China contain an NP gene segment with high identity to that of Ck/SH/F/98. Furthermore, the four RNP genes of the H5N1 virus Dk/SH/35/02 isolated in Shanghai in 2002 shared very high identity with those of Ck/SH/F/98-like variants and also clustered into the same lineage. Taken together, these findings indicate that the Ck/SH/F/98-like viruses in this study may have originated from a single introduction and become a possible donor that provided internal genes for H5N1 viruses since 2001.

**Antigenic analysis of H9N2 isolates**

The H9N2 viruses included in the current study were compared antigenically with representative H9N2 virus from 1994 (Ck/GD/SS/94) and the first isolate from this operation, Ck/SH/F/98. Chicken polyclonal hyper-immune sera against the representative H9N2 virus Ck/GD/SS/94 and Ck/SH/F/98 reacted equally well with all the viruses isolated from this operation (Table 2). Although the HI test via hyper-immune sera against Ck/GD/SS/94 and Ck/SH/F/98 did not allow for differentiation between these two viruses, variation in titres between viruses existed, suggesting a close relationship in antigenicity among these viruses. The reactivity to a panel of mAbs did not discriminate between the earlier H9N2 and these H9N2 variants. However, one of these H9N2 variants, Ck/SH/11/01, demonstrated a unique reactivity pattern; it reacted with only two of six mAbs. We sequenced the HA gene of this virus and revealed the presence of a potential carbohydrate site due to a mutation from Ser to Asn at aa 127 in the HA1 region (data not shown). Based on the presence or absence of this mutational change, we found that all the viruses isolated from eastern China during the past 10 years could be separated into two groups which correlated with the reactivity to the panel of mAbs (data not shown). A mutagenesis study to confirm whether this amino acid change directly contributes to the reactivity profile of mAbs is now under way in our laboratory.

**Pathogenicity of the H9N2 influenza viruses in chickens**

We examined the pathogenicity of these viruses by inoculating eight 6-week-old SPF white Leghorn chickens intravenously with 100 μl allantoic fluid containing the test viruses.
virus. In contrast with the high morbidity and mortality observed for chickens living in the operation, the SPF chickens challenged with most of these viral isolates did not develop severe clinical signs or die during the 3 week observation period. At 21 days post-infection, all of the challenged chickens were seroconverted. However, Ck/SH/7/01 and Ck/SH/1/02 seemed to be more pathogenic than other viruses, as chickens infected with these viruses showed clinical signs of disease, including diarrhoea and facial oedema, with decreased food and water consumption.

**Replication and transmission of H9N2 influenza isolates in chickens**

In order to explore the biological features that enabled the H9N2 viruses to circulate for an extended period of time in an integrated chicken operation, we examined their replication and transmission properties in chickens. Table 3 summarizes the experiment to examine replication and transmission of the viruses in chickens. Most of these variants were shed mainly from the respiratory tract with titres in the range of 4–7 log10 EID50 ml⁻¹ on day 5 post-inoculation. The maximum virus shedding was observed between days 3 and 5 post-inoculation. Viruses were detected in tracheal swabs of directly inoculated birds and physical contact birds as early as 24 h post-contact. It was notable that the ability of these viruses to replicate and be transmitted among the birds was correlated with their pathogenicity. Most of the viruses replicated in the inoculated chickens, but failed to transmit efficiently to the aerosol contact group. However, the more pathogenic Ck/SH/7/01 and Ck/SH/1/02 viruses replicated efficiently in the infected chickens and were transmitted efficiently to the physical contact group and the aerosol contact chickens. Ck/SH/7/01 and Ck/SH/1/02 viruses could be detected in the tracheal and cloacal swabs from the aerosol contact birds by 24 h post-contact; however, lower virus titres were detected in the cloacal swabs compared with the tracheal swabs from both infected and contact birds. Ck/SH/7/01 and Ck/SH/1/02 virus titres were present in the tracheal and cloacal swabs of the birds on day 7 post-infection. The mean antibody titres in the aerosol contact group of Ck/SH/7/01 and Ck/SH/1/02 virus at days 10 and 20 post-inoculation were significantly higher than those of other viruses (Table 3).

**Table 3. Transmission of H9N2 influenza viruses in chickens**

The number of chickens shedding/total number of chickens, and antibody titre are given at the indicated times post-infection.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Method of transmission</th>
<th>No. of chickens shedding/total no. of chickens</th>
<th>Antibody titre*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>3 days Trachea Cloaca 5 days Trachea Cloaca 7 days Trachea Cloaca</td>
<td>10 days 20 days</td>
</tr>
<tr>
<td>Ck/SH/F/98</td>
<td>Direct inoculation</td>
<td>3/3 0/3 3/3 0/3 3/3 0/3</td>
<td>11, 11, 11 11, 11, 9</td>
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<td></td>
<td>Physical contact</td>
<td>1/3 0/3 3/3 0/3 1/3 0/3</td>
<td>3, 4, 0 4, 11, 11</td>
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<tr>
<td></td>
<td>Aerosol contact</td>
<td>1/4 0/4 1/4 0/4 1/4 0/4</td>
<td>2, 0, 0, 0 (Bb) 3, 0, 0, 0 (Bb)</td>
</tr>
<tr>
<td>Ck/SH/2/99</td>
<td>Direct inoculation</td>
<td>3/3 0/3 3/3 1/3 2/3 1/3</td>
<td>3, 7, 6 4, 7, 8</td>
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<td>3, 0, 0 0, 1, 0</td>
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<td></td>
<td>Aerosol contact</td>
<td>0/4 0/4 0/4 0/4 0/4 0/4</td>
<td>0, 0, 0, 0 (Bc) 0, 0, 0, 0 (Bb)</td>
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<tr>
<td>Ck/SH/3/00</td>
<td>Direct inoculation</td>
<td>3/3 1/3 2/3 1/3 1/3 1/3</td>
<td>6, 7, 4 4, 7, 6</td>
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<tr>
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<td>Direct inoculation</td>
<td>3/3 3/3 3/3 2/3 3/3 2/3</td>
<td>11, 11,10 12, 12,11</td>
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<tr>
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<td>9, 10, 9, 11 10,10,11,12</td>
</tr>
<tr>
<td></td>
<td>Aerosol contact</td>
<td>4/4 3/4 2/4 3/4 2/4 2/4</td>
<td>6, 6, 6, 11 (Aa) 10,9,11,11 (Aa)</td>
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<tr>
<td>Ck/SH/14/01</td>
<td>Direct inoculation</td>
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<td>8, 5, 6 7, 7, 6</td>
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<tr>
<td></td>
<td>Physical contact</td>
<td>2/4 0/4 1/4 0/4 1/4 0/4</td>
<td>0, 0, 0, 0 (Bc) 0, 0, 0, 0 (Bb)</td>
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<tr>
<td></td>
<td>Aerosol contact</td>
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<td>0, 0, 0, 0 (Bc) 0, 0, 0, 0 (Bb)</td>
</tr>
<tr>
<td>Ck/SH/1/02</td>
<td>Direct inoculation</td>
<td>3/3 3/3 3/3 3/3 3/3 3/3</td>
<td>10, 10, 11 11, 11, 11</td>
</tr>
<tr>
<td></td>
<td>Physical contact</td>
<td>3/3 3/3 3/3 3/3 3/3 3/3</td>
<td>9, 10, 9 8, 9, 11</td>
</tr>
<tr>
<td></td>
<td>Aerosol contact</td>
<td>4/4 3/4 4/4 3/4 3/4 2/4</td>
<td>2, 2, 2, 2 (Bb) 8, 9, 11,11 (Aa)</td>
</tr>
</tbody>
</table>

*Antibody titre was calculated by 2^N.
†Letters indicate a significant difference between results in the same column (upper case, P<0.01 and lower case, P<0.05).

**Haemagglutination activity with erythrocytes from different animals**

Receptor specificity of the HA protein has been shown to be a determinant of host range for influenza viruses (Connor et al., 1994). Previous work has demonstrated that the receptor specificity of influenza viruses is related to the agglutination of erythrocytes from different animal species.
In order to explore the receptor binding specificity of the H9N2 viruses, the agglutination patterns of these H9N2 variants were systematically analysed with erythrocytes from different animal species (Supplementary Table S1, available in JGV Online). All of these H9N2 variants agglutinate chicken, duck, goose, pigeon, quail, guinea pig, dog and human (type O) erythrocytes, whereas almost all of them did not agglutinate pig erythrocytes. Furthermore, compared with the agglutination patterns with chicken, duck and goose erythrocytes, part of these viruses agglutinate pigeon and quail erythrocytes with much lower titre. Based on the difference in haemagglutination patterns from those of buffalo and goat erythrocytes, most of these H9N2 variants could be discriminated into two further groups. In addition, two 2001 isolates (Ck/SH/11/01 and Ck/SH/12/01) could agglutinate donkey erythrocytes. These results clearly showed that the receptor binding site of these H9N2 variants was, in fact, different. Therefore, direct analysis of receptor specificity of these H9N2 variants using defined glycoconjugates is needed to understand the differences in agglutination patterns of these H9N2 variants.

**Amino acid differences in the HA and NA proteins**

As shown in Supplementary Table S2 (available in JGV Online), amino acid changes in the HA glycoprotein accumulated in a sequential fashion over time. Although some amino acid changes occurred in previously proposed antigenic sites (Ha et al., 2002), the majority of the changes were located in the outer surface of the globular head of HA1. Compared with Ck/SH/F/98, 11 aa differences in the HA protein were found in 2000, 2001 and 2002 isolates. In addition, 11 unique amino acid substitutions in the HA protein from 2002 isolates were distinguishable from those of viruses isolated before 2001. However, the majority of these substitutions occurred in the distal globular head of HA1 rather than the previously proposed antigenic sites and only one change, from Asn to Ser at position 148 (158 in H3 numbering), was located in the proposed antigenic sites. Therefore, direct analysis of receptor specificity of these H9N2 variants using defined glycoconjugates is needed to understand the differences in agglutination patterns of these H9N2 variants.

All of the isolates possessed the same amino acid sequence (Pro-Ala-Arg-Ser-Ser-Arg) at the cleavage site between HA1 and HA2, which is characteristic of the low pathogenic AIVs. Seven potential glycosylation sites, five in HA1 (11, 123, 200, 280 and 287) and two in HA2 (154 and 213), were highly conserved, as is found in most of the isolates from mainland China (Liu et al., 2003; Li et al., 2005). In addition, Ck/SH/11/01 has an additional potential glycosylation site at residue 127, due to the change of Ser to Asn, which was not found in the HA of other viruses in this study.

Analysis of the substitutions of the entire NA gene revealed that amino acid substitutions in NA of the H9N2 viruses during a 5 year period were erratic, while amino acid residues located in the substrate-binding pocket were highly conserved. A highly variable region, where 15 substitutions occurred between residues 19 and 85, was observed. Furthermore, two regions with multiple substitutions were also found. One is a 110 aa region from residues 344 to 455, which are located in the head of NA, where eight amino acid substitutions have occurred. Another multiple mutation region is between residues 125 and 221, where eight substitutions occurred during this 5 year period. Comparison of the substitution rate of amino acids in the NA stalk with that from the entire NA of H9N2 influenza viruses revealed that the rate of amino acid substitutions in the stalk region of the NA is higher than that in the entire coding region or in the head alone, which supports the observations by Xu et al. (2004). The NA proteins from viruses of 2002 differed from those of 1998–2001 isolates by 10 aa. Only one of the four characteristic amino acids, Met-20, from viruses from 2000 was maintained in isolates from 2001. Six potential NA protein glycosylation sites (at positions 69, 86, 146, 200, 234 and 404) from H9N2 viruses in this study were highly conserved. Two isolates of 2002 had an additional potential glycosylation site at residue 246 due to the change of His to Asn, which was not observed in other viruses. Furthermore, all of the NA proteins from the H9N2 viruses examined had a ‘marking’ deletion of 9 nt at positions 206–214 (at aa 62–64) in the stalk of the protein, as previously described (Lu et al., 2005).

**DISCUSSION**

In the present study, we isolated and characterized 22 H9N2 viruses from chicken flocks in a large integrated broiler operation over a 5 year period. One question arises from this: do these viruses originate from a single introduction and maintain a presence in the operation or do they originate from repeated reintroduction? Genetic characterization of 11 representative isolates of these viruses revealed that their HA, NA, M and NS genes all belonged to the Ck/Bf/1/94-like lineage with high identity, while their four RNP genes (PB2, PB1, PA and NP) all clustered together to form a specific Ck/SH/F/98-like lineage. Furthermore, the NA gene of all 11 viruses had a
9 nt deletion between positions 206 and 214. This genetic evidence strongly suggested that all of these viruses are descendents of the first isolates, Ck/SH/F/98, and that they originate from a single introduction.

Interestingly, phylogenetic analysis revealed that the RNP genes of some H5N1 viruses isolated from domestic ducks after 2001 are more closely related to the genes of the Ck/SH/F/98-like H9N2 viruses. For example, one H5N1 isolate, Dk/SH/35/02, a novel genotype in domestic ducks, was also isolated from the suburb of Shanghai. Phylogenetic analysis of the internal genes of Dk/SH/35/02 indicated that the four RNP genes are closely related to those of the Ck/SH/F/98-like H9N2 viruses, while the other genes (M and NS) belong to an H5N1-like virus. In addition, phylogenetic analysis also revealed that a few H5N1 viruses isolated from domestic duck since 2001 contained a Ck/SH/F/98-like RNP gene complex, while the M and NS genes of these H5N1 viruses were similar to the Ck/BJ/1/94-like H9N2 lineage. These findings strongly suggest that interspecies transmission between chickens and domestic ducks may have occurred in this region during the past 10 years.

Chickens are generally considered to be aberrant hosts of influenza viruses because the mutation rates of many chicken viruses are higher than those for viruses isolated from aquatic birds (Suarez, 2000). Furthermore, when an influenza virus infects a new species, infection typically lasts for a short period of time and rarely transmits well enough in the new species to cause an epidemic, due to control efforts or failure of the virus to adapt to the new host (Suarez, 2000; Webster et al., 1992). However, several studies showed that H9N2 influenza viruses have circulated widely in chicken flocks for more than 10 years since their first detection in mainland China in 1994 (Chen et al., 1994), indicating that H9N2 influenza viruses have the ability to circulate in chickens for a long period of time. The results presented in this study also indicate that H9N2 influenza viruses have been circulating in chicken flocks from the same integrated chicken operation for at least 5 years.

Most of the avian influenza viruses are restricted to infecting aquatic birds. However, more and more studies are reporting that H9N2 viruses can replicate and circulate in terrestrial poultry, such as chicken, quail and pigeon. Previous studies have reported that G1-like H9N2 viruses can infect humans and can replicate in the respiratory system of mice (Choi et al., 2004; Guo et al., 2000). The present study demonstrates that H9N2 viruses isolated in eastern China have gradually acquired the ability to replicate efficiently in the respiratory system of chickens and can effectively transmit throughout chicken flocks by aerosol. It is possible that these variants emerged as a result of selective pressure due to vaccination or adaptation in a single host for a long period of time.

The molecular basis of adaptation of influenza A viruses to a new host species is poorly understood. Previous studies have demonstrated that mutation of the polymerase complex is critical for adaptation to the new environment once the virus has been transmitted to a new host (Gabriel et al., 2005). The four RNP genes of these H9N2 viruses are highly related to those from the subtype H5N1 that prevails in poultry in southern China, indicating that they probably shared a recent common progenitor virus. Whether these RNP complexes are necessary for mediating adaptation of the H9N2 virus to chicken should be investigated in future studies. Previous studies reported that the deletion of amino acids in the NA gene of influenza A virus is related to the adaptation of influenza A virus to its host (Alexander, 2000). However, further studies are needed to explore whether a 3 aa deletion at aa 62–65 of the NA gene of these H9N2 variants is related to the adaptation to infecting chickens.

A major determinant of host range is the affinity of the viral HA protein to the sialic acid (SA) receptor of the host cell. A change of preference for the avian influenza virus for s(2, 3)-linked Gal to s(2, 6)-linked Gal for the SA receptor is highly related to host specificity. Stephenson et al. (2003) demonstrated that erythrocytes from different hosts can be used to rapidly define the receptor specificity of influenza viruses because of the differences in the oligosaccharide composition of glycoproteins and glycolipids of erythrocytes. In this study, we found that the chicken H9N2 viruses isolated during the 5 year period differed from each other in their ability to agglutinate erythrocytes from different hosts, suggesting differences in receptor specificities in the H9N2 influenza viruses. Ito et al. (1997) demonstrated that the s(2, 3) linkage and NeuGc, not NeuAc, recognition appear to be essential for agglutination of bovine and equine erythrocytes. Whether the difference in the agglutination pattern of these H9N2 variants to goat and buffalo erythrocytes is related to NeuGc recognition still needs to be determined. Wan & Perez (2007) also demonstrated that the Glu to Leu substitution at sialic acid position 226 in HA allows H9N2 viruses to replicate more efficiently in human airway epithelial cells cultured in vitro. The substitution of Gln for Leu at residue 226 and the change of Ala to Thr at position 190 occurred in the receptor binding site of those H9N2 variants that have previously been reported to be involved in the binding specificity to receptors in host cells (Perez et al., 2003). Therefore, further studies are needed to understand the role of these mutations in the receptor binding site in restricting the host range of these H9N2 variants.

In contrast, it has been reported that avian influenza viruses have not been under constant threat by vaccines and also are not very well matched, antigenically, with the chicken vaccines that are efficacious in poultry (Lipatov et al., 2004; Webster et al., 1992). However, recent studies have demonstrated that the commercial vaccine was not able to completely prevent virus shedding when chickens were challenged with antigenically different isolates (Lee et al., 2004). Our study confirms the previous finding that chickens vaccinated with homologous vaccine may be...
protected against clinical disease resulting from AIV challenge, but it is very difficult to completely stop infection in the field.

In this study, we have demonstrated that recent variants isolated during a specific 5 year period might be derived from the early isolate, Ck/SH/F/98. The H9N2 viruses are poorly pathogenic for chickens under the experimental conditions; however, they often infect birds and compete with other potentially harmful microbes, such as *Staphylococcus*, *Haemophilus* and *E. coli*, pathogens that have caused considerable economic loss to the poultry industry in China (Guo *et al.*, 2000; Kishida *et al.*, 2004). The available evidence indicates that Ck/BJ/1/94-like viruses have become established in chickens as a stable lineage in mainland China (Liu *et al.*, 2003; Li *et al.*, 2005). Furthermore, with their long time adaptation to chickens, the symptoms associated with infection with these viruses are less noticeable but could gradually acquire the ability to spread efficiently in chicken flocks. Once these viruses acquire the ability to replicate and spread efficiently in a human population, H9N2 influenza virus could be likely to become a novel influenza subtype capable of causing human influenza pandemics in the future. Therefore, it is imperative that particular attention is paid to H9N2 viruses of avian origin to avert any future pandemic in humans.

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**REFERENCES**


