

# Influenza A Virus in Taiwan, 1980–2006: Phylogenetic and Antigenic Characteristics of the Hemagglutinin Gene

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Limited amount of information is available in Taiwan on the genetic or antigenic characteristics of influenza A virus prior to the establishment of a Taiwan surveillance network in 2000. Isolates of H1N1 and H3N2 viruses in Taiwan between 1980 and 2006 were studied, and part of the hemagglutinin gene was analyzed due to its importance in terms of viral infection and antibody neutralization. Results from a phylogenetic analysis indicate continuous evolutionary topology in H3N2 isolates, and two distinct H1N1 lineages. Many genetic relationships between vaccine strains and epidemic isolates appearing in Taiwan before other global locations were also observed and recorded in addition to a gradual increase in the number of N-linked glycosylation sites on partial HA1 proteins since 1980. The results from pairwise comparisons of HA1 nucleotide and deduced amino acid sequences indicate shared identities within groups organized according to their bootstrap and *P*-values of approximately 95.5–100% and 95.7–100% in H1N1 and 94.5–100% and 93.2–100% in H3N2 viruses, respectively. Comparisons of amino acid substitutions in the five antigenic regions reveal highly non-synonymous changes occurring in the Sb region of H1N1 and in the B region of H3N2. The results of an antigenic analysis using a hemagglutinin inhibition (HI) test indicate the presence of some epidemic strains 1–2 years earlier in Taiwan than in other parts of the world, as well as higher vaccine mismatch rates. This information supports the need for continuous surveillance of emerging influenza viruses in

Taiwan, which will be useful for making global vaccine decisions. **J. Med. Virol.** 81:1457–1470, 2009. © 2009 Wiley-Liss, Inc.

**KEY WORDS:** H1N1; H3N2; influenza; phylogenetic analysis; hemagglutinin

## INTRODUCTION

Influenza viruses cause symptomatic diseases in about 20% of children and 5% of adults every year [Glezen et al., 1997; Redlberger et al., 2007]. Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses, which is characterized by minus-stranded RNA and a segmented genome. Influenza viruses are divided into subtypes according to the antigenic properties of surface glycoprotein, hemagglutinin (HA), and neuraminidase (NA) [Webster et al., 1992; Saito, 2006]. Influenza viruses

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have caused epidemics and occasional pandemics. The 1918 Spanish, 1957–1958 Asia, and 1968–1969 Hong Kong influenza strains were at the center of three 20th century pandemics [Silverstein, 2006]. Any new influenza A virus subtype poses a threat to human health with significant morbidity and mortality.

Changes in the antigenicity of influenza virus are the results of antigenic drifts and shifts. Antigenic drifts are caused by the low fidelity of viral RNA polymerase and the accumulation of point mutations in the HA and NA genes. Antigenic shifts result from the reassortment of gene segments between human, avian, and swine influenza viruses [Nicholson et al., 2003; Pontoriero et al., 2003; Carrat and Flahault, 2007]. The H1N1 and H3N2 influenza viruses have been co-circulating worldwide since 1977. Due to the high mutation frequency that is characteristic of the influenza virus genome, the continuous monitoring of viral genetic and antigenic changes is considered essential to public health.

Influenza virus HA protein is the primary target of neutralizing antibodies. Influenza viruses can evade host immune systems by mutations on specific antigenic regions that are no longer recognized by neutralizing antibodies. Such mutations are usually generated on the HA1 globular domain, near receptor binding sites [Underwood et al., 1987; Ruigrok et al., 1988]. Results from sequencing analyses of HA1 indicate that amino acid substitutions resulting in antigenic drift primarily accumulate on five antigenic sites in H1N1 (Sa, Sb, Ca1, Ca2, Cb) and H3N2 (A, B, C, D, E) [Gerhard et al., 1981; Wiley et al., 1981]. Previous reports have suggested that more severe epidemics have resulted from antigenic changes in the HA proteins of new antigenic variants [Shih et al., 2005].

Global influenza virus activity is monitored by a surveillance network of laboratories coordinated by the World Health Organization (WHO). Each year WHO makes recommendations for the strain compositions of trivalent influenza vaccines for the northern and southern hemispheres. Suitable vaccine strains are chosen based on antigenic and genetic data for virus strains isolated in previous years [Hsieh et al., 2005; Carrat and Flahault, 2007]. Influenza vaccine efficacy depends on whether currently circulating strains are related antigenically to the vaccine strains. The present study is based on clinical specimens to gather isolates of epidemic H1N1 and H3N2 influenza strains that appeared in Taiwan between 1980 and 2006, and to carry out phylogenetic analyses and antigenic site comparisons to define genetic and antigenic characteristics of the HA gene. Hemagglutinin inhibition (HI) assays were carried out to evaluate antigenicity between the collected isolates and vaccine strains recommended by WHO.

## MATERIALS AND METHODS

### Viruses

Throat swab specimens containing epidemic influenza A strains were collected by physicians and researchers at

the Veterans General Hospital-Taipei between 1980 and 2006. Virus subtypes were determined by immunofluorescent antibody (IFA) staining and real-time RT-PCR. Genetic and phylogenetic analyses were carried out on 28 H3N2 and 35 H1N1 influenza A isolates. All virus isolate data were confirmed by the US Centers for Disease Control (US CDC).

### RT-PCR and Sequencing

Viral RNA was extracted from 140  $\mu$ l of virus-infected MDCK culture fluid with a QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using 1  $\mu$ M of Unil2 primer (5'-AGCAAAGCAGG-3') with SuperScript II reverse transcriptase (Gibco-BRL, Burlington, ON, Canada) as described by Chan et al. [2006] and Hoffmann et al. [2001]. Partial HA1 segments containing major antigenic sites of H1N1 and H3N2 viruses were amplified by PCR using Taq polymerase with the following primers: HA-H1F, 5'-GATGCAGACACAA TATGTAGAGG-3'; HA-H1R, 5'-CNCTACAGAGACA TAAGGATTT-3'; HA-H3F, 5'-CTGTTACCCTTATGATGTGC-3'; and HA-H3R, 5'-GCTYCCATTTGGAGTRATGC-3. Cycling consisted of 95°C for 3 min (1 cycle); 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min (35 cycles); and 72°C for 7 min (1 cycle). Resulting amplicons were analyzed by 1% gel electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). DNA sequencing was performed with a Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction Kit (ABI, Foster City, CA) and ABI-310 Genetic Analyzer (PerkinElmer, Norwalk, CT). Sequences were analyzed using Chromas Lite version 2.01 (Technelysium Pty. Ltd., Helensvale, Australia) and Bioedit Sequence Alignment Editor V.7.0.5.3. All nucleotide sequences were submitted to GenBank.

### Antigenic Characteristics

HI tests were carried out using whole viruses and panels of post-infection ferret sera with representative vaccine strains obtained from the US CDC. Some results were confirmed by the CDC. Any isolate reacting with a reference antiserum at a titer equal to that at which it reacted with the reference homologous virus ( $\pm$ 1- to 2-fold dilution) was said to resemble closely that virus [Kendal et al., 1981; Cox et al., 1983]. This criterion was used to analyze all matches between circulating and vaccine strains [Pontoriero et al., 2003; Hsieh et al., 2005].

### Amino Acid Substitution

Relationships between individual isolates and vaccine strains of the H1N1 and H3N2 influenza A viruses for each year were elucidated by analyzing their amino acid signature patterns. Five major antigenic sites were identified in the HA1 domain of H1N1 (Sa, Sb, Ca1, Ca2, Cb) and five in the same domain of H3N2 (A, B, C, D, E). Deduced amino acid sequences were aligned using BioEdit Sequence Alignment Editor V.7.0.5.3. The

amino acid sequences of A/PR/8/34 and A/HK/1/68 were used as references.

### Phylogenetic Analysis

Phylogenetic trees were constructed using a neighbor-joining (NJ) method based on Kimura's two-parameter distance matrix with 1,000 bootstrap replicates, using the MEGA (version 3.0) and PHYLIP (version 3.5) software packages [Kumar et al., 2004; Lin et al., 2007]. MEGA was used to construct NJ trees and PHYLIP was used with parsimony and maximum-likelihood (ML) methods to verify the topology of taxas shown in the trees. All trees presented in this article are NJ; labels on tree branching points indicate ML-calculated *P*-values.

### N-Linked Glycosylation Site Predictions

Predictions of N-linked glycosylation on H1N1 or H3N2 HA proteins were made using the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Amino acid sequences from yearly isolates were uploaded to the website and total N-linked glycosylation sites were predicted according to the Asn-X-Ser/Thr rule. For partial HA1 segments, sites were predicted within a range of a.a 103–280 for H3N2 and a.a 12–197 for H1N1.

## RESULTS

Subtypes of 63 human influenza A isolates determined by IFA staining and real-time RT-PCR are shown in Table I. Nucleotide sequences of partial HA segments were used to construct phylogenetic trees in order to characterize genetic variations and to determine relationship between vaccine strains and the collected human H1N1/H3N2 influenza viruses. H3N2 phylogenetic analysis results reveal occurrences of antigenic drift every 1–2 years. The tree topologies indicate continuous change patterns. The resulting phylogenetic tree can be divided into 12 groups according to bootstrap values (NJ method) and *P*-values (ML method) (Fig. 1A). In contrast, the H1N1 phylogenetic analysis shows antigenic drift occurring every 1–4 years, with some early isolated strains reemerging more than once (Fig. 1B). For example, the 2005–2006 isolates TW030110-06, TW120202-05, TW031612-06, and TW021011-06 clustered with some 2004 and 2005 isolates and had individual sections that were closer in structure to 1999 Taiwan isolates. The H1N1 phylogenetic tree can be divided into 11 groups and two lineages originating in 1992, with one lineage evolving into 1995–1996 isolates and the other into 1998–2006 isolates (Fig. 1B). Note that the H1N1 and H3N2 strains recommended by WHO for constructing vaccinations clustered frequently with earlier Taiwanese isolates (Fig. 1A,B).

HA1 nucleotide and deduced amino acid sequences were compared within and between groups of isolates and other Taiwanese reference strains. Each group was identified according to its bootstrap and *P*-values. The

results indicate that nucleotide and amino acid identities within each group were 95.5–100% and 95.7–100% in H1N1 and 94.5–100% and 93.2–100% in H3N2, respectively. The identities between each group were 88.5–100% and 84.2–100% in H1N1 and 84.2–100% and 79.7–100% in H3N2, respectively (Tables IIA and IIB). Note that amino acid identities were sometimes lower than nucleotide identities within or between the two groups.

Hemagglutinin proteins constitute receptor-binding and membrane fusion glycoproteins in influenza viruses. The antigenic residues of H1N1 and H3N2 (after aligning deduced amino acid sequences containing major targets for neutralizing antibodies) are shown, respectively, in Figure 2A,B. The greatest variation was noted in Sb (H1N1) and B (H3N2) antigenic sites (Fig. 2A,B). Results from a comparison of HA1 antigenic sites for H1N1 indicate that most 1983–1997 isolates contained Ser(S)73, Phe(F)75, Glu(E)156, and Ile(I)194, and that these changed to Leu(L)73, Ile(I)75, Gly(G)156, and Leu(L)194 in the 1998–2006 isolates. Furthermore, sites 189, 192, 193, 196, and 197 were identified as Gly(G), Arg(R), Ala(A), His(H), and Thr(T)—all dominant in the 1986–2006 isolates, although other amino acid residues occupied these positions prior to 1986 (Fig. 2A). Notably, the HA gene in the 1997–2006 isolates had a characteristic deletion of Lys(K) 134 (data not shown).

The results of an analysis of signature patterns of antigenic sites with H1N1 vaccine strains revealed similarities between the 1983–1985 isolates and A/Brazil/11/78 and A/Chile/1/83; the 1986–1988 isolates and A/Singapore/8/86; the 1990–1996 isolates and both A/Texas/36/91 and A/Bayern/7/95; the 1998–2000 isolates and A/Beijing/262/95; the 2000–2003 isolates and A/New Caledonia/20/99; and the 2006 isolates and A/Solomon Islands/2/06. Distinct similarities were also observed between sections of the 2004–2006 isolates and A/Brisbane/59/2007—the WHO recommendation for the 2008–2009 vaccine strain (Fig. 2A).

The following mutations were observed when comparing HA1 antigenic sites in H3N2 (Fig. 2B): Try(Y)155 (dominant in 1980–1985 isolates) changed to His(H)155 in the 1987–2002 isolates and to Thr(T)155 in the 2002–2006 isolates; Glu(E)156 (dominant in the 1980–1992 isolates) changed to Lys(K)156 and Gln(Q)156 in the 1996–2002 isolates and to His(H)156 in the 2003–2006 isolates; Glu(E)158 (dominant in the 1980–1997 isolates) changed to Lys(K)158 and in some cases to Arg(R)158 in the 1997–2006 isolates; residue 159 was originally Ser(S), but mutated into Tyr(Y) (1987–2004) and Phe(F) (2004–2006); residue 189 in the 1980–1985 isolates mutated from Lys(K) to Arg(R) (1987–1992), Ser(S) (1996–2004), and Asn(N) (2004–2006); and Val(V)196 and Arg(R)197 (found in the 1980–1992 isolates) changed to Ala(A)196 and Gln(Q)197 in the 1997–2006 isolates. Additional changes were observed in 1–3 amino acids at other antigenic sites. Conserved amino acid residues at the HA receptor-binding site in both H3 and H1 influenza A viruses described

TABLE I. Subtypes and Accession Numbers of Influenza Virus Isolates From Clinical Specimens

Isolates	Abbreviation	Specimen	Real-time RT-PCR		Accession no.
			HA	NA	
A/Taiwan/VGHYM0602-17/80	TW060217-80	Throat swab	H3	N2	FJ203729
A/Taiwan/VGHYM0604-08/80	TW060408-80	Throat swab	H3	N2	FJ203730
A/Taiwan/VGHYM0707-02/81	TW070702-81	Throat swab	H3	N2	FJ203728
A/Taiwan/VGHYM0711-20/83	TW071120-83	Throat swab	H1	N1	FJ203702
A/Taiwan/VGHYM0109-12/84	TW010912-84	Throat swab	H3	N2	FJ203727
A/Taiwan/VGHYM0122-09/85	TW012209-85	Throat swab	H3	N2	FJ203725
A/Taiwan/VGHYM0131-05/85	TW012105-85	Throat swab	H3	N2	FJ203726
A/Taiwan/VGHYM0205-16/85	TW020516-85	Throat swab	H1	N1	FJ203701
A/Taiwan/VGHYM0213-12/85	TW021312-85	Throat swab	H1	N1	FJ203700
A/Taiwan/VGHYM1210-03/87	TW121003-87	Throat swab	H3	N2	FJ203724
A/Taiwan/VGHYM0713-10/87	TW071310-87	Throat swab	H3	N2	FJ203723
A/Taiwan/VGHYM1220-03/88	TW122003-88	Throat swab	H3	N2	FJ203722
A/Taiwan/VGHYM0707-10/88	TW070710-88	Throat swab	H1	N1	FJ203698
A/Taiwan/VGHYM0725-08/88	TW072508-88	Throat swab	H1	N1	FJ203699
A/Taiwan/VGHYM1212-03/88	TW121203-88	Throat swab	H1	N1	FJ203697
A/Taiwan/VGHYM0115-09/90	TW011509-90	Throat swab	H1	N1	FJ203695
A/Taiwan/VGHYM0122-01/90	TW012201-90	Throat swab	H1	N1	FJ203696
A/Taiwan/VGHYM0423-11/90	TW042311-90	Throat swab	H1	N1	FJ203694
A/Taiwan/VGHYM0116-02/90	TW011602-90	Throat swab	H3	N2	FJ203720
A/Taiwan/VGHYM1001-06/90	TW100106-90	Throat swab	H3	N2	FJ203721
A/Taiwan/VGHYM0318-03/91	TW031803-91	Throat swab	H1	N1	FJ203693
A/Taiwan/VGHYM0124-01/92	TW012401-92	Throat swab	H1	N1	FJ203692
A/Taiwan/VGHYM0127-08/92	TW012708-92	Throat swab	H1	N1	FJ203691
A/Taiwan/VGHYM0128-01/92	TW012801-92	Throat swab	H3	N2	FJ203719
A/Taiwan/VGHYM0408-05/95	TW040805-95	Throat swab	H1	N1	FJ203690
A/Taiwan/VGHYM0512-01/95	TW051201-95	Throat swab	H1	N1	FJ203688
A/Taiwan/VGHYM0512-02/95	TW051202-95	Throat swab	H1	N1	FJ203689
A/Taiwan/VGHYM0619-01/95	TW061901-95	Throat swab	H1	N1	FJ203687
A/Taiwan/VGHYM0721-12/95	TW072112-95	Throat swab	H1	N1	FJ203686
A/Taiwan/VGHYM0117-02/96	TW011702-96	Throat swab	H1	N1	FJ203685
A/Taiwan/VGHYM0118-02/96	TW011802-96	Throat swab	H1	N1	FJ203683
A/Taiwan/VGHYM0210-04/96	TW021004-96	Throat swab	H1	N1	FJ203684
A/Taiwan/VGHYM0123-04/02	TW012304-02	Throat swab	H1	N1	FJ203682
A/Taiwan/VGHYM0325-06/02	TW032506-02	Throat swab	H3	N2	FJ203718
A/Taiwan/VGHYM0204-01/04	TW020401-04	Throat swab	H3	N2	FJ203715
A/Taiwan/VGHYM0503-13/04	TW050313-04	Throat swab	H3	N2	FJ203716
A/Taiwan/VGHYM0607-09/04	TW060709-04	Throat swab	H3	N2	FJ203714
A/Taiwan/VGHYM1118-16/04	TW111816-04	Throat swab	H3	N2	FJ203717
A/Taiwan/VGHYM1209-01/04	TW120901-04	Throat swab	H3	N2	FJ203712
A/Taiwan/VGHYM0709-08/04	TW070908-04	Throat swab	H3	N2	FJ203711
A/Taiwan/VGHYM1209-10/04	TW120910-04	Throat swab	H3	N2	FJ203713
A/Taiwan/VGHYM0214-01/05	TW021401-05	Throat swab	H3	N2	FJ203706
A/Taiwan/VGHYM0211-01/05	TW021101-05	Throat swab	H3	N2	FJ203710
A/Taiwan/VGHYM0518-26/05	TW051826-05	Throat swab	H3	N2	FJ203709
A/Taiwan/VGHYM0601-41/05	TW060141-05	Throat swab	H3	N2	FJ203708
A/Taiwan/VGHYM0705-05/05	TW070505-05	Throat swab	H3	N2	FJ203707
A/Taiwan/VGHYM1103-11/05	TW110311-05	Throat swab	H1	N1	FJ203681
A/Taiwan/VGHYM1202-02/05	TW120202-05	Throat swab	H1	N1	FJ203680
A/Taiwan/VGHYM0224-09/06	TW022409-06	Throat swab	H1	N1	FJ203678
A/Taiwan/VGHYM0301-10/06	TW030110-06	Throat swab	H1	N1	FJ203679
A/Taiwan/VGHYM0210-11/06	TW021011-06	Throat swab	H1	N1	FJ203677
A/Taiwan/VGHYM0217-05/06	TW021705-06	Throat swab	H1	N1	FJ203676
A/Taiwan/VGHYM0220-18/06	TW022018-06	Throat swab	H1	N1	FJ203675
A/Taiwan/VGHYM0222-05/06	TW022205-06	Throat swab	H1	N1	FJ203674
A/Taiwan/VGHYM0224-11/06	TW022411-06	Throat swab	H1	N1	FJ203673
A/Taiwan/VGHYM0316-12/06	TW031612-06	Throat swab	H1	N1	FJ203672
A/Taiwan/VGHYM0311-04/06	TW031104-06	Throat swab	H3	N2	FJ203703
A/Taiwan/VGHYM0412-17/06	TW041217-06	Throat swab	H1	N1	FJ203671
A/Taiwan/VGHYM0327-11/06	TW032711-06	Throat swab	H3	N2	FJ203704
A/Taiwan/VGHYM0404-14/06	TW040414-06	Throat swab	H1	N1	FJ203670
A/Taiwan/VGHYM0407-04/06	TW040704-06	Throat swab	H1	N1	FJ203669
A/Taiwan/VGHYM0411-15/06	TW041115-06	Throat swab	H3	N2	FJ203705
A/Taiwan/VGHYM0412-16/06	TW041216-06	Throat swab	H1	N1	FJ203668

previously, Ser(S)136, Trp(W)153, His(H)183, and Tyr(Y)195, numbered according to the H3 structure, were also present in the isolates used in this study (data not shown).

The results from amino acid signature analyses of H3N2 vaccine strains indicate similarities between the 1984–1985 isolates and A/Leningrad/360/88; the 1987–1990 isolates and A/Sichuan/02/87, A/Shanghai/11/87, and A/Guizhou/54/89; the 1992 isolates and A/Beijing/353/89; the 1996 isolates and A/Johannesburg/33/94 and A/Wuhan/359/95; the 1997–1998 isolates and A/Sydney/5/97; the 1999–2002 isolates and A/Moscow/10/99; the 2002–2003 isolates and A/Fujian/411/92; the 2004–2005 isolates and A/California/7/04; and the 2005–2006 isolates and A/Wisconsin/67/05 and A/Brisbane/10/07 (Fig. 2B).

HI assays using reference antisera were carried out to identify the antigenic characteristics of H1N1 and H3N2 isolates. As shown in Table III, the H3H2 results show similarities between the 1980–1981, 1984–1985, 1987,

1988, 1990, 1992, 2002, 2004, and the 2005–2006 isolates and A/Bangkok/1/79; A/Philippines/2/82; A/Sichuan/2/87; A/England/427/88; A/Guizhou/54/89 and A/Beijing/353/89; A/Beijing/353/89; A/Panama/2007/99; A/Fujian/411/02 and A/California/7/04; and A/California/7/04 and A/Wisconsin/67/05 strains. In the case of H1N1, the similarities were between the 1983, 1985, 1988–1990, 1991–1992, 1995–1996, the 2002–2006 isolates and A/Brazil/11/78, A/Chile/1/83, A/Singapore/6/86, A/Texas/36/91, A/Bayern/7/95, A/New Caledonia/20/99, and A/Solomon Island/3/06 strains. The data in Table III also show that antigenic relationships between isolates and WHO-recommended vaccine strains were stronger in H1N1 (8/11; 72.7%) than in H3N2 (6/12; 50%), and that N-linked glycosylation sites on partial HA1 segments increased gradually from 1–2 in 1980 to 6–7 in 2006.

After combining these results with existing data from the Clinical Virology Laboratory of the Taipei Veterans General Hospital, as well as from Hsieh et al. [2005] and

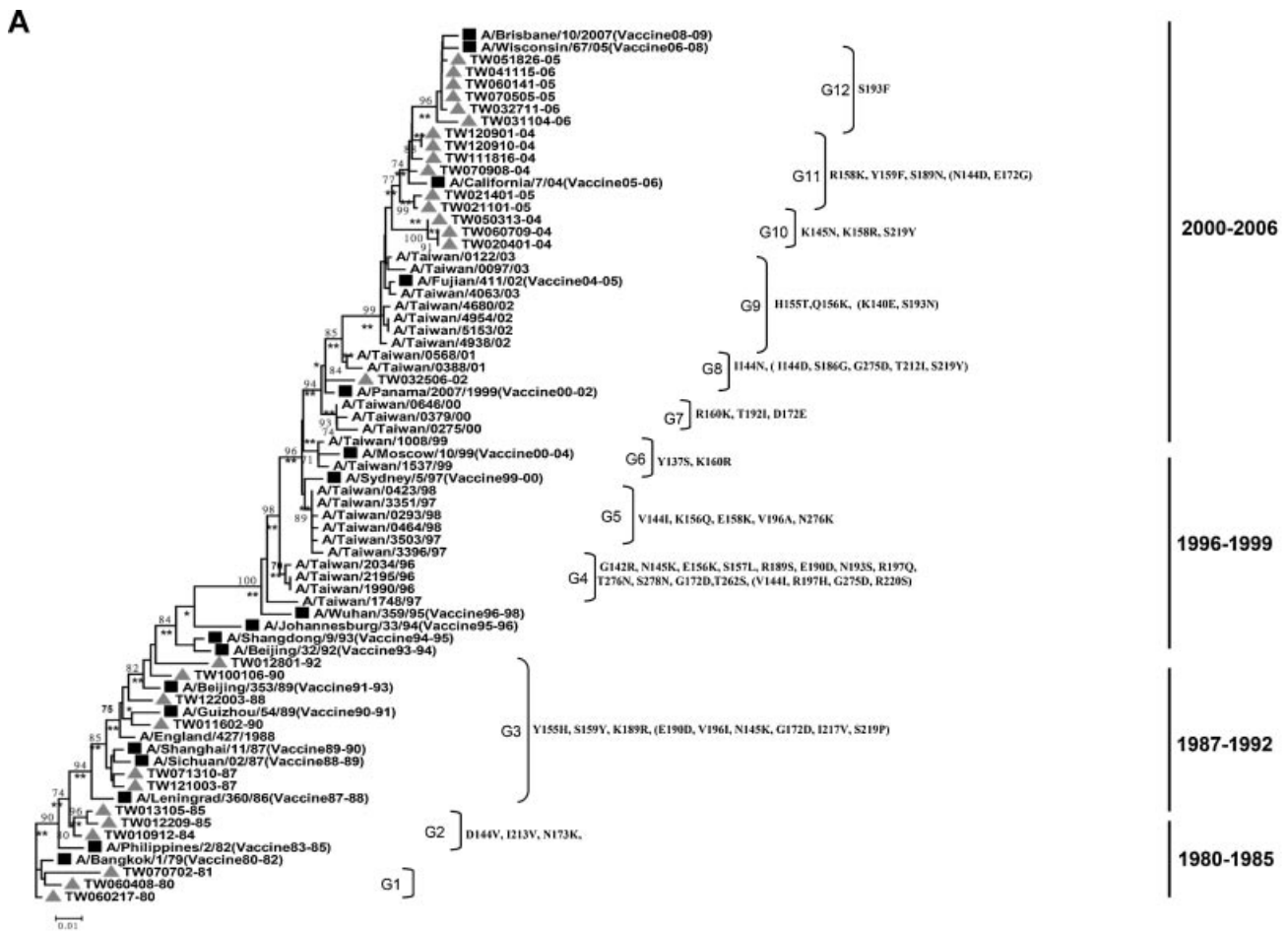


Fig. 1. Phylogenetic analysis of human influenza A virus in Taiwan from 1980 to 2006. Consensus neighbor-joining trees were obtained from 1,000 bootstrap replicates of aligned HA1 sequences from different influenza H3N2 isolates, corresponding to nucleotide positions 356–909 of A/HongKong/1/86 (A). H1N1 isolates correspond to nucleotide positions 46–654 of A/PR/8/34 (B). Results from maximum-likelihood statistical analyses are shown on each branching point.

Bootstrap and *P*-values were used to divide H3N2 influenza virus isolates into 12 groups (G1–G12) and H1N1 isolates into 11 groups (G1–G11). Changes in amino acids on antigenic sites in different periods are labeled, with comparisons of earlier and later periods. Minor amino acid substitutions are displayed within brackets. Squares indicate vaccine strains, and triangles indicate our isolates. \**P* < 0.05; \*\**P* < 0.01.

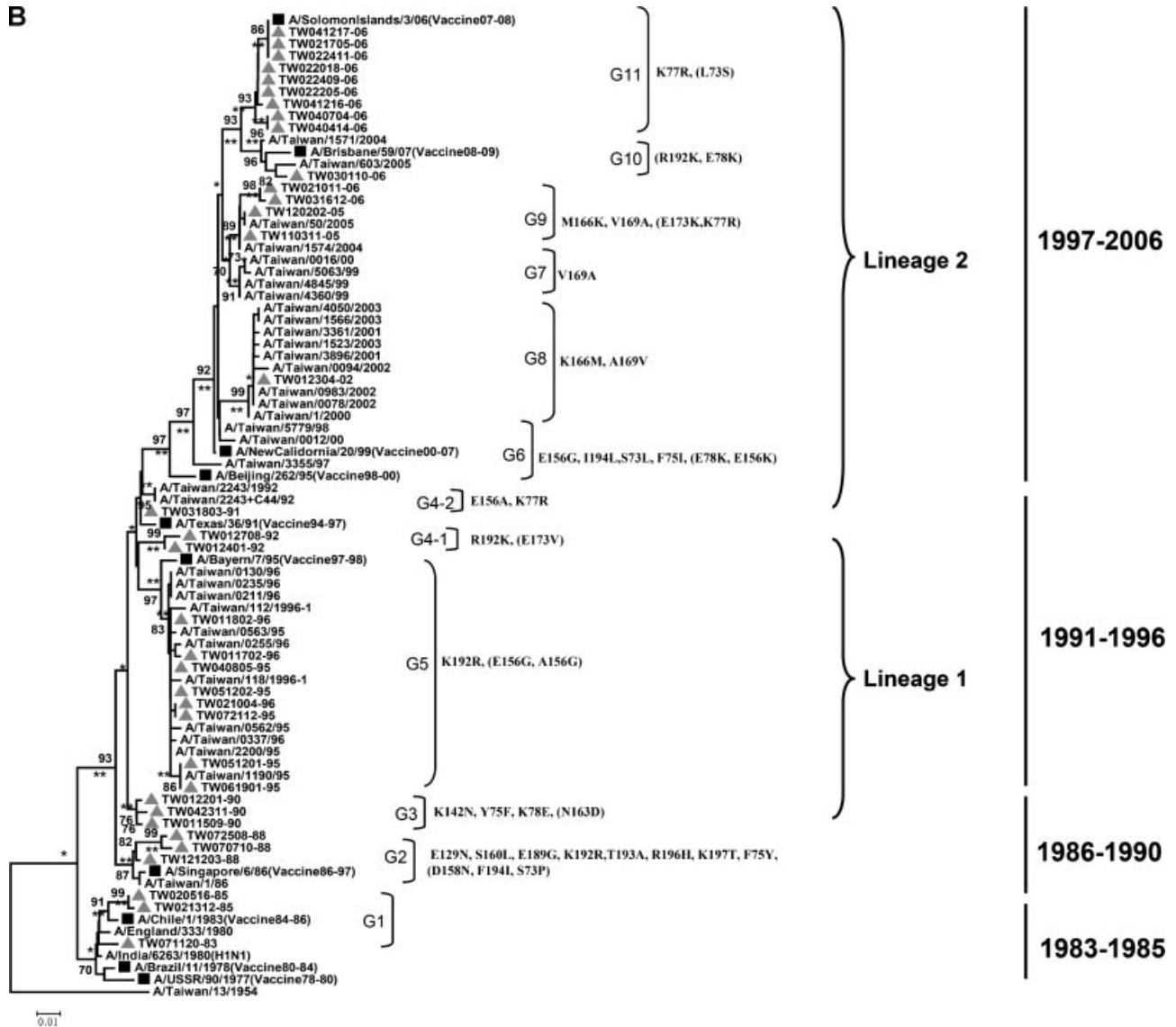


Fig. 1. (Continued)

Shih et al. [2005], comparisons were made with lists of vaccine strains recommended by WHO, world epidemic strains, and Taiwanese isolates. The vaccine match rate for H3N2 (19/28, 67.8%) was lower than for H1N1 (23/28, 82.1%). The data also indicate that some world epidemic strains appeared in Taiwan before appearing in other parts of the world (Table IV).

**DISCUSSION**

The Taiwan Centers for Disease Control (CDC) were founded in 1999, and the surveillance network coordinated by the Taiwan CDC was established the following year [Shih et al., 2005; Lin et al., 2008]. The Veterans General Hospital-Taipei is a major teaching hospital and medical center. Prior to 2000, the hospital's

Department of Virology served as a national reference laboratory for influenza virus isolation and characterization. After 2000, the hospital's specimen collection activity was restricted to the northern region of Taiwan [Shih et al., 2005]. In response to this limitation, influenza A viruses collected and/or examined by other Taiwanese laboratories were included in this analysis. As a result, the viral sequences described in this study represent influenza virus A strains prevalent in Taiwan between 1980 and 2006.

The three most commonly used algorithms in molecular phylogenetic analyses are NJ, ML, and Parsimony (Pars); all three were used to obtain the tree topologies discussed in this article. The phylogenetic tree can be segregated into 12 (H3N2) and 11 (H1N1) groups according to bootstrap values obtained from the NJ

TABLE IIA. Pairwise Comparisons of Influenza H1N1 Virus Hemagglutinin Gene Sequences

	G1		G2		G3		G4		G5		G6		G7		G8		G9		G10		G11		
	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	
G1 (1980–1985)	97.9–99.7	96.3–99.3	97.1–98.5	95.1–98.1	92.1–95.7	92.3–95.5	89–93.9	91.7–93.3	89–90.9	91.5–93.3	88.4–90.3	91.5–93.5	88.4–91.5	90.1–93.3	86.6–90.3	90.7–93.7	87.2–89.6	88.9–91.9	84.2–87.8				
G2 (1986–1988)			98.3–99.3	98.7–99.6	97.1–99.1	95.7–98.9	94.3–96.1	91.5–95.1	93.7–94.3	90.9–91.5	93.5–94.3	90.9–92.7	91.5–94.1	89–92.1	92.1–94.5	88.4–90.9	90.7–93.7	87.2–89.6					
G3 (1990)				98.3–99.3	98.7–99.6	97.1–99.1	95.7–98.1	96.7–97.5	95.7–98.1	91.5–95.1	93.7–94.3	90.9–91.5	93.5–94.3	90.9–92.7	91.5–94.1	89–92.1	92.1–94.5	88.4–90.9					
G4 (1991–1992)					97.5–99.3	96.3–100	95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100	95.7–100	96.7–100				
G5 (1995–1996)						98.3–100	97.5–100	95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100	95.7–100				
G6 (1997–2000)							93.1–95.3	90.9–95.7	95.1–99.3	91.9–93.7	90.3–93.9	93.1–94.7	90.9–93.3	92.9–94.7	90.9–94.5	91.1–94.9	89–93.3	91.7–95.3					
G7 (1999–2000)								95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100					
G8 (2000–2003)									95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100				
G9 (2004–2006)										95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100			
G10 (2004–2006)											95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100		
G11 (2006)												95.5–99.7	95.1–100	99.5–99.7	96.3–100	97.1–97.9	96.9–98.1	97.1–99.1	96.3–100	95.7–100	96.3–98.5	96.7–100	

TABLE IIB. Pairwise Comparisons of Influenza H3N2 Virus Hemagglutinin Gene Sequences

	G1		G2		G3		G4		G5		G6		G7		G8		G9		G10		G11		G12	
	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a	nt	a.a
G1 (1980–1985)	97.3–98.8	96.6–99.4	95.7–97.7	94.3–97.1	91.5–96.4	90.4–95.5	88.4–90.4	84.8–87.0	87.4–89.9	83.1–85.3	87.1–89.5	82.5–85.3	86.1–88.9	80.8–84.2	86.3–88.9	82.0–84.2	85.2–88.0	80.3–83.7	84.2–87.4	80.3–82.5	84.2–87.2	80.3–82.5	84.2–86.9	79.7–83.1
G2 (1986–1988)																								
G3 (1990)																								
G4 (1991–1992)																								
G5 (1995–1996)																								
G6 (1997–2000)																								
G7 (2001–2003)																								
G8 (2004–2005)																								
G9 (2005–2006)																								



method and *P*-values from the ML method. In terms of robustness, a bootstrap value of 70% is cited frequently as a cutoff for cluster reliability [Hillis, 1997, 1998; Lan et al., 2005]. When using the ML method, phylogenetic tree topology is considered significant when *P* < 0.05.

The results of the H3N2 phylogenetic analysis reveal a single evolutionary lineage and a pattern of continuous nucleotide change, identical to recent epidemic and pandemic histories of influenza A viruses resulting from continuous H3N2 antigenic drift [Abed

et al., 2002; Redlberger et al., 2007]. Previous researchers have reported H3 HA gene evolution as a single lineage following the introduction of H3N2 viruses into human populations in 1968; H1N1 viruses in particular represent distinct co-circulating lineages [Hay et al., 2001; Bragstad et al., 2008]. However, the previous data indicate the disappearance and reemergence of H1N1 circulating initially from 1918 to 1957 and reappearing in 1977, with the new strains sharing considerable similarities with those circulating in the 1950s [Hsieh

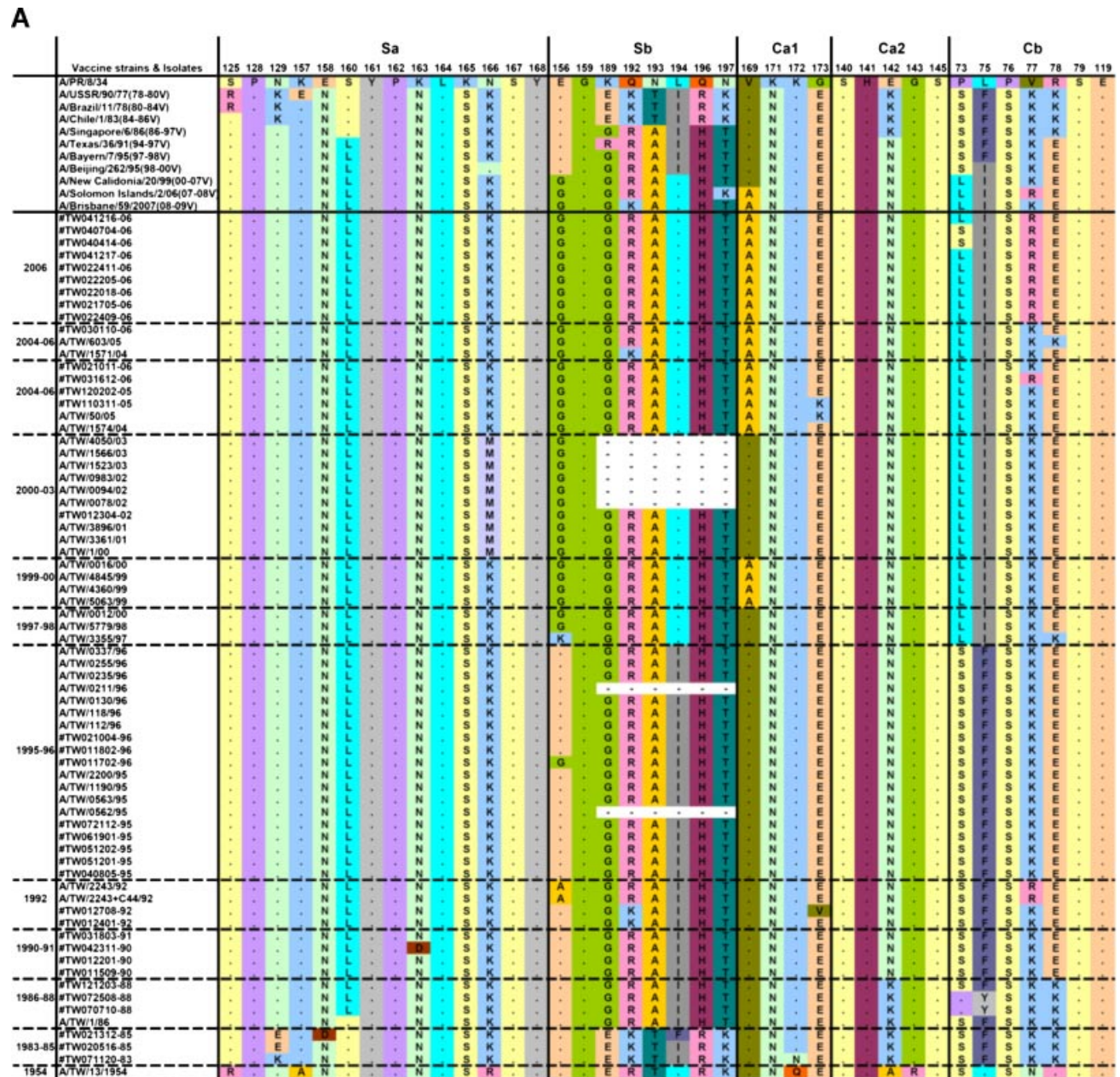


Fig. 2. Amino acid substitution patterns on major antigenic sites of the human influenza A virus in Taiwan from 1980 to 2006. **A:** Synonymous and non-synonymous amino acid changes on antigenic sites Sa, Sb, Ca1, Ca2, and Cb of the HA1 gene of the H1N1 influenza virus, corresponding to residues 12–197 of A/PR/8/34. Periods (labeled with bold dashed lines) were identified based on amino acid signature patterns and phylogenetic tree results. Some Taiwanese reference

strains (non-bold dashed lines) lack information for partial Sb regions. **B:** Synonymous and non-synonymous amino acid changes on antigenic sites A, B, C, D, and E of the HA1 gene of the H3N2 influenza virus, corresponding to residues 103–280 of A/HongKong/1/86. Periods (labeled with bold dashed lines) were identified based on amino acid signature patterns and phylogenetic tree results.



**B**

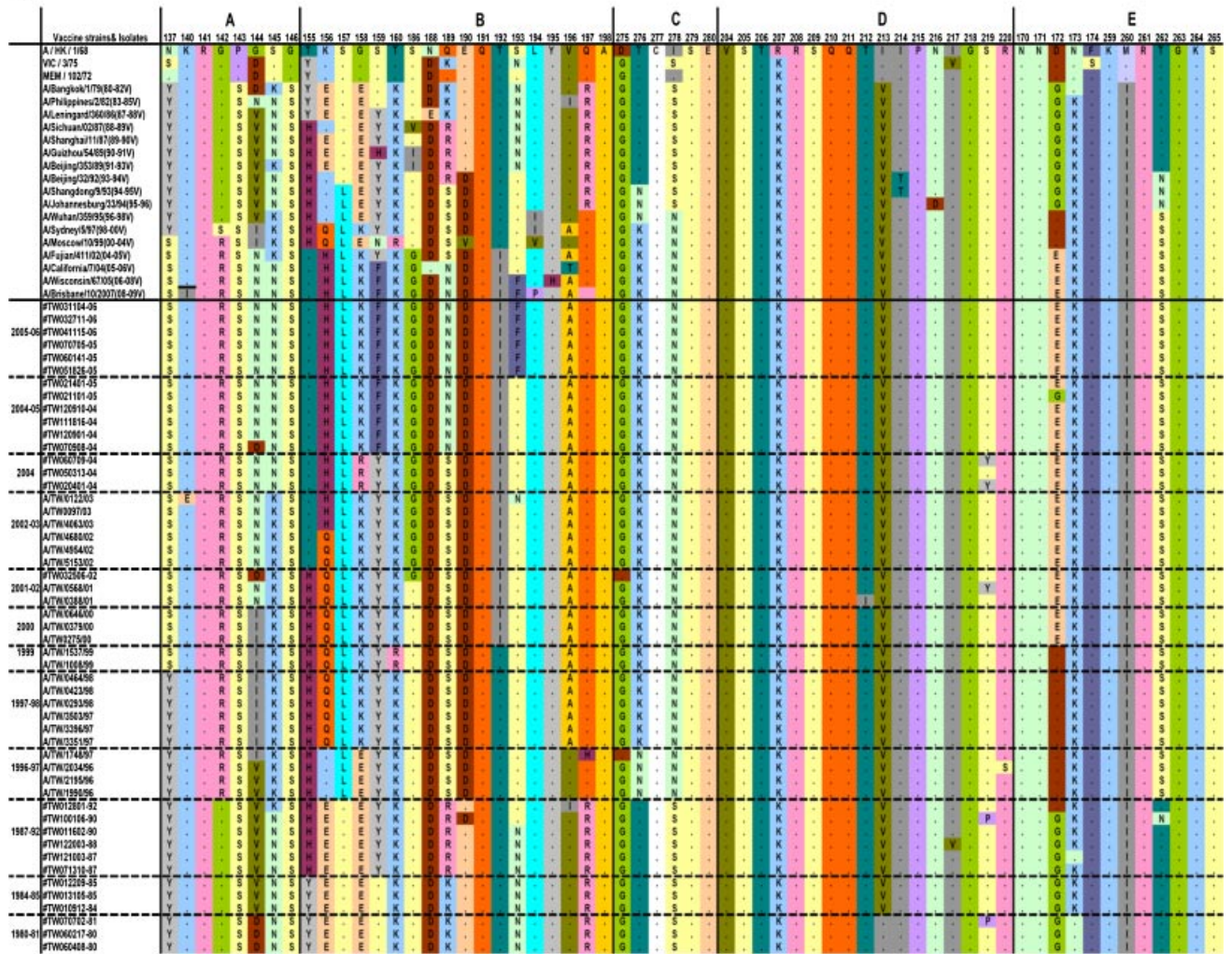


Fig. 2. (Continued)

et al., 2005]. The present results also show two separate H1N1 lineages originating in 1992, supporting the findings of Shih et al. [2005] that H1N1 isolates in Taiwan from 1995 to 2003 can be divided into two major groups.

The results from the phylogenetic analyses and antigenic site comparisons indicate higher evolutionary rates in the five H3N2 antigenic regions compared to their H1N1 counterparts. An average of 1–3 amino acid changes at H3N2 and H1N1 antigenic sites were found over 1- to 2- and 1- to 4-year intervals, respectively. According to Smith et al. [2002], new drift variants of epidemiologic importance generally have four or more amino acid substitutions located in two or more antigenic sites on HA molecules. The present results also indicate that the identities of the amino acid sequences in the HA1 genes of Taiwanese isolates were at times lower than those of the nucleotide sequences. These findings indicate the dominance of non-synonymous change in the antigenic sites of human influenza A viruses, especially in the H3N2 subtype. Jian et al.

[2008] reported significantly more non-synonymous than synonymous nucleotide substitutions at these antigenic sites. Plotkin and Dushoff [2003], who studied influenza A gene sequence evolution for the past two decades, state that HA codons, especially those in antibody-binding regions, express a significant bias toward substitution mutations compared to other internal viral genes. The results from Hsieh et al. [2005] and Tseng et al. [1996] also suggest that among Taiwan epidemic strains, H3N2 shows the highest antigenic variation, followed by type B and H1N1. These findings indicate that non-synonymous changes in the HA1 gene, especially in antigenic regions, have been more frequent in H3N2 than in H1N1 over the past 25 years in Taiwan, perhaps triggering outbreaks and/or epidemics in this country.

HI assays are used most commonly for measuring antigenic differences in epidemic strains, an important aspect of influenza virus surveillance [Sheth and Jhala, 1976; Ikic et al., 1977]. The HI test results of the present study indicate that epidemic strains and vaccine strains

TABLE III. Antigenic Relationships Between Taiwan Epidemic Isolates and Corresponding Vaccine Strains

Year	Number of isolates	Antigenic characterized by HI (-like strain)	Vaccine strains	Number of N-glycosylation sites	Antigenic relation
1980	H3N2: 2	A/Bangkok/1/79	A/Bangkok/1/79	2	Yes
	H1N1: N/A	N/A	A/USSR/90/77	—	N/A
1981	H3N2: 1	A/Bangkok/1/79	A/Bangkok/1/79	1	Yes
	H1N1: N/A	N/A	A/Brazil/11/78	—	N/A
1983	H3N2: N/A	N/A	A/Bangkok/1/79	—	N/A
	H1N1: 1	A/Brazil/11/78	A/Brazil/11/7	5	No
1984	H3N2: 1	A/Philippines/2/82	A/Philippines/2/82	3	Yes
	H1N1: N/A	N/A	A/Brazil/11/78	—	N/A
1985	H3N2: 2	A/Philippines/2/82	A/Philippines/2/82	3	Yes
	H1N1: 2	A/Chile/1/83	A/Chile/1/83	6	Yes
1987	H3N2: 2	A/Sichuan/2/87	A/Mississippi/1/85	3	No
	H1N1: N/A	N/A	A/Christchurch/4/84	—	N/A
1988	H3N2: 1	A/England/427/88	A/Singapore/6/86	—	N/A
	H1N1: 3	A/Singapore/6/86	A/Leningrad/360/86	3	No
	H1N1: 3	A/Singapore/6/86	A/Singapore/6/86	7	Yes
1990	H3N2: 2	A/Guizhou/54/89; A/Beijing/353/89	A/Shanghai/11/87	3	No
	H1N1: 2	A/Singapore/6/86	A/Singapore/6/86	6–7	Yes
1991	H3N2: N/A	N/A	A/Guizhou/54/89	—	N/A
	H1N1: 1	A/Texas/36/91	A/Singapore/6/86	7	Yes
1992	H3N2: 1	A/Beijing/353/89	A/Beijing/353/89	2	Yes
	H1N1: 2	A/Texas/36/91	A/Singapore/6/86	7	Yes
1995	H3N2: N/A	N/A	A/Shangdon/9/93	—	N/A
	H1N1: 5	A/Bayern/7/95	A/Singapore/6/86	7	No
1996	H3N2: N/A	N/A	A/Johannesburg/33/94	—	N/A
	H1N1: 3	A/Bayern/7/95	A/Singapore/6/86	7	No
2002	H3N2: 1	A/Panama/2007/99	A/Moscow/10/99	4	Yes
	H1N1: 1	A/New Caledonia/20/99	A/New Caledonia/20/99	7	Yes
2004	H3N2: 7	A/Fujian/411/02 (3); A/California/7/04 (4)	A/Moscow/10/99	4–5	No
	H1N1: N/A	N/A	A/New Caledonia/20/99	—	N/A
2005	H3N2: 5	A/California/7/04 (2); A/Wisconsin/67/05 (3)	A/Fujian/411/02	5	No
	H1N1: 2	A/New Caledonia/20/99	A/New Caledonia/20/99	6–7	Yes
2006	H3N2: 3	A/Wisconsin/67/05	A/California/7/04	5	No
	H1N1: 12	A/New Caledonia/20/99 (2); A/Solomon Islands/3/06 (10)	A/New Caledonia/20/99	7	Yes; No

N/A, no available data.

Numbers of isolates are displayed within brackets.

were less similar in terms of antigenicity, and that vaccine-like strains often circulated in Taiwan 1–2 years before other global locations (Tables III and IV). The second finding was confirmed by phylogenetic analyses and comparisons of amino acid substitution patterns at antigenic sites, that is, the Taiwanese epidemic strains not only clustered with later vaccine strains, but also displayed similar amino acid signature patterns. In addition, Hsieh et al. [2005] described the initial appearance of strains in Taiwan, including the A/Beijing/262/95(H1N1)-like strain (used for the 1998–1999 vaccine) that was found in France, Senegal, South Africa, and the United States; this strain was isolated in Taiwan in 1996–1997. Shih et al. [2005] also reported on the isolation of the A/Fujian/411/2002(H3N2)-like strain in Taiwan in 2003–2004, 1 year prior to its proposed use as a vaccine strain. In the present study, a comparison of WHO-recommended and Taiwanese epidemic strains for H1N1 and H3N2 produced matching rates of 82.1% and 67.8%, respectively. Compare these with H1N1 vaccine match rates from 1987/1988 to 2003/2004 of 82% for Taiwan and 98.6% for Canada, and with H3N2 vaccine match rates of 53% for Taiwan and 64.9% for Canada [Hsieh et al., 2005].

Other researchers have described a 1- to 2-year time lag between WHO-recommended vaccine strains for northern hemisphere nations and circulating strains in Taiwan and western countries [Hsieh et al., 2005; Shih et al., 2005]. Mismatches between Taiwanese epidemic and vaccine strains may be due to the unique location of Taiwan on the boundary between north and south Asia and its proximity to China, which is considered the epicenter of influenza viruses. New antigenic drift variants may spread into Taiwan by cross-border travel or migrating birds. A second possible explanation is the 6- to 9-month lag time between the selection of vaccine strain and manufacture, a phenomenon also observed in European and North American countries [de Jong et al., 2000; Carrat and Flahault, 2007; Skowronski et al., 2007].

Compared to the results from phylogenetic analyses and antigenic subtypes, the data described in this article suggest a strong association between HA genetic variation and antigenic changes in influenza isolates used to link epidemic and vaccine strains. Due to the limited availability of HI testing equipment in clinical virology laboratories in Taiwan, there may be a greater reliance at present on HA sequencing for evaluating and



TABLE IV. (Continued)

H1N1 vaccine and epidemic strains				H3N2 vaccine and epidemic strains			
Years (Oct. to Sept.)	WHO recommended	World strain	Taiwan isolates (-like)	Years (Oct. to Sept.)	WHO recommended	World strain	Taiwan isolates (-like)
2000/01	A/New Caledonia/20/99	A/New Caledonia/20/99	A/New Caledonia/20/99	2000/01	A/Moscow/10/99	A/Moscow/10/99	A/Panama/2007/99
2001/02	A/New Caledonia/20/99	A/New Caledonia/20/99	A/New Caledonia/20/99	2001/02	A/Moscow/10/99	A/Moscow/10/99	A/Panama/2007/99
2002/03	A/New Caledonia/20/99	A/New Caledonia/20/99	A/New Caledonia/20/99	2002/03	A/Moscow/10/99	A/Moscow/10/99	A/Panama/2007/99
2003/04	A/New Caledonia/20/99	A/New Caledonia/20/99	A/New Caledonia/20/99	2003/04	A/Moscow/10/99	A/Moscow/10/99	A/Fujian/411/02 <sup>a,b</sup>
2004/05	A/New Caledonia/20/99	A/New Caledonia/20/99	A/New Caledonia/20/99	2004/05	A/Fujian/411/02	A/Fujian/411/02	A/Panama/2007/99
2005/06	A/New Caledonia/20/99	A/New Caledonia/20/99	A/Solomon Islands/3/06 <sup>a,b</sup>	2005/06	A/California/7/04	A/California/7/04	A/California/7/04 <sup>a,b</sup>
2006/07	A/New Caledonia/20/99	A/New Caledonia/20/99	A/Solomon Islands/3/06 <sup>a,b</sup>	2006/07	A/Wisconsin/67/05	A/Wisconsin/67/05	A/Wisconsin/67/05

N/A, no available data.

<sup>a</sup>Mismatch between WHO-recommended vaccine and epidemic strains in Taiwan.

<sup>b</sup>Epidemic strain circulated in Taiwan earlier than in other countries and prior to WHO vaccine recommendation.

comparing differences between local epidemic isolates and vaccine strains.

Surface protein glycosylation plays an important role in the biology of many viruses, including the severe acute respiratory syndrome coronavirus (SARS-CoV) [Shih et al., 2006], hepatitis C virus [Goffard et al., 2005], West Nile virus [Hanna et al., 2005], HIV-1 [Bashirova et al., 2001], and influenza viruses [Roberts et al., 1993]. Surface glycosylation may help these viruses enter target cells and evade host immune responses [Fournillier et al., 2001; Abe et al., 2004]. HIV-1 and influenza A viruses have been described as using glycosylated gp120 and HA proteins to interact with and mediate entry via DC-SIGN and mannose molecules on dendritic cells [Reading et al., 2000; Bashirova et al., 2001; Wang et al., 2008]. However, N-linked glycosylation on the HA proteins of influenza viruses has been reported as serving four important biological functions: (a) adding carbohydrates to antigenic sites, which interferes with neutralizing antibody access [Skehel et al., 1984]; (b) influencing virus pathogenicity when it occurs near cleavage sites [Ohuchi et al., 1989]; (c) sialylating oligosaccharide chains near receptor-binding sites, which interferes with viral binding [Ohuchi et al., 1995]; and (d) promoting the proper folding and maintenance of protein conformation or stability [Roberts et al., 1993]. According to the present results, N-linked glycosylation sites on partial HA1 proteins in both H1N1 and H3N2 have gradually increased over time. When describing this same phenomenon, Vigerust et al. [2007] and Zhang et al. [2004] also described human influenza A HA H3 and H1 HA1 accumulating sequons over time, and a gradual increase in N-linked glycosylation in and around the globular head of H3N2 viruses during the past four decades. Vigerust et al. [2007] suggest that the increased glycosylation of influenza viruses may result in decreased virulence.

The ongoing surveillance of influenza virus activity is essential for preventing human pandemics or severe epidemics. With this goal in mind, the Taiwan Centers for Disease Control and Prevention established a national influenza surveillance network in 2000 [Shih et al., 2005; Jian et al., 2008]. However, its work has been hindered by limited information on epidemic strains prior to 2000. This article described the genetic and antigenic characteristics of the HA gene in influenza A isolates from 1980 to 2006 and addresses Taiwan's vaccine mismatch phenomenon. Other gene segments of these isolates are being studied.

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