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Laboratory-Based Surveillance and Molecular Epidemiology of Influenza Virus in Taiwan

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A laboratory-based surveillance network of 11 clinical virological laboratories for influenza viruses was established in Taiwan under the coordination of the Center for Disease Control and Prevention (CDC), Taiwan. From October 2000 to March 2004, 3,244 influenza viruses were isolated, including 1,969 influenza A and 1,275 influenza B viruses. The influenza infections usually occurred frequently in winter in the northern hemisphere. However, the influenza seasonality in Taiwan was not clear during the four seasons under investigation. For example, the influenza A viruses peaked during the winters of 2001, 2002, and 2003. However, some isolated peaks were also found in the summer and fall (June to November) of 2001 and 2002. An unusual peak of influenza B also occurred in the summer of 2002 (June to August). Phylogenetic analysis shows that influenza A isolates from the same year were often grouped together. However, influenza B isolates from the year 2002 clustered into different groups, and the data indicate that both B/Victoria/2/87-like and B/Yamagata/16/88-like lineages of influenza B viruses were cocirculating. Sequence comparison of epidemic strains versus vaccine strains shows that many vaccine-like Taiwanese strains were circulating at least 2 years before the vaccine strains were introduced. No clear seasonality of influenza reports in Taiwan occurred in contrast to other more continental regions.

Influenza virus is a major viral respiratory pathogen that can cause severe illness. It belongs to the family *Orthomyxoviridae*, which is characterized by a segmented, minus-stranded RNA genome. Influenza viruses have been responsible for several human pandemics in recent history, for example, the Spanish H1N1 influenza pandemic in 1918, which killed 20 to 40 million people globally. Influenza epidemics also occur annually in many parts of the world and cause high mortality and morbidity (24, 36, 37, 44, 46, 48). The low fidelity of the viral polymerase complex, as well as host immune selection, accounts for the accumulation of point mutations in hemagglutinin (HA) and neuraminidase genes, producing the antigenic drift on these surface glycoproteins. The antigenic drift plays an important role in the occurrence of influenza epidemics (31). Gene reassortment is the other mechanism attributed to influenza virus mutation. An antigenic shift occurs in the event of a reassortment between two viruses of different surface antigenic subtypes, leading to a novel subtype composition (21, 39, 43). For example, the pandemic influenza A virus subtype H3N2 in 1968 and the subtype H2N2 in 1957 resulted from reassortment between avian and human viruses (2, 28, 47). Because of the high mutation rate of influenza virus, continuously monitoring viral genetic change is essential.

The influenza virus surveillance system has been well established in many countries and has greatly contributed to the control of influenza virus infections. The influenza surveillance work in Taiwan, however, was not well organized until a laboratory-based surveillance network of influenza viruses was established in 2000 under the coordination of the Center for Disease Control and Prevention (CDC)-Department of Health, Taiwan. This network includes 11 clinical virology laboratories geographically distributed in northern, central, southern, and eastern Taiwan. This study described the results of analyzing those influenza viruses collected from this surveillance network.

MATERIALS AND METHODS

Clinical virology laboratories. Eleven clinical virology laboratories participated in this study are distributed in northern, central, southern, and eastern Taiwan. All of the laboratories have passed the proficiency tests of viral diagnosis (organized by the Taiwanese CDC) every half-year, and the principal investigators of the laboratories attended monthly meeting for information sharing and technology discussions. The Clinical Virology Laboratory of Chang Gung Memorial Hospital (Linkou, Taoyuan) has been accredited by the College of American Pathologists' Laboratory Accreditation Program since 2003.

Cases. A total of 32,775 patients who were suspected of having respiratory tract infections during the study period from October 2000 to March 2004 were

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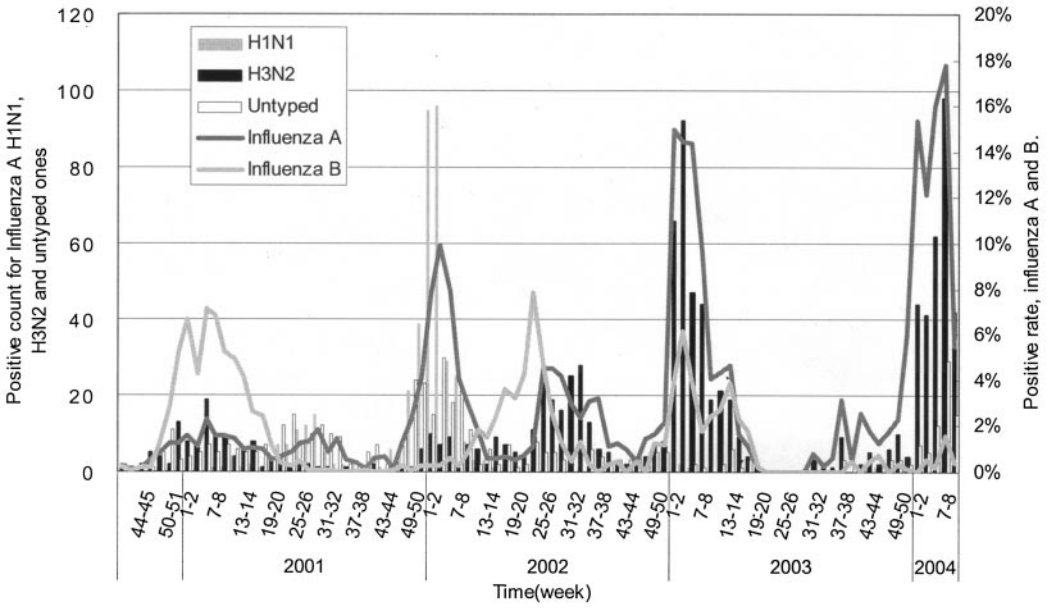

FIG. 1. Positive rate of influenza viral isolation in Taiwan and counts for different influenza A subtypes, 2000 to 2004. Positive count indicates the number of samples positive for influenza virus isolation among all respiratory-tract specimens. The positive rate indicates the percentage of the number of influenza virus positive samples, divided by the total number of respiratory tract specimens.

investigated. The symptoms of respiratory tract infections include cough, sore throat, tonsillitis, pharyngitis, pneumonia, and bronchiolitis. Infected patients with one or more of these symptoms were included in this study.

Specimen collection and transportation. Throat swabs or nasopharyngeal aspirates were obtained from hospitalized patients and outpatients with symptoms of respiratory infections. Throat swabs were collected into transport medium containing 2 ml of Eagle's minimum essential medium (EMEM) (pH 7.2) with gelatin (5 mg/liter), penicillin (400 U/liter), streptomycin (400 μg/liter), gentamicin (50 μg/liter), and amphotericin B (Fungizone) (1.25 μg/liter). Specimens were placed on ice and transported to each clinical virology laboratory within 24 h after collection.

Virus isolation and identification. Respiratory specimens were inoculated onto appropriate tissue cultures (Madin-Darby canine kidney [MDCK], Vero, A549, and rhabdomyosarcoma) to isolate influenza virus or other respiratory tract viruses, such as parainfluenza virus, adenovirus, respiratory syncytial virus, enterovirus, and herpesvirus. Cells were cultured in EMEM (supplemented with 10% fetal bovine serum, penicillin [100 U/ml], streptomycin [100 μg/ml], and amphotericin B [0.25 μg/ml]) and incubated at 35°C with 5% CO_2. Each culture tube was inoculated with 0.2 ml of clinical specimen and incubated for 1 h to allow for adsorption, and then viral growth medium was added. The viral growth medium for Vero, A549, and rhabdomyosarcoma cells was EMEM containing 2% fetal bovine serum and antibiotics; that for MDCK cells was serum-free EMEM with 2 μg of bovine pancreatic crystalline trypsin per ml to promote growth of influenza virus. These culture tubes were incubated at 35°C and examined for cytopathic effect daily for 10 to 14 days (20, 22, 52). Hemadsorption and hemagglutination were done with MDCK cell cultures to detect HA-containing viruses, such as influenza virus and parainfluenza virus, and final identification was performed with a screening kit for respiratory viruses (Chemicon International, Inc.) (22, 26). All of the influenza viruses (3,244 isolates) were typed by immunofluorescent assay by type-specific monoclonal antibodies (Dako, Cambridgeshire, United Kingdom). Furthermore, 1,462 influenza A virus isolates were subtyped by reverse transcription-PCR (RT-PCR) using subtype-specific primers (49, 51); the other 507 influenza A isolates remained to be subtyped. Sixty-one influenza A H1N1 isolates and 152 influenza A H3N2 isolates were randomly selected and subtyped by a hemagglutination inhibition (HI) assay with the World Health Organization (WHO) influenza reagent kit, kindly provided by the Centers for Disease Control and Prevention, Atlanta, Ga.; of these, 54 influenza A H1N1 and 135 influenza A H3N2 isolatges with higher titers were further subjected to sequence analysis.

RNA extraction and RT-PCR for subtyping. The clinical isolates were passed in MDCK cells, and the supernatant was used for viral RNA extraction with the Viral RNA Extraction Miniprep System kit (Viogene, Sunnyvale, Calif.). Viral RNA was amplified into double-stranded DNA by RT-PCR with Ready-To-Go RT-PCR beads (Amersham Biosciences, Piscataway, N.J.) (3, 30). The following RT-PCR program was used in all cases: 42°C for 30 min; 95°C for 5 min; 40 cycles, each consisting of 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; and a final elongation step of 72°C for 10 min. The final product was stored at 4°C.

HI test. A total of 25 μl (100 PFU) of virus suspension was mixed with ferret antisera (kindly provided by the Centers for Disease Control and Prevention) at room temperature for 1 h. The mixture was twofold serial diluted with phosphate-buffered saline. Subsequently, 50 μl of 0.5% guinea pig red blood cells was added at 4°C. The HI result was read after 2 h of incubation.

Nucleotide sequence analysis. The RT-PCR product was purified using the QIAquick Gel Extraction kit (QIAGEN, Valencia, Calif.) and subjected to sequencing. The nucleotide sequence of the purified fragments was determined with an automated DNA sequencer. A 495-nucleotide (nt) HA sequence was obtained for H1N1 isolates from genomic positions 141 to 635, based on A/Puerto Rico/8/34 (H1N1) (NC 002017); a 789-nt sequence was obtained for H3N2 isolates from genomic positions 214 to 1002, based on A/Hong Kong/1/68 (H3N2) (AF348176); and a 393-nt sequence was obtained for influenza B isolates from genomic positions 403 to 795, based on B/Lee/40 (NC 002207). The choice for the study of those specific genomic regions was based on the availability of primers for RT-PCR and subtyping.

Sequence analysis, including pairwise sequence alignment and protein translation, was performed with Lasergene software, version 3.18 (DNASTAR, Madison, Wis.) (5). Multiple sequence alignment was conducted with CLUSTAL W, version 1.81 (41), with a gap-opening penalty of 15 and a gap extension penalty of 6.66. Phylogenetic analysis was performed with PHYLIP (33, 38), version 3.573c, with a Kimura two-parameter distance matrix (program DNADIST) and the neighbor-joining method (program NEIGHBOR). Support for tree topology was determined via bootstrap analysis with 1,000 pseudoreplicate data sets generated with the SEQBOOT program in PHYLIP. A consensus tree was obtained using the CONSENSE program, and the topology was viewed with TreeView, version 1.6.6 (32). Computation of synonymous and nonsynonymous mutations was based on syn-SCAN (19).

Nucleotide sequence accession number. The nucleotide sequence data reported in this work were deposited in the GenBank nucleotide sequence data-

TABLE 1. Epidemic strains in Taiwan versus the vaccine strains through HI tests

Season (mo yr)	Epidemic strain[g]	Vaccine strain[a]
Oct. 1999–Sept. 2000	**A/Panama/2007/99(H3N2)-like**	A/Sydney/5/97(H3N2)-like
	A/Sydney/5/97(H3N2)-like	
	A/New Caledonia/20/99(H1N1)-like	A/Beijing/262/95(H1N1)-like
	B/Beijing/184/93-like	B/Beijing/184/93-like
Oct. 2000–Sept. 2001	A/Panama/2007/99(H3N2)-like	A/Moscow/10/99(H3N2)-like[b]
	A/New Caledonia/20/99(H1N1)-like	A/New Caledonia/20/99(H1N1)-like
	B/Yamanashi/166/98-like	B/Beijing/184/93-like[c]
Oct. 2001–Sept. 2002	A/Panama/2007/99(H3N2)-like	A/Moscow/10/99(H3N2)-like
	A/New Caledonia/20/99(H1N1)-like	A/New Caledonia/20/99(H1N1)-like
	B/Sichuan/379/99-like	B/Sichuan/379/99-like
	B/Hong Kong/330/2001-like	
Oct. 2002–Sept. 2003	A/Panama/2007/99(H3N2)-like	A/Moscow/10/99(H3N2)-like
	No influenza A(H1N1)	A/New Caledonia/20/99(H1N1)-like
	B/Hong Kong/330/2001-like	B/Hong Kong/330/2001-like
Oct. 2003–Sept. 2004[d]	A/Panama/2007/99(H3N2)-like	A/Moscow/10/99(H3N2)-like
	A/Fujian/411/2002(H3N2)-like	
	A/New Caledonia/20/99(H1N1)-like	A/New Caledonia/20/99(H1N1)-like
	B/Hong Kong/330/2001-like	B/Hong Kong/330/2001-like
Oct. 2004–Sept. 2005	N/A[f]	A/Fujian/411/2002(H3N2)-like[e]
		A/New Caledonia/20/99(H1N1)-like
		B/Hong Kong/330/2001-like

[a] Recommended by WHO for the northern hemisphere.
[b] A/Panama/2007/99(H3N2)-like was used for vaccine production.
[c] B/Yamanashi/166/98-like was used for vaccine production.
[d] Data shown are up to March 2004.
[e] A/Wyoming/3/2003(H3N2)-like was used for vaccine production.
[f] N/A, not applicable.
[g] Boldface type indicates strain different from the vaccine strain.

base with accession numbers AY604809 to AY604830, AY604795 to AY604808, and AY604740 to AY604794.

RESULTS

Laboratory surveillance of influenza virus in Taiwan. From October 2000 to March 2004, 3,244 influenza viruses were isolated by this surveillance network, including 1,969 influenza A viruses and 1,275 influenza B viruses. Among influenza A viruses, 420 were H1N1 subtype and 1,042 were H3N2, and the others remained to be subtyped. Figure 1 shows the positive rate of influenza isolation, and the subtyping result for influenza A viruses. In Taiwan, the four seasons were generally recognized as spring (March to May), summer (June to August), fall (September to November), and winter (December to February). The average temperatures during spring, summer, fall, and winter in Taiwan from 2000 to 2004 were 21.5, 28.3, 23.4, and 16.1°C. The influenza seasonality was not clear during the four seasons under investigation. For example, the influenza A viruses peaked during the winters of 2001, 2002, and 2003. However, some isolated peaks were also found in the summer and fall of 2001 and 2002. An unusual peak of influenza B also occurred in the summer of 2002. It is shown that influenza B was the major circulating strain during the 2000–2001 season. Meanwhile, influenza A H1N1 was the major circulating strain for 2001 and 2002. Subsequently, influenza A H3N2 became dominant during the last two seasons. Table 1 lists the result of the HI test for the isolates during recent seasons. The H1N1 epidemic strain in Taiwan for the reported seasons was A/New Caledonia/20/99 (H1N1)-like, which was the selected vaccine strain since the winter of 2000. In fact, A/New Caledonia/20/99(H1N1)-like strain was reported circulating in Taiwan as early as 1998 (16). A partial mismatch of the epidemic strain versus the vaccine strain was also noted for several seasons, including A/Panama/2007/99(H3N2)-like viruses in the 1999–2000 season, B/Hong Kong/330/2001-like viruses in the 2001–2002 season, and A/Fujian/411/2002 (H3N2)-like viruses in the 2003–2004 season (listed in boldface type in Table 1).

HA sequence analysis of the epidemic strains. Figure 2A shows the phylogenetic relationships of HA for H1N1 strains, including 38 selected Taiwanese strains from 1995 to 2003 and nine reference strains. Influenza A H1N1 HA sequences of isolates from 1995 to 2000 were obtained from Chang Gung Memorial Hospital and were previously reported before the establishment of the influenza surveillance network (1). Among the Taiwanese influenza A H1N1 isolates from 2001 to 2004, sequences of the first two isolates every month in winter (December to February) and the first isolate every month in other seasons were chosen for phylogenetic analysis. The sequence identity among the Taiwanese isolates was 92.1 to 100%, based on a 492- to 495-nt HA segment. The most dissimilar pairs occurred between one 2002 isolate, A/Taiwan/0032/02, and three isolates gathered from 1995 to 1996, A/Taiwan/1190/95, A/Taiwan/0255/96, and A/Taiwan/0342/96. Eleven isolates

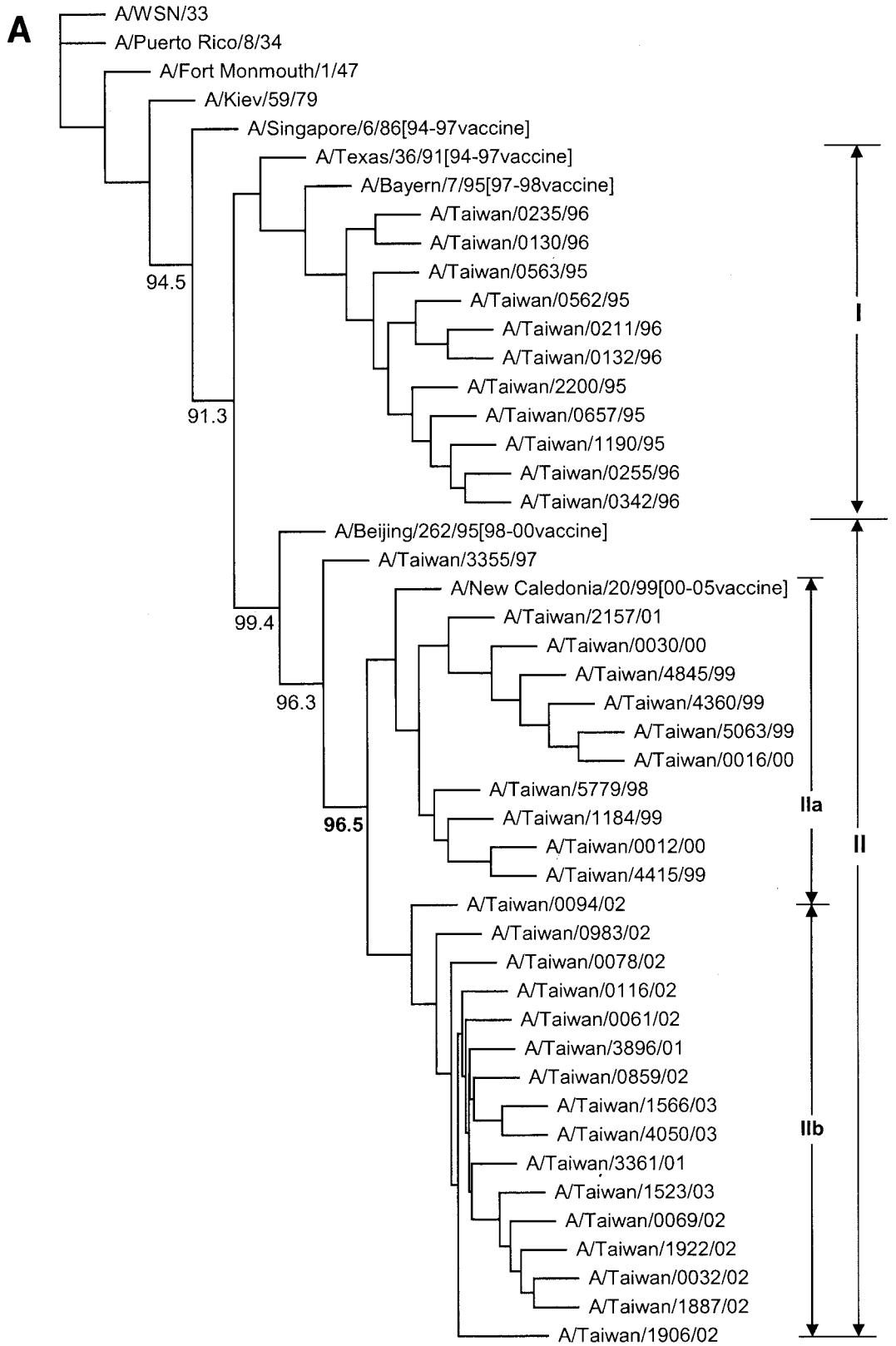

FIG. 2. Sequence analysis of influenza A (H1N1) viruses in Taiwan. (A) Phylogenetic relationship of influenza A H1N1 subtype. HA nucleotide sequences used are 495-nt long, from positions 141 to 636 based on A/Puerto Rico/8/34(H1N1). (B) Percent identities of Taiwanese influenza A H1N1 isolates versus vaccine strains from 1995 to 2003. White arrows indicate that those strains were isolated at the corresponding season(s) when respective vaccine strains were selected by the WHO, and black arrows indicate the strains with high similarity with the vaccine strains before they were formally selected by the WHO.

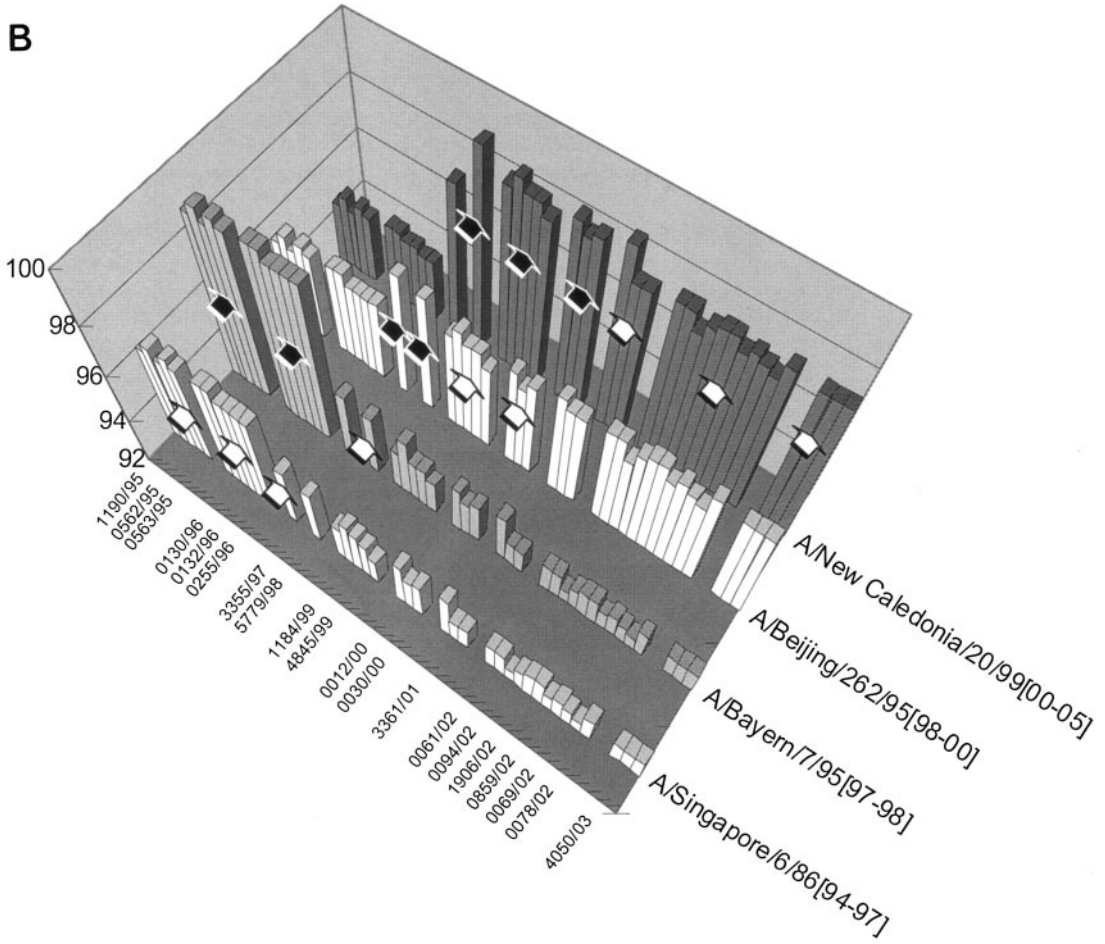

FIG. 2—*Continued.*

gathered from 1995 to 1996 shown in Fig. 2A as group I clustered with A/Bayern/7/95 (8, 9), the 1997–1998 vaccine strain, with a nucleotide sequence identity from 98.5 to 98.9%. The other 27 1997–2003 Taiwanese strains formed group II, with 96.7 to 100% identity. There was only one 1997 isolate, A/Taiwan/3355/97, which was less similar to A/Beijing/262/95 (96.9%, 1998–2000 vaccine strain) (10, 11) and closer to A/New Caledonia/20/99 (97.9%, 2000–2005 vaccine strain) (12–15). This 1997 isolate was found to disjoin from the remaining Taiwanese isolates in group II. Ten 1998–2001 isolates, shown in Fig. 2A as subgroup IIa, clustered with A/New Caledonia/20/99, with a nucleotide sequence identity from 98.5 to 99.7%. The more recent 16 2001–2003 isolates were separated into subgroup IIb with a slightly lower nucleotide sequence identity of 97.7 to 98.3% to A/New Caledonia/20/99. One 2001 isolate, A/Taiwan/2157/01 in subgroup Iia, was 98.9 to 99.1% identical to the other three 2000 isolates in the same subgroup. However, this isolate was less similar (96.5%) to the two 2001 isolates found in subgroup IIb. Using the 1995 Taiwanese isolates as a baseline, both the numbers of synonymous and nonsynonymous changes for subsequent sequences within the 495-nt HA segment under investigation were found sharply increased from 2 substitutions in 1996 to over 20 synonymous substitutions and 10 nonsynonymous substitutions after 1997 (data not shown). In contrast to the 11 previous 1995–1996 Taiwanese H1N1 isolates, all 27 recent (1997–2003) isolates exhibited a characteristic deletion mutation Lys (K) at amino acid 134 of the HA gene, as previously reported (17). Another 11 notable site mutations also occurred, including R changing to L between C_{52} and L_{53}, T_{56} to I, S to L between E_{77} and L_{78}, F_{79} to I, A_{88} to V, KES to KGS between P_{125} and S_{126}, T_{137} to S, K_{149} to R, E_{156} to G, K_{166} to M, and S_{186} to P. Except for the mutations KES to KGS between P_{125} and S_{126} and K_{166} to M, which were observed from 2001 to 2002, all other eight mutations occurred from 1996 to 1997. Among these changes, variations at positions 78, 125, 156, and 166 have been reported to affect antigenicity (6, 23). Figure 2B compares the pairwise identities of the Taiwanese HA segments being investigated with the H1N1 vaccine strains used in recent years. Strains from the same year were grouped together. The blocks indicated by white arrows represent the percentage of identities between epidemic strains (strains isolated during the seasons when the corresponding vaccine strains were introduced) and vaccine strains. A black arrow indicates a high identity (better match) between epidemic and vaccine strains. Interestingly, the Taiwanese 1995–1996 strains (indicated by black arrows) closely matched A/Bayern/7/95 (97.4 to 97.8%), 2 years before its selection as a vaccine strain in the 1997–1998 season. Sim-

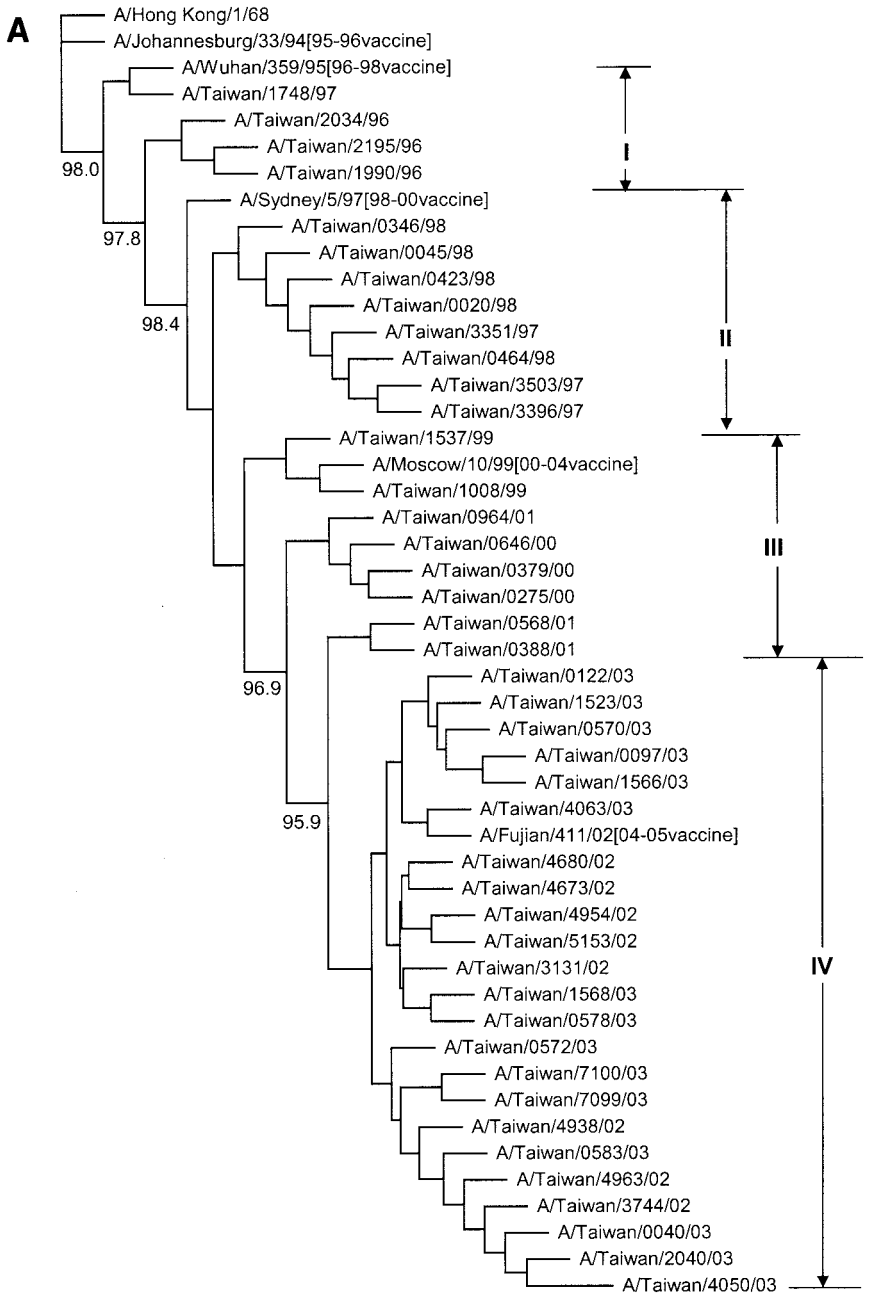

FIG. 3. Sequence analysis of influenza A (H3N2) viruses in Taiwan. (A) Phylogenetic relationship of influenza A H3N2 subtype. HA nucleotide sequences used are 789 nt long, from positions 214 to 1002 based on A/Hong Kong/1/68(H3N2). (B) Percent identities of Taiwanese influenza A H3N2 isolates versus vaccine strains from 1996 to 2003. The 2003–2004 vaccine strain suggested by WHO was A/Moscow/10/99, although A/Panama/2007/99 (vaccine strain for 2000–2002) was widely used for vaccination.

ilar observations were made for the 1997–1998 Taiwanese strains compared with A/Beijing/262/95 (1998–2000 vaccine strain with 96.8 to 97.0% identity), and for the 1998–2000 Taiwanese strains compared with A/New Caledonia/20/99 (2000–2005 vaccine strain with 98.6 to 99.8% identity).

Figure 3A shows the phylogenetic relationships of H3N2 isolates, including 43 1996–2003 Taiwanese isolates, and six reference strains. Taiwanese H3N2 isolates from 1996 to 2000 included here were previously reported from Chang Gung Memorial Hospital (16). Taiwanese H3N2 isolates obtained from 2001 to 2004 were chosen as the first two isolates every month in winter (December to February) and the first isolate every month in other seasons. The nucleotide sequence identity in terms of HA genes among Taiwanese 1996–2003 H3N2 isolates was 94.1 to 100%, based on a 789-nt HA segment, which slightly exceeded that among the 38 Taiwanese H1N1 isolates (92.1 to 100%) being investigated. The most distinct pair was between A/Taiwan/1748/97 and A/Taiwan/0097/03. Four 1996–1997 isolates were clustered to group I and were 98.4 to 98.6% identical to the 1996–1998 vaccine strain A/

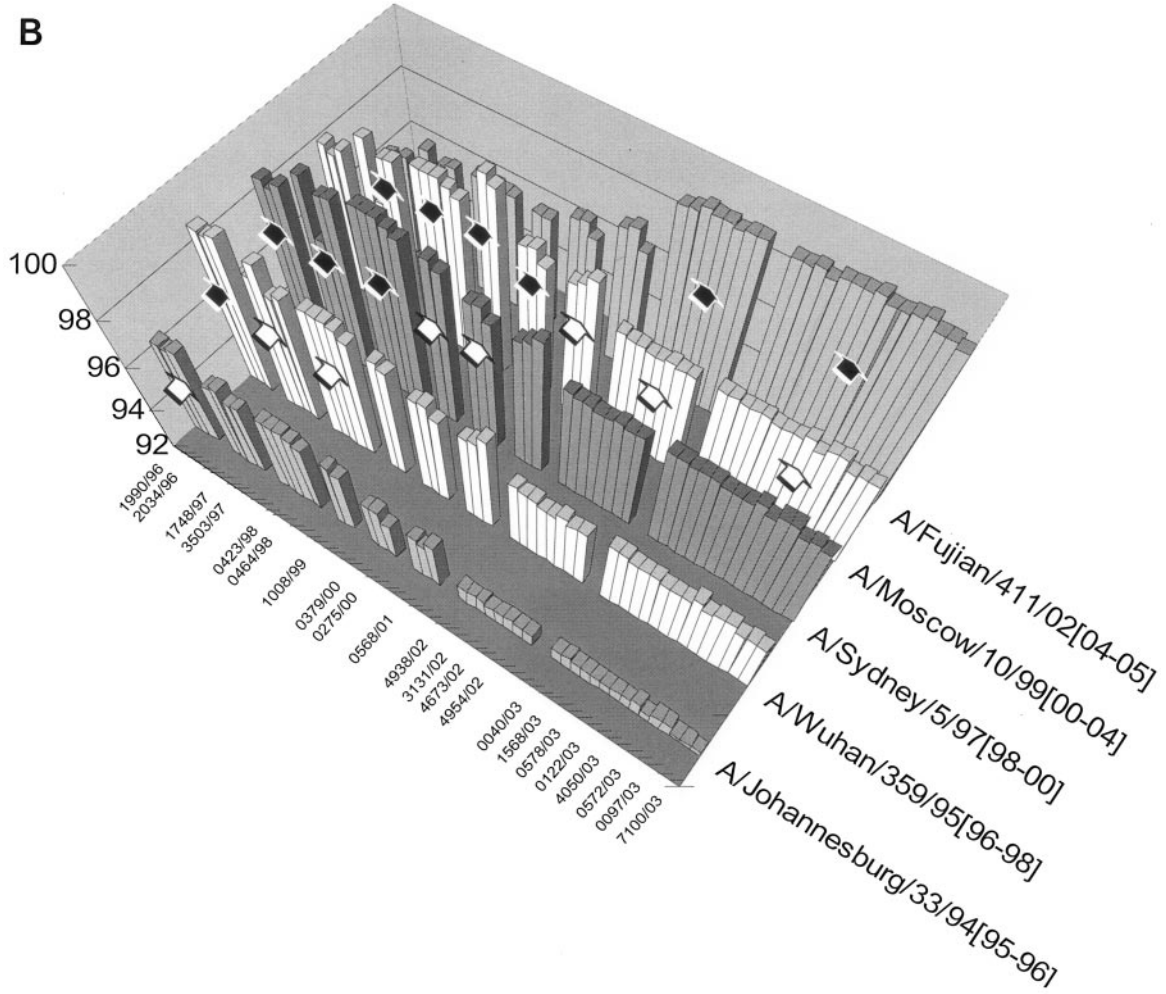

FIG. 3—*Continued.*

Wuhan/359/95 (7). Eight isolates gathered from 1997 to 1998 clustered to group II, which was 99.1 to 99.4% identical to the 1998–2000 vaccine strain A/Sydney/5/97 (9–11). Eight isolates (1999–2001) were clustered in group III, together with the 2000–2004 vaccine strain A/Moscow/10/99 (13–15) with 97.7 and 99.9% identity. All the remaining 23 2002–2003 isolates were clustered in group IV and were 99.2 to 99.7% identical to the 2004–2005 vaccine strain A/Fujian/411/02. Using the 1996 Taiwanese isolates as a baseline, the number of nucleotide changes within the 789-nt HA segment under investigation was found to gradually increase from 2 synonymous substitutions in 1997 to 10 in 2003 and from approximately 10 nonsynonymous substitutions in 1997 to approximately 25 in 2003 (data not shown). Interestingly, the number of nonsynonymous substitutions for these H3N2 isolates was found to be consistently higher than those synonymous one, while in H1N1 the number of synonymous substitutions was found to be higher after 1997. Nineteen noteworthy site mutations were identified from the aligned amino acid sequences: K_{62} to E, P_{103} to Q, V_{144} to I, K_{156} to Q, E_{158} to K, V_{196} to A, and N_{276} to K occurred from 1996 to 1997; R_{57} to Q, Q_{103} to P, and Y_{137} to S occurred from 1998 to 1999; D_{172} to E and T_{192} to I occurred from 1999 to 2000; I_{144} to N and G_{225} to D occurred from 2000 to 2001; and H_{75} to Q, E_{83} to K, A_{131} to T, H_{155} to T, S_{186} to G, V_{202} to I, and W_{222} to R occurred from 2001 to 2002. Notably, at position 103, P was changed to Q from 1996 to 1997, remained Q in 1997 and 1998, and changed back to P from 1998 to 1999. At position 144, V was changed to I between 1996 and 1997, which was further changed to N from 2000 to 2002. At position 156, K was changed to Q from 1996 to 1997, which was further changed to H in 2003. All of the amino acid positions of HA (H3) mentioned above are located in the HA antibody-combining sites except 225 (within the receptor binding sites), 202, and 222. Among these changes, variations at positions 62, 144, 156, 158, 172, 196, and 276 have been reported to affect viral antigenicity (1, 25, 27). Pairwise identities of the investigated HA segment for these Taiwanese strains versus the recently used H3N2 vaccine strains were shown in Fig. 3B. Similar to the data shown in Fig. 2B, strains shown in Fig. 3B were grouped by year, and white arrows indicate the percentage of identities between local isolates and their corresponding vaccine strains. Figure 3B clearly demonstrates that many vaccine-like Taiwanese strains (indicated by black arrows) were circulating at least 2 years before the vaccine strains were formally

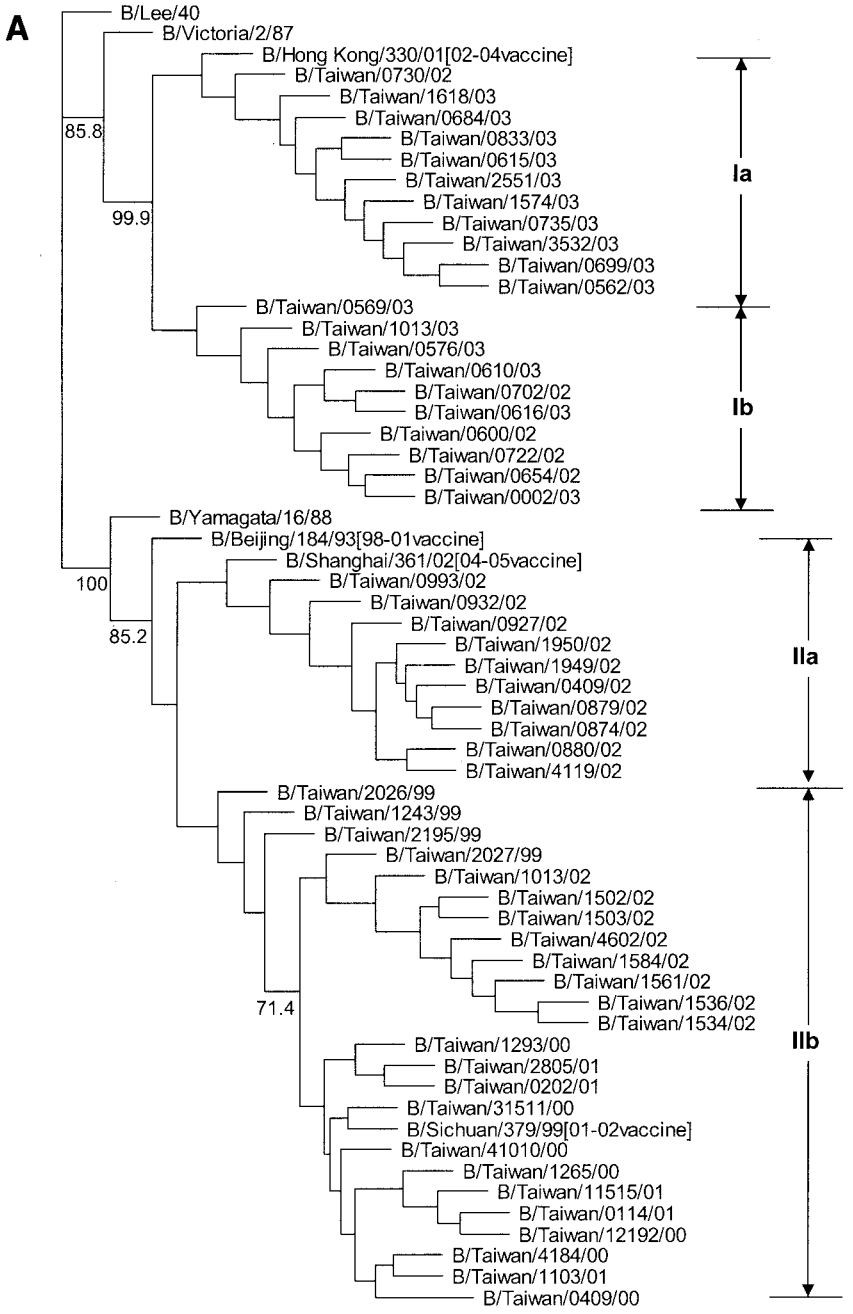

FIG. 4. Sequence analysis of influenza B viruses in Taiwan. (A) Phylogenetic relationship of influenza B virus. HA nucleotide sequences used are 393 nt long, from positions 403 to 795 based on B/Lee/40. (B) Percent identities of Taiwanese influenza B isolates versus vaccine strains from 1999 to 2003.

introduced, based on their high percent identities to the corresponding vaccine strains. For example, the Taiwanese strains from 1996 to 1997 maintained a high percent identity (97.2 to 99.4%) to A/Sydney/5/97 (1998–2000 vaccine strain), while the 1996–2000 strains matched well (96.7 to 99.9%) to A/Moscow/10/99 (2000–2004 vaccine strain). Also found was a surprisingly high percent identity (99.2 to 99.7%) for the 2002–2003 Taiwanese strains to A/Fujian/411/02, which was recently chosen as the 2004–2005 vaccine strain.

Figure 4a shows the phylogenetic relationships for influenza B isolates, including 55 Taiwanese strains from 1999 to 2003 and seven reference strains. Two reference strains—B/Victoria/2/87 and B/Yamagata/16/88—were included, since they have been known as two major antigenic-genetic lineages of influenza B viruses circulating worldwide since the mid-1980s (13). Taiwanese influenza B isolates from 1999 to 2000 were previously available from Chang Gung Memorial Hospital. Taiwanese influenza B isolates from 2001 and 2003 were chosen as the first two isolates every month in winter (December to February) and as the first isolate every month in other

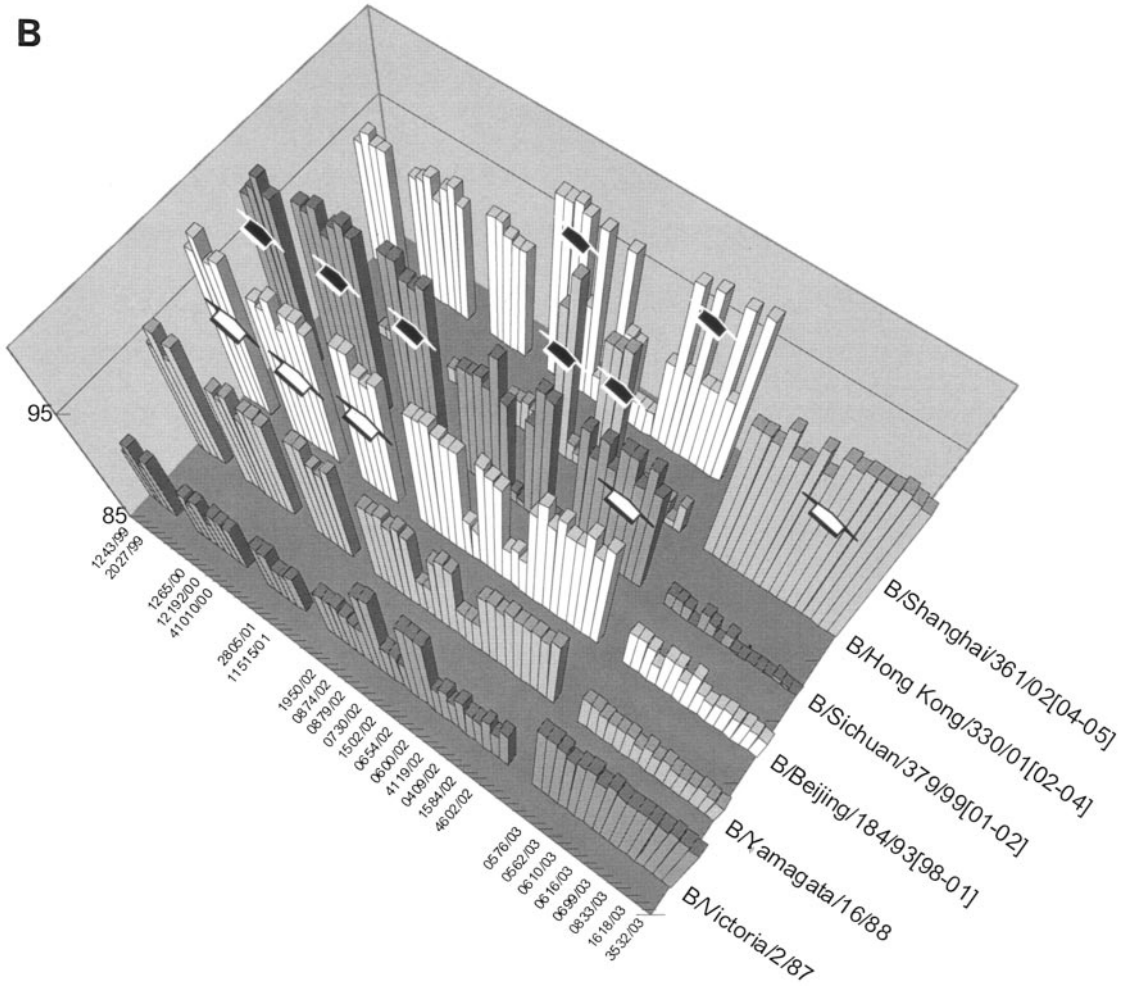

FIG. 4—*Continued.*

seasons. Taiwanese influenza B isolates in 2002, due to the distinct pattern that they showed almost throughout the year, were chosen as the first two isolates in every month. Pairwise identities among these 55 Taiwanese strains, based on a 396-nt HA segment, ranged from 84.1 to 100%. The most dissimilar pairs occurred between one 2000 isolate, B/Taiwan/12192/00, and two 2003 isolates, B/Taiwan/3532/03 and B/Taiwan/0615/03. Notably, the Taiwanese influenza B isolates accumulated significant variation in terms of HA nucleotide sequences (15.9% from 2000 to 2003), compared to the Taiwanese influenza A isolates (7.9% for H1N1 from 1995–1996 to 2002, and 5.9% for H3N2 from 1997 to 2003). Twenty-one Taiwanese 2002–2003 isolates were in line with the B/Victoria/2/87 lineage and formed group I, of which 11 were clustered together with the 2002–2004 vaccine strain B/Hong Kong/330/01 (14, 15) as group Ia. The percent identities between these 11 isolates and B/Hong Kong/330/01 were between from 98.4 and 99.4%. The other 10 Taiwanese influenza B isolates apparently were grouped into a separate group, Ib, with significant bootstrap support (999 of 1,000), while the percent identities with B/Hong Kong/330/01 were slightly lower, ranging from 96.7 to 97.2%. Ten other 2002 Taiwanese influenza B isolates, together with the 2004–2005 vaccine strain B/Shanghai/361/02 (15), clustered into group IIa. The percent identities for these 10 isolates in comparison to B/Shanghai/361/02 ranged from 97.7 to 98.7%. Fig. 4A shows group IIb, containing four 1999 Taiwanese isolates, seven 2000 isolates, seven 2001 isolates, and eight 2002 isolates, along with a vaccine strain, B/Sichuan/379/99 (2001–2002 season). These group II isolates were in line with the lineage of B/Yamagata/16/88. Notably, they (including three vaccine strains) all experienced a characteristic deletion of N at position 189 of HA protein, as did B/Yamagata/16/88. On the other hand, all strains in cluster I retained this amino acid, as B/Victoria/2/87 did. Using the 1999 Taiwanese isolates as a baseline, the number of nucleotide changes within the 396-nt HA segment under investigation was found to have accumulated from approximately 5 for both the synonymous and nonsynonymous substitutions, to both >25 in 2003 (data not shown). Unlike in H1N1 or H3N2 isolates, these Taiwanese influenza B viruses had equivalent numbers of synonymous and nonsynonymous substitutions within the five seasons reported. In particular, the numbers of substitutions for those 2002 isolates exhibited a high-and-low oscillating profile of between 10 and 25. Other notable site mutations from 16

1999–2001 sequences to 16 2003 sequences (excluding all 2002 isolates) include K_{142} to R or H, T_{147} to N, Q_{148} to H, R_{162} to K, L_{163} to I, A_{172} to V or I, S_{174} to N, K_{175} or R_{175} to G, S_{176} to N, R_{188} to K, P_{198} to S, V_{201} to I, H_{205} to Y, K_{209} to E, E_{210} to G, K_{223} to E, K_{227} to A, N_{228} to K, N_{234} to K, I_{245} to V, and D_{258} to N. Among these amino acid changes, frequent variations have been reported to occur at positions 162 and 175 (4, 35). On these mutation sites in this report, the Taiwanese 2002 isolates were found to contain alternating amino acids from both influenza B lineages. This phenomenon complies with the observation illustrated in Fig. 4A, where the 2002 Taiwanese isolates were found to cluster into distinct groups, as well as the high-and-low number of substitutions profile described earlier. Coordinates used here were based on the HA segment of B/Lee/40. Figure 4B displays the percent identities between the analyzed Taiwanese influenza B isolates and six reference strains. The white arrows (Fig. 4B) showed that the circulated influenza B strains closely matched the corresponding vaccine strains, except that five 2002 isolates were found much less identical to the 2001–2002 vaccine strain B/Sichuan/379/99 (88.2 to 89.2%) than other 2002 isolates (94.4 to 95.9%). Rather, these five influenza B isolates were found to be highly identical (96.7 to 99.5%) to B/Hong Kong/330/01 (2002–2004 vaccine strain). Similar to the observations of the influenza A H1N1 and H3N2 scenarios, most circulated Taiwanese influenza B viruses were found closely matched to the vaccine strains introduced 1 or 2 years later. For example, the Taiwanese strains from 1999–2001 closely matched (97.0 to 99.2%) the 2001–2002 vaccine strain B/Sichuan/379/99. Among those 23 reported Taiwanese 2002 strains, 10 were even most similar (97.7 to 98.7%) to the 2004–2005 vaccine strain B/Shanghai/361/02.

DISCUSSION

The influenza HI test illustrates how the local strains reacted to the vaccine strains. Over the four reported influenza seasons, some mismatches were noted between the Taiwanese endemic strains and the vaccine strains. For example, A/Panama/2007/99(H3N2)-like and A/New Caledonia/20/99(H1N1)-like strains were found in Taiwan during the 1999–2000 season, while the vaccine strains were not introduced until the next season. B/Hong Kong/330/2001-like strains were also detected in the 2001–2002 season and were selected to be the vaccine strains 1 year later. Similarly, A/Fujian/411/2002(H3N2)-like strains were isolated in the 2003–2004 season and were only recently proposed as the 2004–2005 vaccine strains. A vaccine manufacturing process generally takes 6 to 9 months after the vaccine strain is selected in approximately each February for the northern hemisphere, based on the epidemic strain circulated in the previous season. As a result, it is normal to see a 1-year lag before a vaccine strain becomes available. This phenomenon has also been reported in United States (7–12, 20, 22, 26) and European countries (27).

While the resources available for performing HI tests are often limited to many virology laboratories, HA sequence comparison between the vaccine strains and local Taiwanese strains provides an alternate way of evaluating how the endemic strains conform to the vaccine strains. This presented approach provides a quantitative description of influenza epidemiology over the often-used representation of phylogenetic relationships. It has been shown that the HA sequences correlate well with the influenza antigenic properties and can be used to link the endemic strains and vaccine strains (34). As shown in Fig. 2B, the A/Bayern/7/95(H1N1)-like strain (a 1997–1998 vaccine strain) prevailed in Taiwan in 1995 and 1996, and the A/New Caledonia/20/99(H1N1)-like strain (a 2000–2005 vaccine strain) prevailed from 1997 to 2000. Figure 3B reveals the A/Sydney/5/97(H3N2)-like strain (a 1998–2000 vaccine strain) showing up in Taiwan as early as 1996, as well as A/Moscow/10/99(H3N2)-like strain (a 2000–2004 vaccine strain) from 1997 and A/Fujian/411/02(H3N2)-like strain (a 2004–2005 vaccine strain) from 2002. As mentioned, it is normal to see a 1-year lag before a vaccine strain becomes available. Phylogenetic study of recent human influenza isolates from many other countries also revealed the 1-year lag phenomenon (27). However, sequence analyses of Taiwanese strains in the past 10 years have shown that epidemic strains often become the vaccine strains as many as 2 to 3 years later. It would be interesting to study how many vaccine-like strains had circulated in Taiwan 2 or 3 years ahead before the vaccine strain was introduced.

As shown in Fig. 4B, all 1999–2001 Taiwanese influenza B strains exhibited high homology (95.2 to 98.2%) to the vaccine strain B/Beijing/184/93. However, these Taiwanese strains were also found highly similar (97.0 to 99.2%) to B/Sichuan/379/99, which was a vaccine strain chosen for the 2001–2002 season. Notably, during 2002, some isolates closely matched B/Shanghai/361/02 (2004–2005 vaccine strain), some matched B/Hong Kong/330/01 (2002–2004 vaccine strain), while the others matched B/Sichuan/379/99. Note that the Taiwanese 2002 influenza B isolates illustrated in Fig. 4B are arranged in ascending order according to the time when they were isolated. No apparent boundary exists for these strains in terms of their isolation times, reflecting the fact that they were found clustering separately with different vaccine strains (see also Fig. 4A). Restated, the 2002 influenza B isolates were found to mix with the two well-known genetic lineages (clusters I and II, with B/Victoria/2/87 and B/Yamagata/16/88, respectively). It was reported as early as in 1999 that B/Victoria/2/87-like strains cocirculated with B/Beijing/184/93-like strains in some Asian countries but not in the United States (8). Our data in 2002 illustrated this cocirculation.

In the United States, influenza type A (H3N2) viruses generally occur earlier in the winter season than A (H1N1), with outbreaks often beginning in December and peaking during or after the Christmas holidays. Influenza B transmission in the United States usually occurs later, often as a separate wave going into March or April (29). In Netherland, the influenza epidemics usually occur in winter (45). In Japan, winter (December to February) is the major season for influenza, and usually the peak occurs in February (42). In mainland China, the peak of epidemic of influenza in northern area is in winter, from December to January. However, three peaks in spring (April to May), summer (June to August), and winter (December to January) in the southern area (50) have been observed. In Hong Kong, influenza-like illness occurs throughout the year, peaking from March to May (18). In the tropics with no obvious winter season, influenza infections have been observed mainly during the rainy seasons in Asian, African, and South

American countries (40). In this report, we found that the influenza seasonality in Taiwan was not clear during the past four seasons under investigation, which is probably because Taiwan is an island in the subtropical zone with insignificant temperature changes between seasons.

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