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Original Article

Surveillance of avian and swine influenza in the swine population in Taiwan, 2004

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Background and Purpose: We conducted serological and virological surveillance of pig farms in Taiwan from areas epidemic for low pathogenic avian influenza virus (AIV), H5N2 subtype, in order to determine the prevalence of AIV and swine influenza virus (SIV) in 2004.

Methods: Pig sera from 9833 animals from 1974 farms in 9 counties were examined using agar gel precipitation (AGP) to screen for the presence of antibody against influenza A virus. AGP-positive sera were subjected to hemagglutination-inhibition test against H1, H3, H5 and H7 AIV subtypes and H1 and H3 SIV subtypes. Nasal swabs from 881 pigs were also examined for the presence of SIV by virus isolation in specific pathogen-free embryonated chicken eggs. Virus isolates were identified by reverse transcriptase-polymerase chain reaction followed by DNA sequencing of hemagglutinin and neuraminidase genes.

Results: The AGP test on sera revealed the presence of antibodies against influenza A virus in 62.6% of farms and in 37.7% of pig sera. SIV antibodies to subtype H1 and H3 were found in 10.8% and 65.8% of sera, respectively. There were two peaks of the serological prevalence of SIV in pigs: one between January and February, and the other in October. By contrast, hemagglutinin tests against H5 and H7 AIV subtypes were negative in all sera, while there was a very low positive rate against H1 and H3 AIV subtypes. One H1N2 and one H3N1 viral isolate were obtained from nasal swabs of pigs. Phylogenetic analysis of hemagglutinin and neuraminidase genes revealed both isolates were reassortants of both classical and recent SIVs.

Conclusions: Different subtypes of SIV co-circulate among swine from different farms within the same county and may cause clinical outbreaks of the disease in Taiwan.

Key words: Influenza A virus; Influenza in birds; Phylogeny; Sequence analysis, DNA; Serology

Introduction

Influenza A virus has a broad host range that can include humans, mammals and birds [1,2]. Based on the difference in antigenicity of hemagglutinin (HA) and neuraminidase (NA), influenza A virus can be subdivided into 16 HA subtypes and 9 NA subtypes [3]. Despite the diversity of influenza A virus, historic records show that only H1N1, H2N2 and H3N2 subtypes infected humans before 1997. Similarly, only H5 and H7 subtypes of avian influenza virus (AIV) became highly pathogenic strains that caused epizootic disease and massive deaths in domestic poultry [4]. All combinations of the HA and NA subtypes of AIV, however, were isolated from asymptomatic migratory waterfowl and seashore birds [2]. Wild birds have long been considered as subclinical carriers which serve as the major reservoir of genes for all subtypes of influenza A viruses [5]. Owing to the different receptor requirements between AIV and human influenza viruses, AIV preferentially binds to the N-acetylneuraminic acid-alpha2,3-galactose linkage on sialyloligosaccharides, while human influenza viruses prefers the N-acetylneuraminic acid-alpha2,3-galactose linkage [6].

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In general, neither AIV nor human influenza virus has difficulty crossing the species barrier to infect humans, or vice versa [6]. However, studies of pandemic strains of human influenza virus revealed that some segments of virus genes from AIV may transfer to mammalian influenza virus through genetic reassortment [7]. This might result in a new generation of emerging virus which could cause a pandemic. Historical examples include the 1918 Spanish flu, Asia influenza H2N2 subtype in 1957, and Hong Kong influenza H3N2 subtype in 1968 [8].

Phylogenetic studies have shown that the 1957 pandemic H2N2 virus evolved from the circulating H1N1 virus, which acquired the H2, N2, and PB1 genes from avian species [9]. Similarly, the 1968 pandemic H3N2 virus evolved from the circulating H2N2 virus, which acquired H3 and PB1 genes from birds [2]. For these examples, pigs were postulated to play a critical role as 'mixing vessels' for gene reassortment of both avian and human influenza viruses [2,10]. More importantly, serologic studies indicated that swine influenza virus (SIV) could infect humans directly [11,12].

Direct human infection by AIV was first reported in 1997 in an outbreak in which six out of 18 people infected with the H5N1 subtype died [13,14]. The homology of the genes from viral isolates in these patients was 95-99% of those in chickens [10]. To avoid the spread of this disease in humans, the government of Hong Kong slaughtered at least four million chickens. The H9N2 subtype of quail influenza virus was also reported to infect humans in Hong Kong in 1999 but caused mild respiratory illness [10]. Although direct contact with AIV can cause serious infection in humans, it is not the only way for the virus to break through the host-range barrier. A study of pig sera in southeastern China detected antibodies of several subtypes of AIV, i.e. H4, H5, and H9 [15]. In addition, pigs could be experimentally infected with highly pathogenic H5N1 AIV with mild clinical presentation [16].

Although Taiwan is still an H5N1-free area, low pathogenic strains of H5N2 subtype have been isolated from migratory birds and also from domestic poultry in nine counties [17]. The potential for low pathogenic strains of AIV to evolve into highly pathogenic strains continues to increase. In addition, many pig farms are located in H5N2 virus epidemic counties. Pigs may acquire H5N2 subtype or other AIV from domestic poultry in these areas and thereby serve as 'mixing vessels'. The aim of this study was to investigate the prevalence of AIV H1, H3, H5, H7 and SIV H1, H3 subtypes in the swine population in Taiwan.

Methods

Specimen collection

A total of 9883 serum samples were collected from 1974 pig farms located in 9 counties in Taiwan from January to November 2004. Serum samples were randomly collected weekly from 200-300 pigs at pig farms. All sera underwent agar gel precipitation (AGP) test for influenza viruses. The antibody titers of AGP test-positive samples were further analyzed by hemagglutination-inhibition (HI) test. For virus isolation, nasal swabs were also randomly collected from pigs at farms with serum samples positive for SIV.

Serological procedures

AGP and HI tests were done according to the standard procedures recommended by the World Health Organization with modification [18]. AGP test was performed in 1% Noble agarose (Difco Laboratories, Detroit, MI, USA) prepared in distilled water containing 8% sodium chloride and 0.1% sodium azide. The medium was autoclaved at about 121°C for 15 min, cooled to 60°C, and then 2.5-mL amounts were dispensed into 35-mm diameter plastic petri dishes (BD Falcon™; BD Biosciences, San Jose, CA, USA) which were stored in plastic bags at 4°C until use. Six wells, 4 mm in diameter, evenly positioned around and 9 mm (center to center) from a central 4-mm-diameter well, were cut with a template. Test and control sera were added to the outer wells and virus antigen was added to the central well. After incubation at 37°C for 24 h, precipitin bands were recorded by visual examination with indirect light. Hemagglutination activity titrations and HI assays were performed by standard procedures [18]. Briefly, the human serum samples were treated with receptor-destroying enzyme (Denka Seiken Co. Ltd., Tokyo, Japan) at 37°C for 18 h to eliminate nonspecific inhibitors of hemagglutination, after which the samples were tested for HA-specific antibodies using a standard HI assay [18]. The following strains of avian and classical swine influenza A viruses were employed as antigens: A/Duck/Yilan/106/86(H1N3), A/Duck/Ukraine/1/63(H3N8), A/Chicken/Changhua/031209/2003(H5N2), A/Duck/Tainan/A45/2002(H7N7), A/Swine/Changhua/199-3/2000(H1N1), A/Swine/Pingtung/199-2/2002(H3N2). All HI assays were run simultaneously, and HI titers were defined as the

reciprocal of the highest dilution of serum that completely inhibited hemagglutination of 4 HA units of the virus with a 0.5% solution of chicken red blood cells.

Virus isolation

One portion of each sample, chosen at random, was thawed and 0.2-mL volumes were inoculated into the allantoic cavity of two 11-day-old specific pathogen-free embryonated hen eggs, which were purchased from the Branch Institute of Animal Drugs Inspection of the Animal Health Research Institute, Taipei, Taiwan. Penicillin (10,000 units/mL) and streptomycin (10,000 μg/mL) were added to the inoculum. The eggs were incubated at 35°C for 48 h. The allantoic fluids were tested for hemagglutination activity with chicken erythrocytes. A second passage in eggs was performed in the same manner. All positive samples from this second passage were stored at –70°C for further identification.

RNA extraction

Virion RNA was extracted using the commercial RNA isolation reagent, TRIReagent® LS (Molecular Research Center, Inc., Cincinnati, OH, USA) as described previously [19]. Briefly, one volume (0.25 mL) of virus-infected cells was mixed with three volumes (0.75 mL) of TRIReagent® LS. After the sample was homogenized, 0.2 mL of chloroform was added and the sample was shaken vigorously for 15 sec and then stored at room temperature for 2 to 3 min. The resulting mixture was centrifuged at 12,000 *g* for 15 min. The aqueous phase was collected and viral RNA precipitated using isopropanol and then washed with 70% ethanol. Finally, the purified RNA was dissolved in 9 μL sterilized deionized water.

Subtyping by reverse transcriptase-polymerase chain reaction

Detection of NP gene and H1, H3, N1 and N2 subtyping was done by multiplex reverse transcriptase-polymerase chain reaction (RT-PCR), according to a previously described procedure [20,21]. Briefly, 9 μL of the RNA preparation was mixed with 16 μL of a buffer containing 50 mM potassium chloride, 10 mM Tris-hydrochloride (pH 8.4) 2.5 mM magnesium chloride and 0.02% gelatin, 1 mM each of deoxynucleotide triphosphates, 2 units of ribonuclease inhibitor, 50 pmol of oligonucleotide and 1 μL of avian myeloblastosis virus reverse transcriptase (ribonuclease H minus; Promega Corporation, Madison, WI, USA) [5-10 units/μL]. The mixture was incubated at 42°C for 60 min to yield cDNA. For PCR, 100 μL of reaction mixture contained amplification buffer (10 mM Tris-hydrochloride [pH 8.3] 50 mM potassium chloride, 2.5 mM magnesium chloride, 0.02% gelatin), 20 pmol each of the primers, 1 μL (4 units) of KlenTaq DNA polymerase (Clontech, Palo Alto, CA, USA) and 25 μL of cDNA solution. The amplification reaction was performed at 94°C for 0.5 min, 55°C for 30 sec, and 72°C for 1 min for 30 cycles in a DNA thermal cycler (GeneAmp PCR System 9700; Perkin-Elmer, Foster City, CA, USA). One-tenth of the amplified product was applied to 1.5% agarose gel and stained with ethidium bromide. The PCR products were separated by agarose gel electrophoresis and visualized under a ultraviolet illuminator.

Cloning and sequencing of HA and NA gene

The PCR-derived dsDNA was ligated into the pGem-T vector (Promega) and transformed into *Escherichia coli* JM109. The positive clones were selected and cultured in L-broth containing 100 μg/mL ampicillin (Sigma, St. Louis, MO, USA) and incubated at 37°C overnight. The bacteria were centrifuged at 3000 rpm for 15 min. The pellet was treated with the Wizard Minipreps DNA purification system (Promega) to extract the plasmid DNA which was used as a template for automated sequencing on an Applied Biosystems 373A automated DNA sequencer (Perkin-Elmer), which employed cycle sequencing dye terminator chemistry. T7 and Sp6 primers were used to sequence the HA gene.

Sequence data and phylogenetic analyses

Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA, Version 3.1; Institute of Molecular Evolutionary Genetics, University Park, PA, USA) analytical package [22]. Phylogenetic tree construction was performed based on the neighbor-joining method and bootstrap analysis (n = 1000) to determine the best-fitting tree for the HA gene [23]. In addition to the sequence data determined in this study, previously reported nucleotide sequences and sequences from the GenBank database were also used in phylogenetic tree construction.

Nucleotide sequence accession numbers

The nucleotide sequence data determined in this study were deposited in the DDBJ (DNA Data Bank of Japan, Mishima, Shizuoka, Japan), EMBL (European

Molecular Biology Laboratory; European Bioinformatics Institute, Cambridge, UK) and NCBI (National Center for Biotechnology Information; National Library of Medicine, National Institutes of Health, Bethesda, MD, USA) sequence databases under the accession numbers DQ447184, DQ447185, DQ447186 and DQ447187 for the DNA sequences of H1, H3, N1, and N2 genes of A/swine/Taiwan/CO935/2004(H1N2) and A/swine/Taiwan/0408/2004(H3N1), respectively.

Results

Serological surveillance

9883 serum samples were collected from 1974 pig farms located in 9 counties where poultry farms with H5N2-infected birds have been reported. To detect specific antibody of influenza A virus, all sera were subjected to AGP tests. The overall serum-positive rate in AGP tests was 37.7% (3724/9883), while the total pig farm-positive rate was 62.6% (1235/1974). The positive rates in pig sera ranged from 9.1% to 99.3% during January to November, 2004 (Table 1).

The results revealed that the epidemic season had two peaks (Fig. 1). The first peak was in January and February, and was followed by a dramatic decline in March and April. The rates then increased gradually in May and reached the second peak in October, and then declined again in November. Geographic distribution data showed that positive rates were rather evenly distributed in eight out of nine counties. The exception, in the county of Taoyuan, was attributed to the collection of only 18 serum samples as opposed to an average of 1233 serum samples (150-2360) in the other counties (Table 1). This lower sample number may have led to the underestimation of actual rates in this county. Analysis of data on the number of farms with a positive result and the number of farms examined for the presence of influenza A virus antibody revealed a similar seasonal distribution pattern to that obtained using the number of serum samples tested (Fig. 1). On average, the presence of antibody to influenza A virus was detected in more than half of the pig farms tested in each county.

1234 AGP-positive sera were subjected to HI tests using AIV with subtypes H1, H3, H5 and H7, and SIV with subtypes H1 and H3. All sera were negative on HI tests against AIV subtypes H5 and H7 (data not shown). Low serum-positive rates were found against AIV H1 (1.9%, 23/1234) and H3 (1.0%, 13/1234), respectively (data not shown). By contrast, high serum-positive rates were found against SIV H3 (65.8%, 812/1234) and SIV H1 (10.8%, 133/1234) [Table 2]. HI antibody

Table 1. Prevalence rate of swine influenza in nine counties of Taiwan

County	Number of positive sera/number of sera examined											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Total (%)
TY	0	0	0	0	3/18	0	0	0	0	0	0	3/18 (16.7)
TC	0	0	8/25	5/25	1/10	4/10	2/25	5/20	9/15	7/15	0/5	41/150 (27.3)
CH	140/195	175/175	72/150	19/170	21/130	37/80	45/245	79/190	83/170	116/220	15/385	802/2110 (38.0)
NT	0	45/45	28/75	1/35	10/60	5/20	1/65	26/80	44/90	67/120	4/170	231/760 (30.4)
YL	139/160	163/165	65/165	7/130	26/130	25/80	66/200	85/190	102/180	141/270	63/390	882/2060 (42.8)
CY	5/5	20/20	19/40	4/40	6/25	8/15	4/45	24/45	11/20	25/45	3/70	129/370 (34.9)
TN	102/125	128/130	64/235	12/135	38/215	26/110	66/255	119/255	92/180	184/340	29/380	860/2360 (36.4)
KS	5/5	35/35	92/245	24/80	37/145	26/155	66/195	76/135	68/145	116/175	30/190	575/1505 (38.2)
PT	0	5/5	6/20	4/35	22/60	18/40	26/85	36/75	34/50	37/60	13/120	201/550 (36.5)
Total (%)	391/490 (79.8)	571/575 (99.3)	354/955 (37.1)	76/650 (11.7)	164/793 (20.3)	149/510 (29.2)	276/1115 (24.8)	450/990 (45.5)	443/850 (52.1)	693/1245 (55.7)	157/1710 (9.1)	3724/9883 (37.7)

Abbreviations: TY= Taoyuan; TC = Taichung; CH = Changhua; NT = Nantou; YL = Yunlin; CY = Chiayi; TN = Tainan; KS = Kaohsiung; PT = Pintung

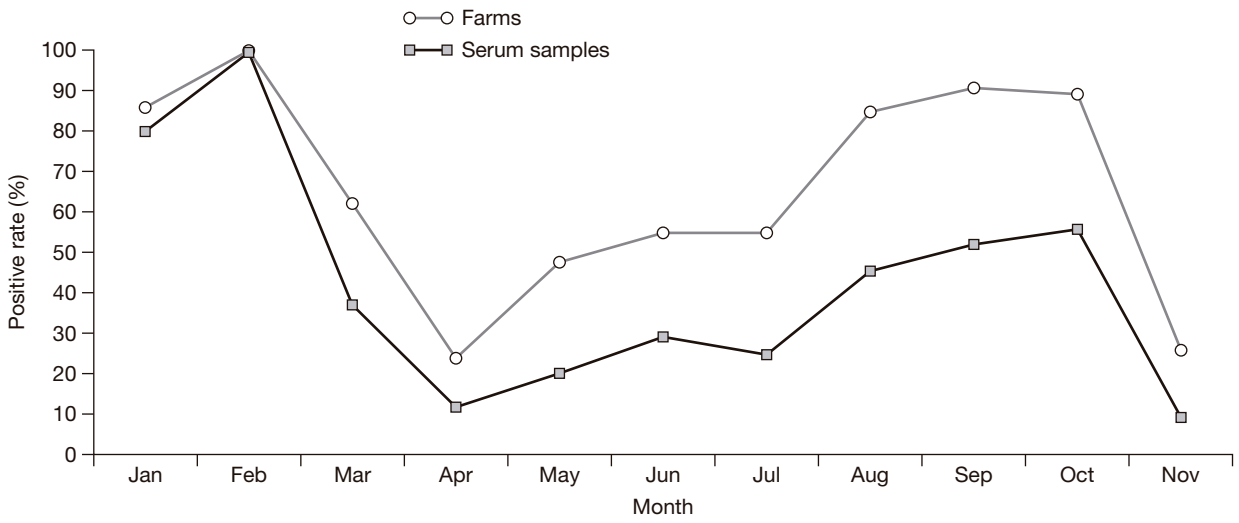

Fig. 1. Positive rates of agar gel precipitation antibody against swine influenza in swine from January to November, 2004.

positivity against swine H3 subtype ranged from 23.5% to 100% during January to September, but dropped dramatically in March, when positive rates of HI antibody against swine H3 subtype reached only 23.5% (Fig. 2). In contrast, low percentages of HI antibody were found against swine H1 (6-18.9%) and duck H1 (0.9-6.6%) subtypes throughout the study period.

Virus gene detection, isolation and subtyping

Viral gene detection, isolation and subtyping were performed on 881 pig nasal swabs collected from slaughterhouses. The specimens were subjected to RT-PCR and specific pathogen-free embryonated egg inoculation. Seven out of 881 nasal swabs were positive for influenza A virus nucleoprotein gene by RT-PCR (data

Table 2. Prevalence rate of H1 and H3 subtypes of swine influenza in nine counties of Taiwan

County	Subtype	Number of positive sera/number of sera examined											
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Total (%)
TY	H1	0	0	0	0	0/1	0	0	0	0	0	0	0/1 (0.0)
	H3	0	0	0	0	1/1	0	0	0	0	0	0	1/1 (100.0)
TC	H1	0	0	0/3	0/1	1/1	0/1	1/1	0/1	0/3	0/2	0	2/13 (15.4)
	H3	0	0	1/3	1/1	1/1	1/1	1/1	1/1	1/3	1/2	0	8/13 (61.5)
CH	H1	1/30	5/35	2/23	0/8	2/12	2/12	6/26	1/32	5/28	0/38	0/10	24/254 (9.4)
	H3	28/30	35/35	5/23	8/8	12/12	11/12	21/26	15/32	13/28	10/38	9/10	167/254 (65.7)
NT	H1	0	0/9	0/8	0/1	1/4	0/1	0/1	0/12	1/15	0/22	0/3	2/76 (2.6)
	H3	0	9/9	1/8	1/1	3/4	1/1	1/1	4/12	9/15	9/22	3/3	41/76 (53.9)
YL	H1	4/29	9/33	5/24	2/4	3/12	3/10	6/26	3/34	3/34	4/49	5/32	47/287 (16.4)
	H3	28/29	33/33	8/24	4/4	11/12	10/10	23/26	18/34	20/34	23/49	32/32	210/287 (73.2)
CY	H1	0/1	2/4	0/5	0/1	1/3	0/3	0/2	2/8	0/4	2/6	0/2	7/39 (17.9)
	H3	1/1	4/4	1/5	1/1	3/3	3/3	1/2	5/8	2/4	3/6	2/2	26/39 (66.7)
TN	H1	0/23	3/26	2/24	1/6	1/20	0/10	3/29	2/42	2/36	1/59	1/19	16/294 (5.4)
	H3	23/23	26/26	2/24	6/6	17/20	9/10	19/29	22/42	18/36	18/59	18/19	178/294 (60.5)
KS	H1	0/1	1/7	5/30	0/7	1/13	2/14	5/25	1/25	2/24	5/34	2/16	24/196 (12.2)
	H3	1/1	7/7	10/30	7/7	13/13	13/14	16/25	15/25	12/24	19/34	15/16	128/196 (65.3)
PT	H1	0	1/1	0/2	0/3	2/9	1/5	1/11	2/14	3/10	1/11	0/8	11/74 (14.9)
	H3	0	1/1	0/2	3/3	8/9	4/5	7/11	5/14	10/10	7/11	8/8	53/74 (71.6)
Total (%)	H1	5/84	21/115	14/119	3/31	12/75	8/56	22/121	11/168	16/154	13/221	8/90	133/1234 (10.8)
		(6.0)	(18.3)	(11.8)	(9.7)	(16.0)	(14.3)	(18.2)	(6.5)	(10.4)	(5.9)	(8.9)	
	H3	81/84	115/115	28/119	31/31	69/75	52/56	89/121	85/168	85/154	90/221	88/90	812/1234 (65.8)
		(96.4)	(100.0)	(23.5)	(100.0)	(92.0)	(92.9)	(73.6)	(50.6)	(55.2)	(40.7)	(97.8)	

Abbreviations: TY= Taoyuan; TC = Taichung; CH = Changhua; NT = Nantou; YL = Yunlin; CY = Chiayi; TN = Tainan; KS = Kaohsiung; PT = Pintung

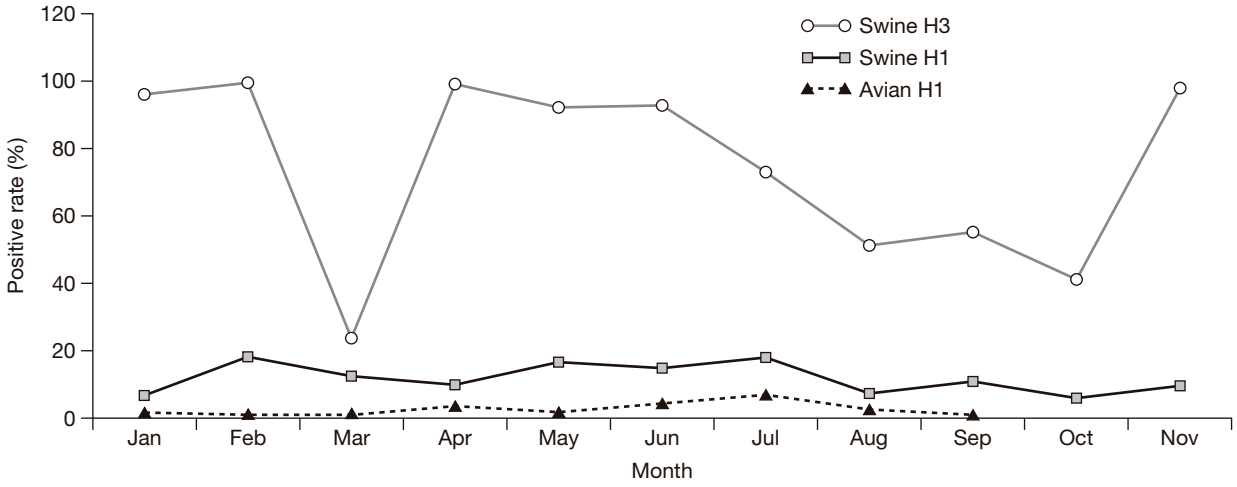

Fig. 2. Positive rates of hemagglutination-inhibition antibody against swine influenza H3, H1 and avian influenza H1 subtypes from January to November, 2004.

not shown). Two viruses with subtypes H1N2 and H3N1 were isolated from different farms in Taichung County. RT-PCR was used to identify the HA and NA subtypes of these isolates and revealed that one belonged to H1N2 and the other belonged to H3N1. According to the format host, place of origin, serial number and isolation year, the nomenclature for these two isolates was A/swine/Taiwan/CO935/2004(H1N2) and A/swine/Taiwan/0408/2004(H3N1), respectively.

Phylogenetic analysis

The entire nucleotide sequences of the HA and NA genes of the A/swine/Taiwan/CO935/2004(H1N2) and A/swine/Taiwan/0408/2004(H3N1) isolates were compared with selected influenza viral sequences from swine, avian and human influenza viruses to form phylogenetic trees, using the neighbor-joining method. The topologies of the phylogenetic trees are shown in Fig. 3, Fig. 4, Fig. 5 and Fig. 6. The analysis revealed that the HA gene of A/swine/Taiwan/CO935/2004(H1N2) was similar to that of A/swine/Ontario/11112/04(H1N1) and A/swine/St-Hyacinthe/106/91(H1N1) [Fig. 3], while the NA gene of A/swine/Taiwan/CO935/2004(H1N2) was similar to that of A/swine/Pingtung/92-2/2003(H1N2) and A/Swine/Pingtung/199-2/2002(H3N2). The N2 gene of all of these strains was close to that of A/Hong Kong/46/80(H3N2)-like strains (Fig. 4). Unlike the phylogenetic results of A/swine/Taiwan/CO935/2004(H1N2), the HA gene of A/swine/Taiwan/0408/2004(H3N1) was similar to that of A/swine/Changhua/72-10 clone B5/2003(H3N1), A/swine/Chiai/77-10/2001(H3N1) and also A/swine/Pingtung/199-2/2002(H3N2). The H3 gene of all of these strains belonged to the A/Hong Kong/46/80(H3N2)-like strains (Fig. 5). In contrast, the NA gene of A/swine/Taiwan/0408/2004(H3N1) was very similar to that of A/swine/Changhua/72-10 clone B5/2003(H3N1), A/swine/Chiai/77-10/2001(H3N1) and also A/swine/Taichung/2000-8/2002(H1N1). The N1 gene of all of these strains was close to A/swine/Ontario/23866/04(H1N1) and related strains (Fig. 6).

Discussion

According to data reported by the Council of Agriculture in Taiwan, 11,894,736 pigs were bred in 2004. In addition, 377,959,000 chickens, 32,247,000 ducks, 6,542,000 geese and 386,000 turkeys were slaughtered for food. This huge population of pigs and poultry poses a risk of human acquisition of zoonotic infections, as both poultry and pigs are natural hosts of AIV. Interspecies transmission of AIV and SIV may occur in poultry, pigs and humans [24]. To provide data for use in the prevention and control of outbreaks of the influenza virus, this study undertook large-scale surveillance of AIV and SIV in the pig population in 2004.

Over 8000 serum samples were analyzed for antibodies against influenza virus in this study. To reduce labor requirements, the AGP test was used to perform the serological screening. The results of AGP testing indicated that two influenza seasons occurred in the pig population in 2004; the first occurred at the end of winter (from January to March) and the second in the summer and fall (from April to October). Each of these seasons was followed by a rapid decline in the

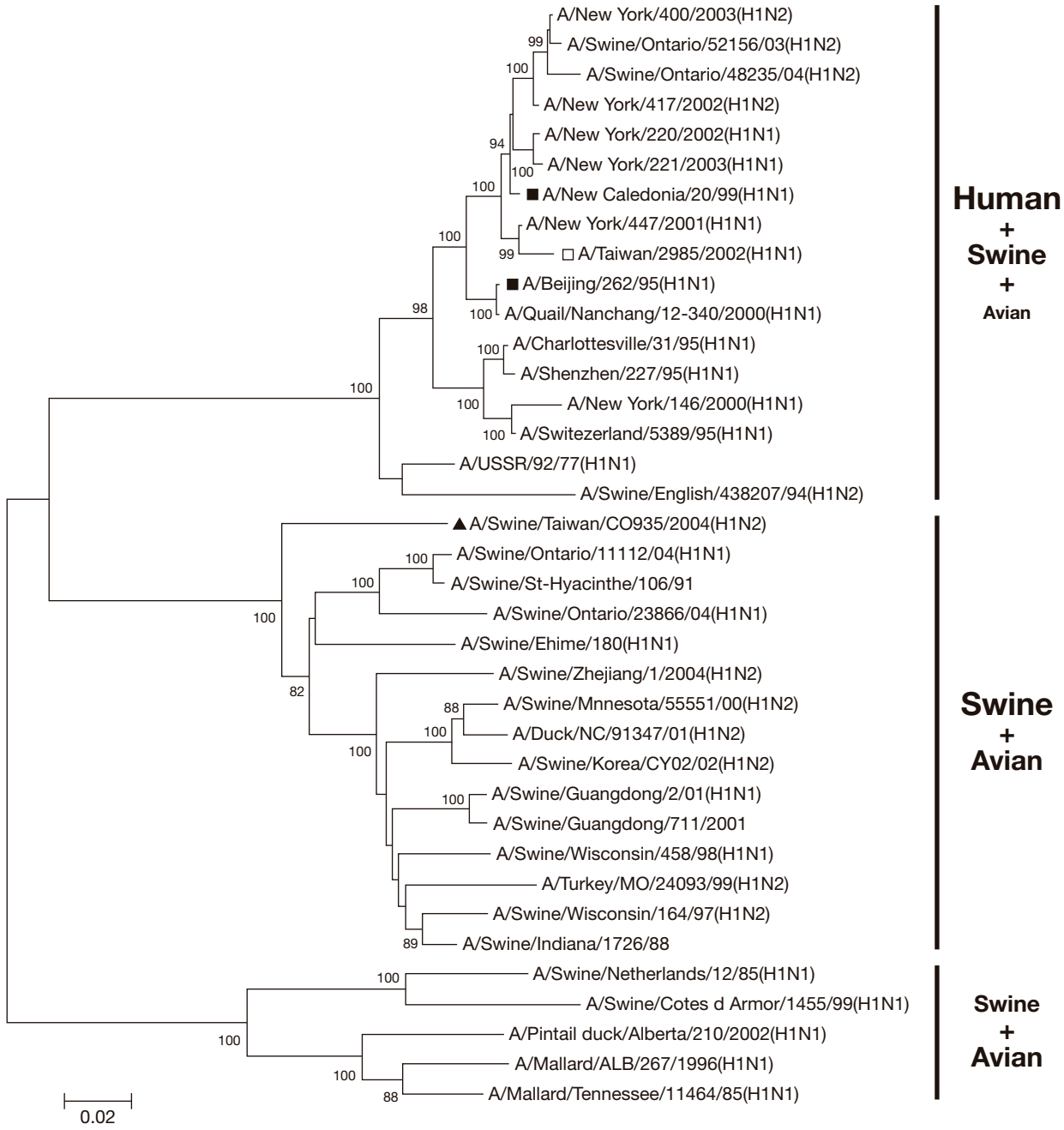

Fig. 3. Evolutionary tree for hemagglutinin gene of A/swine/Taiwan/CO935/2004(H1N2) of swine influenza virus isolates in 2004. ■ = human vaccine strains; □ = human current strains; ▲ = swine isolates in this study. "Human + Swine + Avian" or "Swine + Avian" means the clade of the tree contains a mixed population of human strains, swine strains and avian strains, or swine strains and avian strains of influenza A virus. The tree was constructed using the neighbor-joining method with bootstrap-resampling (n = 1000), as described in the text. The number at each branch point indicates the percentage probability that the resultant topology is correct. The lengths of the horizontal lines are proportional to the nucleotide changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor.

subsequent month. The influenza season in the human population occurs from November to March; the SIV season in the pig population appears to have a close co-relationship. Since the results of this study relate to only a single year, consecutive surveillance of swine populations is needed to confirm these findings and to provide data to facilitate efficient efforts to prevent and control swine influenza in Taiwan.

The HI test results indicated that most of the AGP-positive sera contained antibody to SIV of the

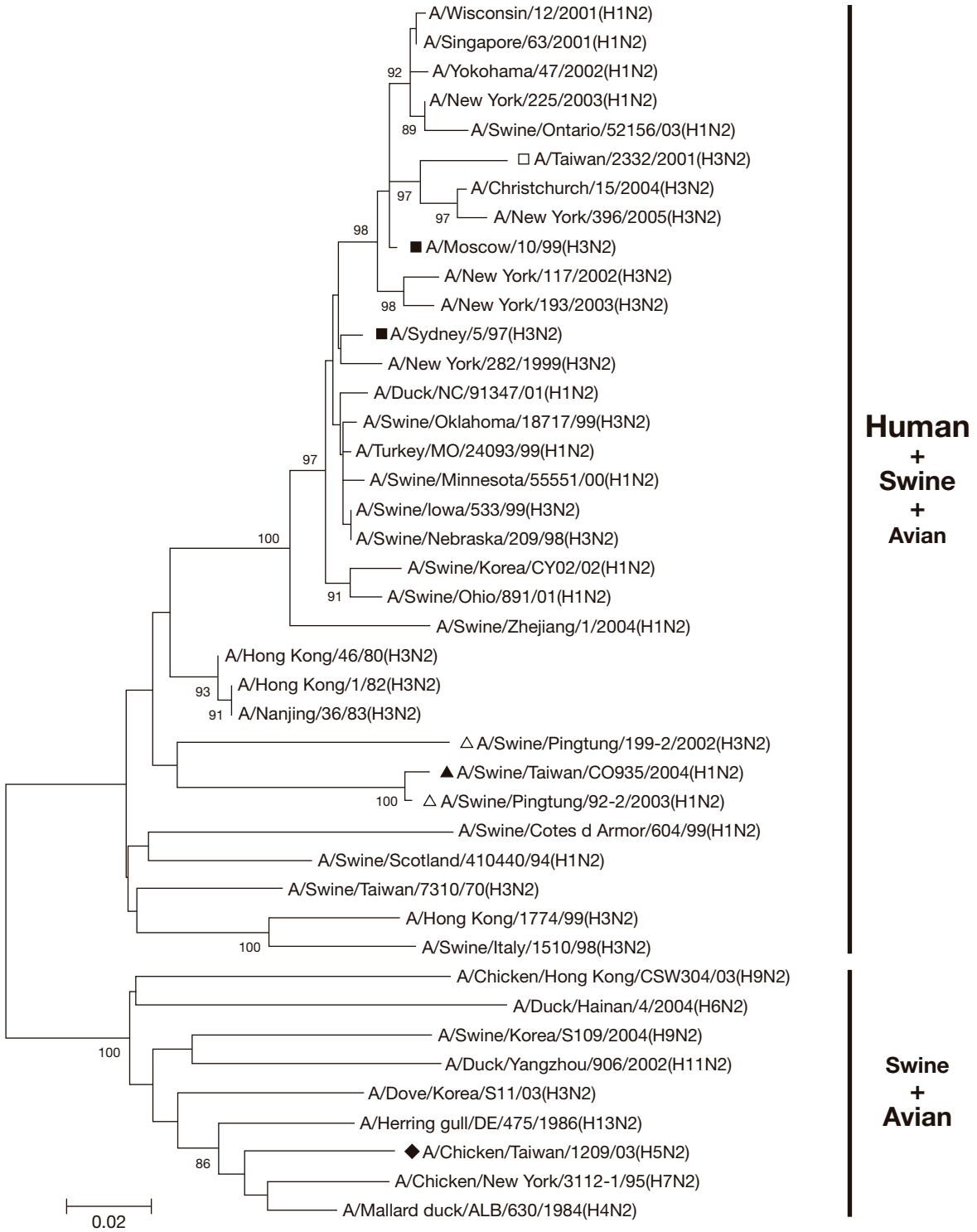

Fig. 4. Evolutionary tree for neuraminidase gene of A/swine/Taiwan/CO935/2004(H1N2) of swine influenza virus isolates in 2004. □ = human current strains; ■ = human vaccine strains; △ = swine local strains; ▲ = swine isolates in this study; ◆ = low pathogenic avian influenza epidemic strain isolated in chickens. "Human + Swine + Avian" or "Swine + Avian" means the clade of the tree contains a mixed population of human strains, swine strains and avian strains, or swine strains and avian strains of influenza A virus. The tree was constructed using the neighbor-joining method with bootstrap-resampling (n = 1000), as described in the text. The number at each branch point indicates the percentage probability that the resultant topology is correct. The lengths of the horizontal lines are proportional to the nucleotide changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor.

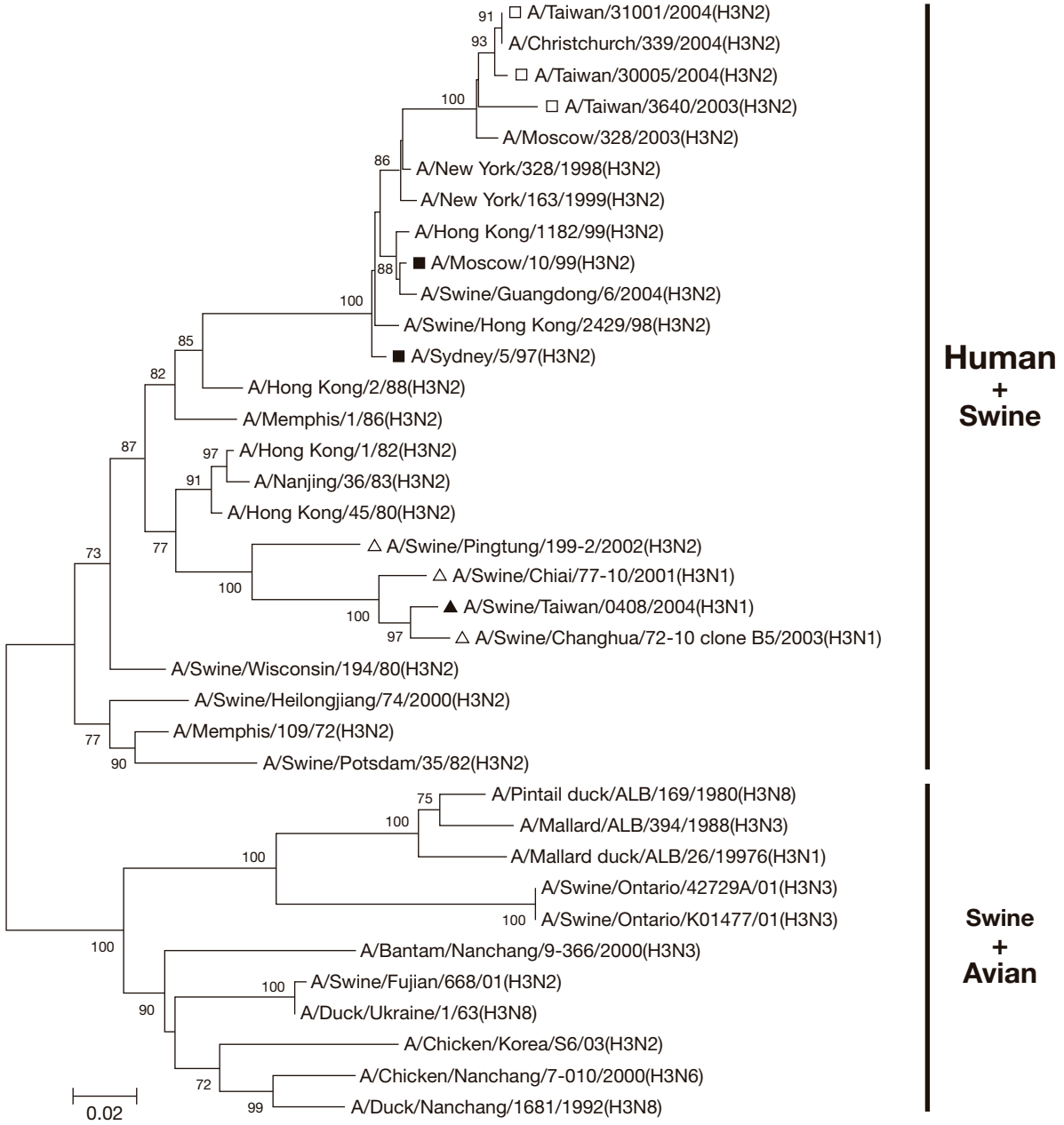

Fig. 5. Evolutionary tree for hemagglutinin gene of A/swine/Taiwan/0408/2004(H3N1) of swine influenza virus isolates in 2004. □ = human current strains; ■ = human vaccine strains; △ = swine local strains; ▲ = swine isolates in this study. "Human + Swine" means the clade of the tree contains both human strains and swine strains; "Swine + Avian" means the clade of the tree contains swine strains and avian strains of influenza A virus. The tree was constructed using the neighbor-joining method with bootstrap-resampling (n = 1000) as described in the text. The number at each branch point indicates the percentage probability that the resultant topology is correct. The lengths of the horizontal lines are proportional to the nucleotide changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor.

H3 subtype during January to September, except for the month of March. This finding implies that subtypes other than the early classical swine H1N1 subtype and the recent classical swine H3N2 subtype of SIV may have been prevalent in March. Concurrent circulation of different subtypes of SIV was reported in Spain [25]. Recently, four combinations of HA and NA subtypes of SIV were reported to have co-circulated in Taiwan, including A/Swine/ChangHua-Taiwan/199-3/2000 (H1N1), A/Swine/TaiChung-Taiwan/200-8/2002(H1N1), A/Swine/TaiNan-Taiwan/46-4/2005(H1N2), A/Swine/TaiNan-Taiwan/103-11/2003(H3N1), and A/Swine/

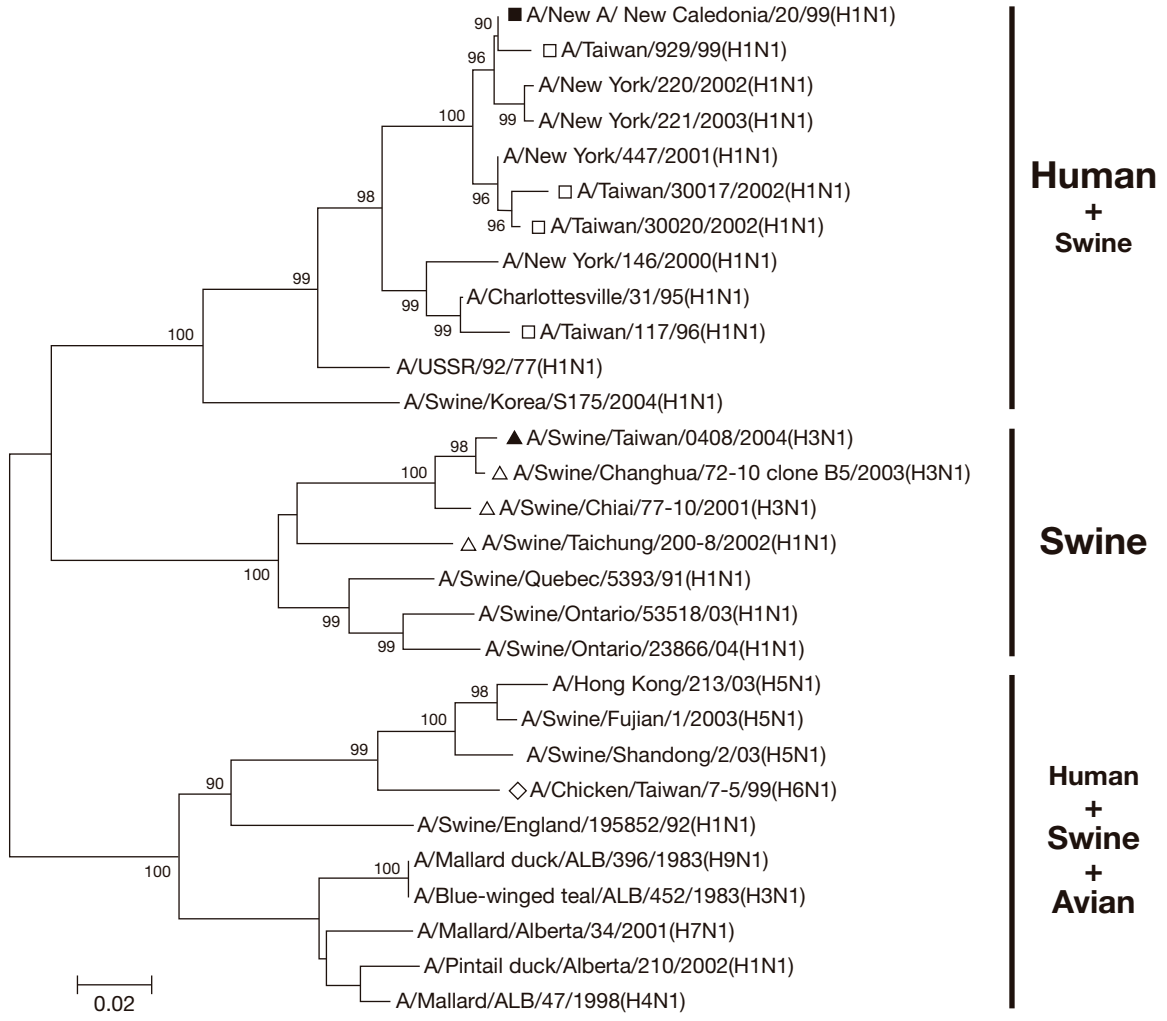

Fig. 6. Evolutionary tree for neuraminidase gene of A/swine/Taiwan/0408/2004(H3N1) of swine influenza virus isolates in 2004. ■ = human vaccine strains; □ = human current strains; ▲ = swine isolates in this study; △ = swine local strains; ◇ = recent avian influenza virus in the chicken population in Taiwan. "Human + Swine" means the clade of the tree contains both human strains and swine strains; "Human + Swine + Avian" means the clade of the tree contains a mixed population of human strains, swine strains and avian strains of influenza A virus. The tree was constructed using the neighbor-joining method with bootstrap-resampling (n = 1000), as described in the text. The number at each branch point indicates the percentage probability that the resultant topology is correct. The lengths of the horizontal lines are proportional to the nucleotide changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor.

PingTung-Taiwan/199-2/2002(H3N2) [26]. Despite viral strains belonging to the same HA subtype, their antigenicity may be different. In the HI test for antibody against SIV in this study, only A/Swine/Changhua/199-3/2000(H1N1) and A/Swine/Pingtung/199-2/2002(H3N2) were selected as virus antigens. Since A/Swine/Changhua/199-3/2000(H1N1) belongs to an early classical swine H1N1 subtype while A/Swine/Pingtung/199-2/2002(H3N2) belongs to a recent classical swine H3N2 subtype (personal communication by Dr. Ching-Ping Tsai), these results from serological testing reveal a role of these subtypes in the epidemiological picture of SIV in the pig population in Taiwan. Compared with human influenza virus, however, the diversity of SIV seems to be more complex. Both H1N1 and H3N2 subtypes were further classified to classical swine H1N1, 'avian-like' H1N1 and 'human-' and 'avian-like' H3N2 subtypes [27]. Thus, further data are needed regarding the presence different strains of SIV in pigs. At present, administration of SIV vaccine in the pig population remains prohibited in Taiwan. The serological results from this study revealed that the majority of pig farms were SIV-positive in at least nine out of fifteen counties in the main island of Taiwan, with a high degree of antigenic diversity of SIV.

In this study, only two strains of SIV H1N2 and H3N1 subtypes were recovered from 881 nasal swabs, in contrast to a high AGP-positive result in pigs. This very low virus isolation rate may be attributable to inactivation of the virus in the specimens during long-distance transportation and storage processes. In addition, since inoculation of specimens in specific pathogen-free chicken embryonated eggs has been proved to be a more sensitive method for SIV isolation than MDCK cell culture [28], keeping the samples at a low temperature before virus inoculation in chicken embryonated eggs might have improve the virus isolation rate. Another reason for the low virus isolation rate might relate to the random collection of nasal swabs from pigs, albeit from pig farms that were seropositive for SIV.

Recently, phylogenetic analysis has been shown to be a useful tool to investigate genetic reassortment of SIV [29,30]. Phylogenetic study indicated that avian-like SIV was generated in pigs in European countries [31]. In addition, SIV was shown to be able to reassort with AIV and human influenza virus to produce triple reassortants [32]. Reassortants which possessed different pathogenic and antigenic properties co-circulated in North America at the same time [33]. Furthermore, AIV H3N3 and H1N1 subtypes were isolated from Canadian pigs [30]. In the present study, phylogenetic analysis of HA and NA genes of the isolates revealed that the HA gene of A/swine/Taiwan/CO935/2004(H1N2) was acquired from a recently epidemic strain, i.e., A/swine/Ontario/11112/04(H1N1), while the NA of the virus was closely related to the old human influenza virus, i.e., A/Hong Kong/46/80 (H3N2). In contrast, the HA gene of another isolate, A/swine/Taiwan/0408/2004(H3N1), was acquired from the old human influenza virus, while the NA gene of that virus was closely related to recent epidemic strains. Frozen evolution was reported in a recent study of equine influenza virus [34]. It could be noticed the HA and NA genes of our isolates showed a similar phenomenon. Finally, our phylogenetic results also indicated that A/swine/Taiwan/CO935/2004(H1N2) and A/swine/Taiwan/0408/2004(H3N1) belonged to human-swine reassortants.

In conclusion, this study showed that specific antibody against influenza A virus could be detected in the majority of the pig population in Taiwan in 2004. Only a minority of tested pigs showed a very low antibody-positive rate against AIV or avian-like influenza virus. The findings that human-swine reassortants of SIV were co-circulating in pigs implies that these animals play an important role as intermediate hosts for AIV and human influenza virus. Low pathogenic AIVs, including H5N2 and other subtypes in domestic poultry and migratory birds are still prevalent in Taiwan, indicating the high potential for HPAI virus infection in pigs which might lead to human transmission. To avoid human-swine transmission of influenza viruses, vaccination of swine farm residents and employees with human influenza vaccine should be considered.

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