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Emergence of Avian H1N1 Influenza Viruses in Pigs in China

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Avian influenza A viruses from Asia are recognized as the source of genes that reassorted with human viral genes to generate the Asian/57 (H2N2) and Hong Kong/68 (H3N2) pandemic strains earlier in this century. Here we report the genetic analysis of avian influenza A H1N1 viruses recently isolated from pigs in southern China, a host suspected to generate new pandemic strains through gene reassortment events. Each of the eight gene segments was of avian origin. Phylogenetic analysis indicates that these genes form an Asian sublineage of the Eurasian avian lineage, suggesting that these viruses are an independent introduction into pigs in Asia. The presence of avian influenza viruses in pigs in China places them in an optimal position for transmission to humans and may serve as an early warning of the emergence of the next human influenza virus pandemic.

As the world awaits the next influenza virus pandemic, an event which is considered inevitable, studies have focused on China for early signs indicative of the emergence of the next pandemic strain (15, 25, 30, 31). Although a human influenza virus pandemic has not occurred for many years, influenza A viruses continue to arise from the aquatic avian reservoir and cause extensive outbreaks in both avian and nonhuman mammalian species. Recent examples are the highly pathogenic H5N2 subtype in poultry in Mexico (10) and the H3N8 subtype in horses in China (6), reminding us of the potential for interspecies transmission and production of serious disease in their new hosts. It is now 28 years since the Hong Kong/68 (H3N2) pandemic in humans and 19 years since the reappearance of the Russian (H1N1) strain in 1977. The most catastrophic influenza virus pandemic occurred in 1918, when the so-called Spanish influenza virus claimed over 25 million lives worldwide (3). Because this virus could not be isolated in 1918, it is not possible to establish its molecular characteristics; however, analysis of its descendants suggests that it acquired all of its genes from an avian influenza virus and was introduced intact into the human population (5). Since counterparts of this and other pandemic viruses still exist in nature, the likelihood of new devastating outbreaks cannot be ignored.

The search for indicators of the next human influenza virus pandemic has produced provocative clues. The Asian/57 (H2N2) and Hong Kong/68 (H3N2) pandemic viruses acquired two or three gene segments from the Eurasian avian influenza virus reservoir (12, 19). Thus, on each occasion reassortment occurred between a Eurasian avian and human influenza virus to generate a pandemic strain. How did this reassortment event occur? Since human cells do not have receptors for avian influenza viruses, an intermediate host possessing receptors for each of these viruses might be needed. Pigs are leading candidates for this role (20). Recent studies indicate that they possess receptors for both the α2-3Gal (avian) and α2-6Gal (human) epitopes (11a) and can therefore be experimentally infected with both avian and human influenza viruses (13).

Although transmitted to pigs relatively often (32), human influenza viruses rarely become established in the swine population (32, 33). One exception is an H3N2 influenza virus related to the strain that caused the Hong Kong pandemic in 1968 (29); however, it is still uncertain whether the virus was introduced into pigs before or after its introduction into humans. We do not know how often avian influenza viruses have been transmitted to pigs; one instance occurred in Europe in 1979 when an avian H1N1 virus appeared in the pig population and eventually became established as the chief cause of influenza virus in these animals (16, 21). This virus has since been found to have reassorted on two occasions with H3N2 human influenza viruses circulating among pigs in The Netherlands, giving rise to reassortants with six avian influenza virus gene segments capable of infecting children (2).

Since the first isolation of a human influenza virus in 1933, each of the pandemic influenza viruses has originated in China. The Asian/57 H2N2 pandemic strain was first detected in the eastern Guizhou and eastern Yunnan provinces, while the Hong Kong/68 H3N2 strain emerged through Hong Kong from Guangdong Province and the Russian/77 H1N1 strain reappeared in northern China. These findings have contributed to the hypothesis that China serves as an epicenter for the generation of pandemic viruses (28). In order to assess whether pigs in China could be a possible source of pandemic-type reassortants, a year-long surveillance study in Hong Kong of pigs from southern China was undertaken (5a). Avian-like influenza viruses were isolated, and molecular analysis was done to determine their sources, whether from pigs in Europe or perhaps directly from birds in southern China itself.

MATERIALS AND METHODS

Sampling of pigs for viruses. Each week from July 1993 to June 1994, tracheal swab samples were taken at random from domestic pigs at an abattoir in Hong Kong. Around 60 samples (1 per pig) were collected on each weekly visit, resulting in a total of 3,100 specimens for analysis. All of the young adult pigs that were sampled originated from China and appeared healthy. According to the records of the abbatoir, most of the pigs came from the Hunan, Jiangxi, Guizhou, and Guangdong Provinces of southern China. These pigs would have travelled by road or train for up to 3 days, which would have caused conditions of stress and the opportunity for virus spread and amplification. A small group came from Henan Province in central eastern China. Swab samples were collected in medium 199 containing antibiotics (9), held at 0°C during transport, and within 3 to 4 h of their arrival in the laboratory, injected into embryonated chicken eggs (23).

Virus isolation and serological analysis. Nine- to 11-day-old embryonated chicken eggs, inoculated allantoically with 0.2 ml of a swab specimen, were incubated at 35°C for 72 h. The allantoic fluids were then harvested and tested for hemagglutinin (HA) activity (34). Fluids that lacked detectable HA activity

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TABLE 1. Serologic characterization of H1N1 swine isolates from southern China with monoclonal and polyclonal antibodies

Virus	HA inhibition titer with the indicated antibody							
	Monoclonal antibody to A/NJ/8/76					Polyclonal antiserum to:		
	6/1	36/3	40/3	72/3	117/2	Dk/Alb/35/76	NJ/8/76	Dk/HK/717/79
A/Sw/HK/168/93[a]	<[b]	<	<	<	<	<	<	<
A/Sw/HK/176/93[a]	<	<	<	<	<	200	<	150
A/Sw/HK/181/93[c]	76,800	6,400	6,400	102,400	102,400	400	1,600	300
A/NJ/8/76	300	<	51,200	38,400	51,200	300	4,800	200
A/Dk/Alb/35/76	<	<	<	<	<	800	<	200
A/Dk/HK/717/79	<	<	<	<	<	100	<	600
A/USSR/77	<	<	<	<	<	200	200	<

[a] Representatives of 10 of 11 avian-like isolates from pigs in 1993.
[b] <, HA inhibition titer of less than 100.
[c] Representatives of 514 classic isolates from pigs in 1993 to 1994.

were passaged in a blind manner an additional time. Positive isolates were characterized by HA inhibition and neuraminidase (NA) inhibition tests according to recommendations of the World Health Organization (34). HA and NA inhibition tests were performed with monospecific antisera to the HA and NA proteins as well as with postinfection ferret antisera (27); a panel of monoclonal antibodies against the HA of the influenza A/New Jersey/8/76 virus strain was also used for antigenic characterization (8).

Serological analysis of the pigs was conducted during the year-long study; around 25 pig serum samples were collected every two weeks for seroepidemiological studies and were examined by HA inhibition analysis.

Analysis of viral RNA. Nucleotide sequencing studies were performed on representative viral isolates from Chinese pigs, on avian viruses previously identified in Hong Kong (Table 1), and on reference avian and swine strains from the repository at St. Jude Children's Research Hospital. Viral RNA was extracted directly from infected allantoic fluid, as described previously (1). For genotyping and phylogenetic analysis, the RNAs were reverse transcribed and amplified by PCR.

PCR products were purified by a commercially available system (Wizard PCR Preps; Promega). The 5′ ends of primers specific for the gene under study were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Sequences were obtained by fmol sequencing as previously described (14). The entire lengths of the HA1 genes of related viruses were sequenced, as well as 87 to 95% of the nucleotide sequences of the nucleoprotein (NP), matrix (M), and nonstructural (NS) genes. Smaller portions of the PB1, PB2, PA, and NA genes were sequenced.

Partial sequence data for all eight viral genes were analyzed by the Fast-DB program to identify the closest matches with sequences in GenBank. The HA1, PB1, M, NS, and NP sequences were phylogenetically analyzed with PAUP (phylogenetic analysis using parsimony) software, version 2.4, from David Swofford of the Illinois Natural History Survey, Champaign (4).

RESULTS

In view of the established circulation of avian influenza viruses in European pig populations, the extensive virologic surveillance in Hong Kong of pigs that had been under way from 1976 to 1982 (27, 29) was renewed. In the 1976 to 1982 studies, analysis of 624 gene segments of swine influenza viruses from China failed to show any evidence of avian influenza virus genes (30). During a 1-year period, from July 1993 to June 1994, 525 influenza viruses were isolated from 3,100 apparently healthy pigs originating from southern China (16.9% isolation rate) and their antigenicities were determined (5a). Antigenic screening of the isolates with monospecific antisera to 14 HA subtypes revealed that all of the viruses were of the H1 subtype (results not shown). Further analysis with monoclonal antibodies to A/New Jersey/8/76 (H1N1) and strain-specific antisera distinguished two different groups: 514 viruses reacted with the monoclonal antibody panel and with sera specific to A/New Jersey/8/76 (H1N1), while the remaining 11 viruses, which had been isolated in September 1993, failed to react with the monoclonal antibodies, the majority of these (10 of 11) producing low titers with antisera to A/Duck/Alberta/35/76 (H1N1) and A/Duck/Hong Kong/717/79 (H1N3) (Table 1). Most of the H1N1 isolates—the group of 514 viruses—were antigenically indistinguishable from each other, and their reactivity patterns were indistinguishable from currently circulating classic swine H1N1 influenza viruses (results not shown). Of the remaining 11 H1N1 viruses, 10 gave indistinguishable reactivity patterns (not shown) while 1 (A/Swine/Hong Kong/168/93) failed to react with any of the sera tested. Thus, by serologic criteria, these viruses are more closely related antigenically to avian influenza H1N1 viruses than to classic swine H1N1 influenza viruses.

The 11 avian-like H1N1 influenza viruses were isolated in September 1993; of these, a single isolate was detected on 20 September 1993 and the remaining 10 isolates were detected on 27 September 1993. The remaining 514 classic swine isolates were isolated throughout the year, with a peak isolation rate in March.

When subjected to NA inhibition testing with monospecific sera to the nine NA subtypes, each of the 525 viruses reacted with N1 antisera (not shown). The 525 viruses could not be distinguished from each other in these assays and were designated as belonging to the N1 subtype.

Serological analysis of 636 pig serum samples collected during the year-long analysis revealed that 13% of pigs had antibodies to swine H1N1 strains (e.g., A/New Jersey/8/76) and that 2.5 and 3.0% had antibodies to the H3N2 strains A/Victoria/3/75 and A/Hong Kong/1/93, respectively, suggesting that while H3N2 viruses were not isolated, they were probably circulating at low levels in the pig populations of southern China.

Genotyping of swine H1N1 influenza viruses from southern China. To characterize each of the viral gene segments and determine their hosts of origin, partial sequences of all eight segments of the 11 viruses that showed an antigenic relationship to avian strains and eight segments of four viruses of the group with an antigenic relationship to classic swine influenza virus were determined (Table 2). Each of the 11 isolates had identical nucleotide sequences in all of the genes sequenced, except the HA gene of A/Sw/HK/168/93, which had two base changes, one at residue 520 (G→A), resulting in an amino acid substitution at residue 146 (Arg→Gln), and the other at residue 528 (C→T), which did not result in an amino acid change. Additionally, there was a point mutation in PB1 at residue 1365 that was silent. Analysis of the isolates representative of the classical swine viruses showed that the portions of the genes analyzed were similar to each other but not identical.

To identify close homologies of the gene segments, 240 to 1,397 nucleotides of each gene of a representative virus (A/Sw/HK/168/93) from the avian-like group and A/Sw/HK/273/94 from the classical swine group were compared with sequences

TABLE 2. Extent of nucleotide sequence homology between the new H1N1 isolates and reference strains

Gene segment	A/Sw/HK/168/93[a]			A/Sw/HK/273/94[b]			% Homology between A/Sw/HK/168/93 and A/Sw/HK/273/94
	Sequence analyzed	Most closely related strain from GenBank	Homology (%)	Sequence analyzed	Most closely related strain from GenBank	Homology (%)	
PB1	1222–1481	A/Dk/HK/412/78	92	1251–1490	A/Sw/Ontario/2/81	97	78
PB2	977–1262	A/Ruddy Turnstone/NJ/35/85	94	977–1304	A/Sw/Tenn/24/77	92	88
PA	34–311	A/Ruddy Turnstone/NJ/35/85	94	27–362	A/Sw/Tenn/26/77	94	81
HA	41–1153	A/Dk/Aus/749/80	92	41–1153	A/Sw/Iowa/17672/88	98	75
NP	36–1432	A/Sw/Germany/2/81[c]	94	36–1432	A/Sw/Beijing/94/91	99	82
NA	251–666	A/Parrot/Ulster/73	91	252–492	A/Sw/Wisconsin/3523/88	97	79
M	26–1001	A/Dk/Nanchang/1749/93	98	26–1001	A/Sw/Iowa/17672/88	98	89
NS	35–867	A/Dk/Nanchang/1944/93	93	35–867	A/Sw/Iowa/17672/88	98	85

[a] Represents avian-like swine isolates.
[b] Represents classic swine isolates.
[c] Of avian origin.

available in GenBank. Each of the eight gene segments of the group of 11 viruses was most closely related to the gene segments of avian influenza viruses. The extent of homology ranged from 98% for the M gene of A/Duck/Nanchang/1749/92 (H11N2), a duck virus recently isolated in China (31), to 91% for the NA gene of A/Parrot/Ulster/73 (H7N1). Since few gene sequences from recently isolated Eurasian avian viruses are available for comparison in GenBank, the actual homology between the Hong Kong isolates and avian strains may be closer than estimated.

Analysis of four of the 514 isolates without avian-like antigenic features showed high homology to classic swine influenza viruses (92 to 99% for each of the eight gene segments). The high homology, up to 99% for the NP gene of A/Sw/Beijing/94/91 (H1N1), emphasizes the high genetic similarity among the swine influenza viruses in this region of the world. At this time, only the NP gene of A/Sw/Beijing/94/91 is available for comparison. When the homologies of the gene segments of viruses in the two antigenic groups (e.g., A/Sw/HK/168/93, representing the avian-like H1N1 viruses, versus A/Sw/HK/273/94, representing the classical swine H1N1 viruses) were compared, the values were uniformly low (Table 2), confirming the separation of the viruses into the two groups.

Phylogenetic relationships of the H1N1 influenza viruses from southern China. To characterize the gene segments in the avian-like H1N1 influenza viruses more precisely, we constructed phylogenetic trees using the majority of the sequence of the NP, M, and NS genes, a portion of the PB1 gene, and the entire HA1 region of the HA gene, from one or two avian-like and one or two classic swine viruses (see the figure legends for details).

The HA1 tree is rooted to the H2 subtype and comprises two distinct lineages: one including only mammalian isolates and the other including only avian isolates or isolates of avian origin (Fig. 1). The mammalian lineage is further divided into two sister groups, one of which contains human H1N1 strains dating from 1933 (when the first human influenza virus [A/WSN/33] was isolated) to the present while the other contains classic swine strains, such as A/Swine/Iowa/30 and strains currently circulating in the United States (A/Swine/Nebraska/92) and in China (A/Swine/Beijing/91). The avian lineage is divided into American (i.e., North America) avian isolates and Eurasian isolates, the latter comprising Asian and European sublineages. The HA1 molecule of representative Hong Kong swine viruses showed relationships to either mammalian or avian lineages: A/Sw/HK/172/93 and A/Sw/HK/273/94 strains lie within the classic swine lineage and A/Sw/HK/168/93 and A/Sw/HK/176/93 lie within the Asian avian sublineage.

Phylogenetic analyses of the PB1, NP, M, and NS genes (Fig. 2) show clear divisions of each of these genes into different lineages. Influenza viruses exemplified by A/Sw/HK/168/93 are on the Eurasian avian branch of the trees, and those exempli-

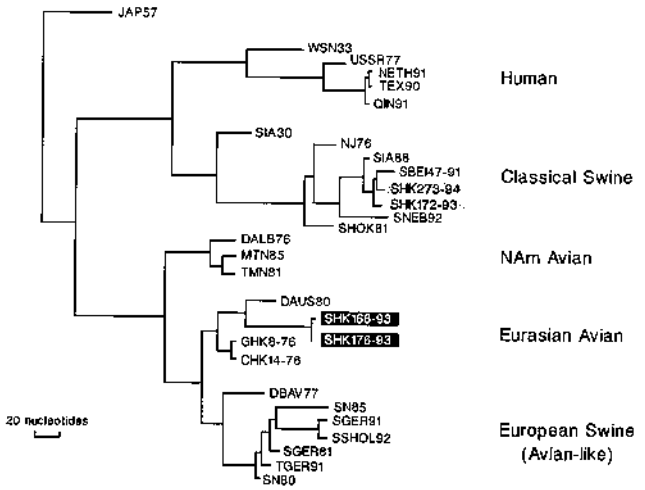

FIG. 1. Phylogenetic tree for the HA1 genes of influenza A viruses based on 981 bp from residues 84 to 1064. The nucleotide tree is rooted to H2 HA1. The complete nucleotide sequences of the HA1 segments of the HA genes of four influenza viruses from pigs in China (boxed) were added to the H1 HA1 sequences available in GenBank and were analyzed with the PAUP software program, version 2.4 (David L. Swofford, Illinois Natural History Survey), which relies on a maximum parsimony alogorithm. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing branches and labels. The abbreviations are as follows: JAP57, A/Japan/305/57; WSN33, A/WSN/33; USSR77, A/USSR/90/77; NETH91, A/Netherlands/813/91; TEX90, A/Texas/22/90; QIN91, A/Qingdao/28/91; SIA30, A/Swine/Iowa/15/30; NJ76, A/New Jersey/11/76; SIA88, A/Swine/Iowa/17672/88; SBEI47-91, A/Swine/Beijing/47/91; SHK273-94, A/Swine/Hong Kong/273/94; SHK172-93, A/Swine/Hong Kong/172/93; SNEB92, A/Swine/Nebraska/1/92; SHOK81, A/Swine/Hokkaido/2/81; DALB76, A/Duck/Alberta/35/76; MTN85, A/Mallard/Tennessee/11464/85; TMN81, A/Turkey/Minnesota/1661/81; DAUS80, A/Duck/Australia/749/80; SHK168-93, A/Swine/Hong Kong/168/93; SHK176-93, A/Swine/Hong Kong/176/93; GHK8-76, A/Goose/Hong Kong/8/76; CHK14-76, A/Chicken/Hong Kong/14/76; DBAV77, A/Duck/Bavaria/2/77; SN85, A/Swine/Netherlands/12/85; SGER91, A/Swine/Germany/8533/91; SSHOL92, A/Swine/Schleswig-Holstein/1/92; SGER81, A/Swine/Germany/2/81; TGER91, A/Turkey/Germany/3/91; and SN80, A/Swine/Netherlands/3/80. NAm, North American.

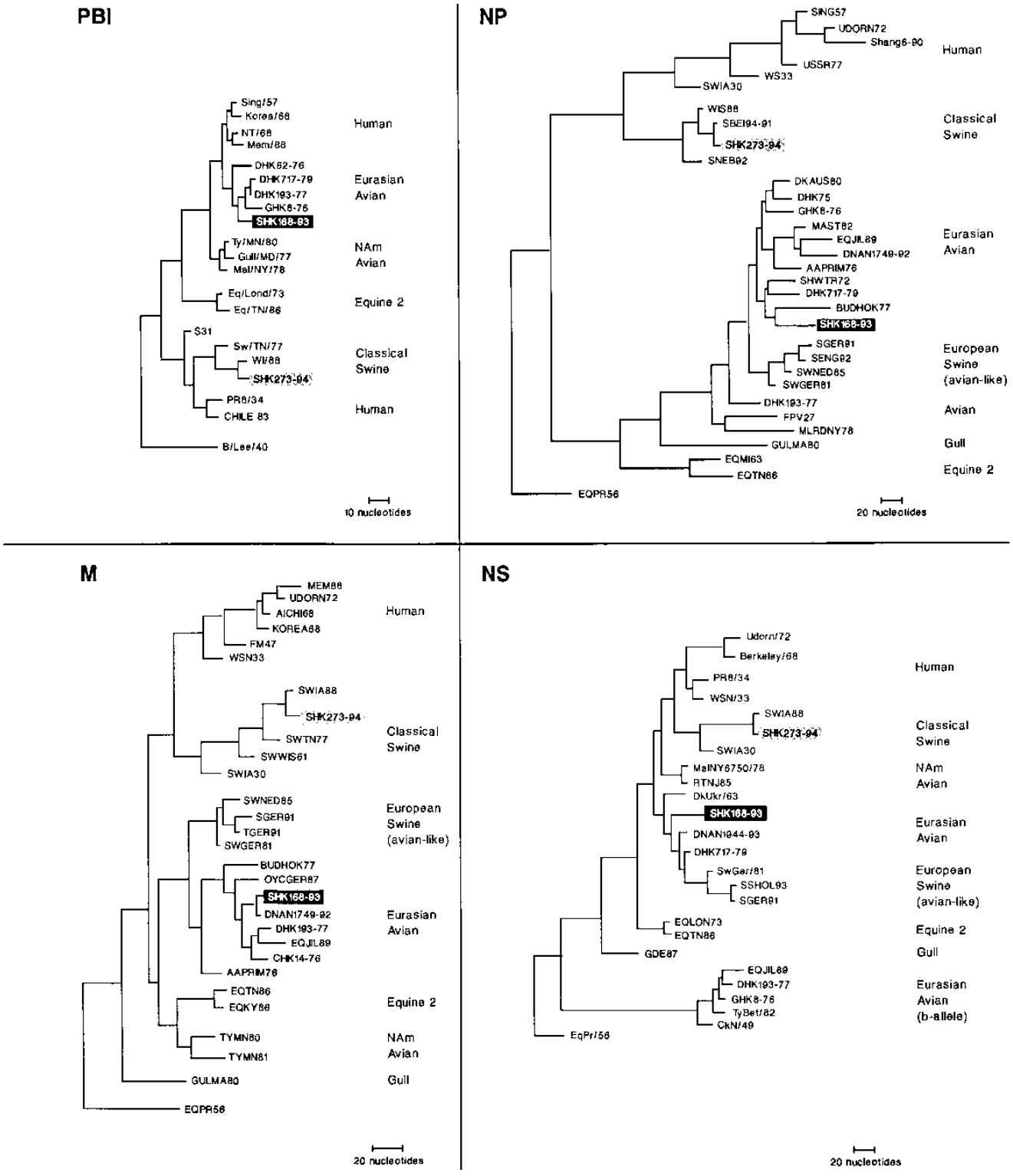

FIG. 2. Phylogenetic trees for the internal genes—PB1, NP, M, and NS—of H1N1 influenza viruses from pigs in southern China. Phylogenetic relationships were determined with the PAUP software program (Fig. 1). The nucleotide trees of the NP, M, and NS genes are rooted to A/Equine/Prague/1/56 (H7N7). The PB1 tree is rooted to B/Lee/40. The nucleotide sequences used in the analyses were as follows: PB1 (216 bp), residues 1251 to 1466 (9% of the gene); NP (1,387 bp), residues 46 to 1432 (87% of the gene); M (976 bp), residues 26 to 1001 (95% of the gene); and NS (808 bp), residues 35 to 842 (91% of the gene). The names of the viruses corresponding to the abbreviations used in the trees can be found in the following references: 5 (NP, M, NS), 11 (M), 12 (PB1), and 16 (NS). For viruses not found in these publications, the names and abbreviations (in alphabetical order) are as follows: AAPRIM76, A/Anas acuta/Primorje/695/76 (H3N2); CHILE83, A/Chile/1/83 (H1N1); Shang6-90, A/Shanghai/6/90 (H3N2); CHK14-76, A/Chicken/Hong Kong/14/76 (H1N1); DHK62-76, A/Duck/Hong Kong/62/76 (H11N2); DHK193-77, A/Duck/Hong Kong/193/77 (H1N2); DHK717-79, A/Duck/Hong Kong/717/79 (H1N3); DNAN1749-92, A/Duck/Nanchang/1749/92 (H11N2); DNAN1944-93, A/Duck/Nanchang/1944/93 (H7N4); EQJIL89, A/Equine/Jilin/1/89 (H3N8); GDE87, A/Laughing gull/Delaware/2838/87 (H7N2); GHK8-76, A/Goose/Hong Kong/8/76 (H1N1); OYCGER87, A/Oystercatcher/Germany/87 (H1N1); SBEI94-91, A/Swine/Beijing/94/91 (H1N1); SENG92, A/Swine/England/195852/92 (H1N1); SGER91, A/Swine/Germany/8533/91 (H1N1); SHK168-93, A/Swine/Hong Kong/168/93 (H1N1); SHK273-94, A/Swine/Hong Kong/273/94 (H1N1); SNEB92, A/Swine/Nebraska/1/92 (H1N1); SSHOL93, A/Swine/Schleswig-Holstein/1/93 (H1N1); SW31, A/Swine/1976/31 (H1N1); and TGER91, Turkey/Germany/3/91 (H1N1). NAm, North American.

fied by A/Sw/HK/273/94 are on the classical swine branch of the trees. This confirms the homology studies for each of these genes and firmly establishes that these gene segments are of avian origin.

The remaining question is whether the phylogenetic analysis of these genes can provide information on the derivation of the avian-like H1N1 viruses (e.g., A/Sw/HK/168/93). Did these Chinese swine viruses of avian origin spread from Europe, where related viruses are currently circulating, or do they constitute a different introduction of influenza virus from the avian influenza virus reservoir into pigs in China? The largest amount of information available for analysis is from the HA1 and NP genes; the swine viruses in Europe of avian origin (e.g., A/Sw/Germany/81) form a lineage separate from that of the swine virus of avian origin in China (e.g., A/Sw/Hong Kong/168/93) (Fig. 1 and 2). In the NP tree, the most closely related virus to the A/Sw/Hong Kong/168/93 virus is A/Budgerigar/Hokkaido/77, and these viruses show a sister group relationship with avian strains isolated earlier from Hong Kong (e.g., A/Duck/Hong Kong/717/79). It is interesting that A/Equine/Jilin/89, which is of Asian avian origin (6), is more closely related to the avian swine isolates from China than those from Europe.

The M gene phylogenetic tree has topology similar to that of the NP phylogenetic tree and shows a clear separation of European avian-like viruses into one sublineage and a separation of Chinese avian-like viruses into another sublineage. Insufficient numbers of recent Eurasian PB1 and NS gene segments are available in GenBank to permit fine resolution of the origins of viruses in swine in the Eurasian landmass. Overall, the available information indicates that the avian influenza viruses in swine in China are not direct descendants of avian influenza viruses in European pigs and probably constitute separate introductions into pigs from avian influenza viruses from Asia.

DISCUSSION

Antigenic and genetic analyses of influenza viruses isolated from pigs in Hong Kong in 1993 have established that two different groups of H1N1 viruses were cocirculating among pigs that originated in southern China. One group belonged to the classic swine lineage, and the other belonged to the Eurasian avian lineage. These studies provide convincing evidence that an avian influenza virus spread from the avian reservoir to pigs in southern China.

Earlier studies of influenza viruses from pigs in the geographical region of southern China, including Taiwan, from 1976 to 1982, revealed cocirculating H1N1 and H3N2 subtypes (26, 27) and detected a low incidence of reassortants (30) but no evidence for avian influenza virus gene segments. During the present study, no H3N2 viruses were detected in pigs, suggesting that these viruses may be circulating at very low levels in pigs in China.

The isolation of 10 avian-like influenza viruses from pigs with identical nucleotide sequences in their HAs on one sampling occasion and with three nucleotide differences in a single isolate on another occasion raises the question of whether they were derived from a single source. It can be postulated that the 10 identical viruses originated from a single infected pig and that during the three days of transport and stress, this isolate was amplified, infecting at least 10 other pigs. The single isolate with three nucleotide differences probably originated from the same region in China 1 week earlier. Since only 1 of every 150 to 170 pigs being slaughtered was sampled on any one day, it is possible that additional pigs were also infected with these avian-like influenza viruses. This raises the possibility of a limited focus of avian-like influenza viruses in pigs in China and the likelihood that the virus has the capacity for pig-to-pig spread. The serological findings that 13% of pigs had detectable antibodies to H1N1 influenza viruses confirms the presence of these viruses in pigs from China. The isolation rate of 16.9% probably reflects the amplification of influenza viruses during transport and stress. The failure of either the avian-like or the classical swine H1N1 viruses to cause an epizootic in the transported pigs is not at all surprising, for there was insufficient time for development of clinical signs and this finding is in keeping with the results of earlier studies (26, 27).

The detection of H1N1 influenza viruses of avian origin in Chinese pigs raises an intriguing question. Did these viruses spread to Asia from Europe or did they emerge from a separate introduction of an avian H1N1 influenza virus into pigs in China? Analysis of the HA1 and NP phylogenetic trees of which sufficient sequences are available for detailed analysis supports the second possibility (Fig. 1 and 2). The avian influenza viruses that continue to circulate in European pigs—e.g., A/Swine/Germany/81 (17)—form a European sublineage separate from the Asian sublineage of avian influenza viruses from China in both the HA1 and NP phylogenetic trees. Analysis of the phylogenetic tree of the M gene segments supports this contention in that the avian influenza virus in European pigs forms a sister group relationship with the avian viruses in Chinese pigs (Fig. 2). Analysis of the PB1 and NS phylogenetic trees places the avian-like swine influenza viruses from China within the European avian lineage; however, the relatively small number of sequences of Asian avian isolates available in GenBank does not permit discrimination within the lineage. The available evidence supports the notion that the avian influenza viruses detected in pigs in China in 1993 were an independent introduction into pigs and probably did not originate in Europe.

Because of the novel nature of the avian H1N1 influenza viruses from Chinese pigs, the possibility that they may have been laboratory contaminants of H1N1 avian strains previously isolated in Hong Kong should be considered. Three facts argue against this. One, during the year of pig surveillance, only recent swine isolates were handled in the laboratory. Two, there is a close phylogenetic relationship of the NP gene to A/Duck/Hokkaido/77 and of the M gene to A/Duck/Nanchang/1749/93 (viruses not available in Hong Kong). Three, the current H1 isolates form an Asian sister group relationship with an earlier H1N1 virus from Hong Kong in 1976 (i.e., A/Goose/HK/8/76) in the phylogenetic tree (Fig. 1). Taken together, this information supports the avian derivations of these recent H1N1 isolates. Their frequency in pigs in China is not known but might be established by serologic studies that are under way.

The classic swine influenza viruses circulating in southern China in 1993 are most closely related to H1N1 viruses circulating among pigs in northern China (7), as illustrated by the HA1 and NP phylogenetic trees and the close relationships of A/Swine/Hong Kong/273/94 (H1N1) and A/Swine/Beijing/47/91 (H1N1). Thus, classic swine influenza viruses are circulating in pigs in China in northern (7), central (Henan and Jiangxi), and southern (Guizhou and Guangdong) provinces.

Should isolation of an avian influenza virus in a postulated intermediate host (pigs) in southern China raise concern over the possible emergence of a new human pandemic strain? Since an H1N1 influenza virus is currently circulating among humans, it is improbable that this avian H1N1 strain could spread to an immune population. Nonetheless, the detection of an avian influenza virus in pigs within a hypothetical influenza

epicenter from which all human pandemics of this century are thought to have emerged is highly significant. What future circumstances could give rise to an avian-like pandemic virus? The acquisition, by reassortment, of different surface glycoproteins of any avian influenza virus present in ducks in the epicenter would be a key element. Antibody to avian influenza virus HA has been detected in farming people in the Chinese countryside, where ducks enter or reside in the home (18, 24, 31). Viruses of the H4 and H7 subtypes are of interest here. Another key element would be the acquisition of H2N2 surface glycoproteins. Although this subtype is much less frequent than other avian subtypes in China, it does occur (22, 23). However, recent surveillance of ducks has not been done. Whether this avian H1N1 virus first detected in 1993 is a long-term resident of Chinese pigs or a recent introduction that will become established in the swine populations of China, as was the case in Europe after its introduction in 1979 (20), remains to be established. Meanwhile, influenza virus surveillance of pigs and ducks, particularly pigs, should be a high priority, as it may provide the first warning of an impending pandemic of human influenza virus.

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REFERENCES

1. **Bean, W. J., G. Sriram, and R. G. Webster.** 1980. Electrophoretic analysis of iodine-labeled influenza virus RNA segments. Anal. Biochem. **102:**228–232.
2. **Claas, E. C. J., Y. Kawaoka, J. C. de Jong, N. Masurel, and R. G. Webster.** 1994. Infection of children with avian-human reassortant influenza virus from pigs in Europe. Virology **204:**453–457.
3. **Crosby, A. W.** 1989. America's forgotten pandemic: the influenza of 1918. Cambridge University Press, Cambridge.
4. **Fitch, W. M., and J. S. Farris.** 1974. Evolutionary trees with minimum nucleotide replacements from amino acid sequences. J. Mol. Evol. **3:**263–278.
5. **Gorman, O. T., W. J. Bean, Y. Kawaoka, I. Donatelli, Y. Guo, and R. G. Webster.** 1991. Evolution of influenza A virus nucleoprotein genes: implications for the origin of H1N1 human and classical swine viruses. J. Virol. **65:**3704–3714.

5a. **Guan, Y., P. H. Li, and K. F. Shortridge.** Unpublished data.

6. **Guo, Y., M. Wang, Y. Kawaoka, O. Gorman, T. Ito, T. Saito, and R. G. Webster.** 1992. Characterization of a new avian-like influenza A virus from horses in China. Virology **188:**245–255.
7. **Guo, Y., R. G. Webster, and Y. H. Zhuge.** 1992. Swine (H1N1) viruses isolated from pigs in China and studies on the origin of isolates. Chin. J. Clin. Exp. Virol. **6:**347–353.
8. **Hinshaw, V. S., D. J. Alexander, M. Aymard, P. A. Bachmann, B. C. Easterday, C. Hannoun, H. Kida, M. Lipkind, J. S. Mackenzie, K. Nerome, G. C. Schild, C. Scholtissek, D. A. Senne, K. F. Shortridge, J. J. Skehel, and R. G. Webster.** 1984. Antigenic comparisons of swine-influenza-like H1N1 isolates from pigs, birds and humans: an international collaborative study. Bull. W. H. O. **62:**871–878.
9. **Hinshaw, V. S., R. G. Webster, and B. Turner.** 1980. The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Can. J. Microbiol. **26:**622–629.
10. **Horimoto, T., E. Rivera, J. Pearson, D. Senne, S. Krauss, Y. Kawaoka, and R. G. Webster.** 1995. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology **213:**223–230.
11. **Ito, T., O. T. Gorman, Y. Kawaoka, W. J. Bean, and R. G. Webster.** 1991. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J. Virol. **65:**5491–5498.

11a. **Kawaoka, Y.** Personal communication.

12. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A virus in the 1957 and 1968 pandemics. J. Virol. **63:**4603–4608.
13. **Kida, H., T. Ito, J. Yasuda, Y. Shimzu, C. Itakura, K. F. Shortridge, Y. Kawaoka, and R. G. Webster.** 1994. Potential for transmission of avian influenza viruses to pigs. J. Gen. Virol. **75:**2183–2188.
14. **Krisnan, B. R., R. W. Blaksley, and D. E. Berg.** 1991. Linear amplification DNA sequencing from single phage plaques and bacterial colonies. Nucleic Acids Res. **19:**1153.
15. **Lin, Y. P., L. L. Shu, S. Wright, W. J. Bean, G. B. Sharp, K. F. Shortridge, and R. G. Webster.** 1994. Analysis of the influenza virus gene pool of avian species from southern China. Virology **198:**557–566.
16. **Ludwig, S., U. Schultz, J. Mandler, W. M. Fitch, and C. Scholtissek.** 1991. Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology **183:**566–577.
17. **Ludwig, S., L. Stitz, O. Planz, H. Van, W. M. Fitch, and C. Scholtissek.** 1995. European swine virus as a possible source for the next influenza pandemic? Virology **212:**555–561.
18. **Markwell, D. D., and K. F. Shortridge.** 1982. Possible waterborne transmission and maintenance of influenza viruses in domestic ducks. Appl. Environ. Microbiol. **43:**110–116.
19. **Scholtissek, C., W. Rohde, V. von Hoyningen, and R. Rott.** 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87:**13–20.
20. **Scholtissek, C., U. Schultz, S. Ludwig, and W. M. Fitch.** 1993. The role of swine in the origin of pandemic influenza, p. 193–207. *In* C. Hannoun et al. (ed.), Options for the control of influenza. II. Elsevier Science Publishers, New York.
21. **Schultz, U., W. M. Fitch, S. Ludwig, J. Mandler, and C. Scholtissek.** 1991. Evolution of pig influenza viruses. Virology **183:**61–73.
22. **Shortridge, K. F.** 1979. H2N2 influenza viruses in domestic ducks. Lancet **i:**439.
23. **Shortridge, K. F.** 1982. Avian influenza A viruses of southern China and Hong Kong: ecological aspects and implications for man. Bull. W. H. O. **60:**129–135.
24. **Shortridge, K. F.** 1992. Pandemic influenza—a zoonosis? Semin. Respir. Infect. **7:**11–25.
25. **Shortridge, K. F.** 1995. The next pandemic influenza virus? Lancet **346:**1210–1212.
26. **Shortridge, K. F., A. Cherry, and A. P. Kendal.** 1979. Further studies of the antigenic properties of H3N2 strains of influenza A isolated from swine in South East Asia. J. Gen. Virol. **44:**251–254.
27. **Shortridge, K. F., A. P. King, and R. G. Webster.** 1987. Monoclonal antibodies for characterizing H3N2 influenza viruses that persist in pigs in China. J. Infect. Dis. **155:**577–581.
28. **Shortridge, K. F., and C. H. Stuart-Harris.** 1982. An influenza epicentre? Lancet **ii:**812–813.
29. **Shortridge, K. F., R. G. Webster, W. K. Butterfield, and C. H. Campbell.** 1977. Persistence of Hong Kong influenza virus variants in pigs. Science **196:**1454–1455.
30. **Shu, L. L., Y. P. Lin, S. M. Wright, K. F. Shortridge, and R. G. Webster.** 1994. Evidence for interspecies transmission and reassortment of influenza A viruses in pigs in southern China. Virology **202:**825–833.
31. **Shu, L. L., N. N. Zhou, G. B. Sharp, S. Q. He, T. J. Zhang, W. W. Zou, and R. G. Webster.** An epidemiological study of influenza viruses among Chinese farm families with household ducks and pigs. Epidemiol. Infect., in press.
32. **Webster, R. G., and Y. Kawaoka.** 1994. Influenza—an emerging and re-emerging disease. Semin. Virol. **5:**103–111.
33. **Wentworth, D. E., B. L. Thompson, X. Xu, H. L. Regnery, A. J. Cooley, M. W. McGregor, N. J. Cox, and V. S. Hinshaw.** 1994. An influenza A (H1N1) virus, closely related to swine influenza virus, responsible for a fatal case of human influenza. J. Virol. **68:**2051–2058.
34. **World Health Organization Collaborating Centers for Reference and Research on Influenza.** 1982. Concepts and procedures for laboratory-based influenza surveillance, B-17–B-44. World Health Organization Collaborating Centers for Reference and Research on Influenza, Centers for Disease Control, Atlanta, Ga.