

Evidence for Interspecies Transmission and Reassortment of Influenza A Viruses in Pigs in Southern China

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The Asian/57, Hong Kong/68, and Russian/77 pandemics of this century appeared or reappeared in China. Interspecies transmission and genetic reassortment of influenza viruses have been implicated in the origin of these human pandemic influenza viruses. Pigs have been suspected to be the "mixing vessel" where reassortment occurs. To investigate this possibility, 104 porcine influenza viruses collected at random from Southern China from 1976 to 1982, including 32 H3N2 isolates and 72 H1N1 isolates, were studied using dot blot hybridization, partial sequencing, and phylogenetic analysis. There were 29 of 32 H3N2 isolates characteristic of viruses originally derived from humans; the other 3 isolates were reassortants containing genes from porcine and human influenza viruses. Phylogenetic analyses of the polymerase B1 (PB1) genes showed that interspecies transmission from humans to pigs has happened multiple times in pigs in Southern China. All 72 H1N1 isolates were of porcine origin characteristic of classical porcine H1N1 influenza virus. Analysis of 624 genes of porcine influenza viruses from Southern China failed to detect any evidence for avian influenza virus genes. This contrasts to what is currently found in Europe, where the majority of porcine influenza virus isolates are of avian origin. © 1994 Academic Press, Inc.

Influenza viruses are negative sense RNA viruses with single-stranded genomes composed of eight segments. The viruses can be divided into a number of subtypes based on differences in the surface glycoproteins, hemagglutinin (HA) (14 subtypes) and neuraminidase (NA) (9 subtypes). All known subtypes of influenza viruses are found among wild avian species that are the primary reservoirs for the virus (Hinshaw and Webster, 1982). In general, influenza viruses are species specific; however, whole viruses may occasionally be transmitted from one species to another and genetic reassortment between viruses from two different hosts can create a virus that is infectious to a third host. In pigs in different regions of the world there are influenza viruses from three hosts including classical swine influenza virus (H1N1), avian-like H1N1 influenza viruses, and human-like H3N2 influenza viruses (Schultz *et al.*, 1991); the term avian- or human-like denotes that these viruses were originally detected in birds or humans and later transmitted to pigs.

Previous studies have shown that interspecies transmission and genetic reassortment of viruses are associated with the appearance of pandemic influenza (Scholtissek *et al.*, 1978). The human influenza pandemic

strains of 1957 [Asian/57 (H2N2)] and 1968 [Hong Kong/68 (H3N2)] were the result of reassortment: three genes (HA, NA, and PB1) of the Asian/57 strain were of avian influenza virus origin, and the remaining genes were of human virus origin (Scholtissek *et al.*, 1978; Webster and Laver, 1972); the Hong Kong/68 pandemic strain obtained its HA and PB1 genes from an avian virus and the other genes were characteristic of human influenza viruses (Kawaoka *et al.*, 1989). Phylogenetic analysis of the gene segments of these viruses reaffirms the earlier sequence analysis (Webster *et al.*, 1992). These reassortments may have occurred as a result of transfer of an avian virus to a human host already infected with a human strain. However, the available evidence indicates that influenza viruses of avian origin do not undergo productive replication in humans. Only limited replication has been detected following high-titer inoculation of volunteers with influenza of avian origin (Beare and Webster, 1991). Therefore, a more likely explanation for the reassortment events responsible for the 1957 and 1968 pandemic viruses is that mixing between human and avian influenza viruses occurred in another animal that served as a "mixing vessel." Pigs have been suggested to be such an intermediate host wherein influenza genomes of human, porcine, and avian origins can mix (Scholtissek *et al.*, 1985).

Pigs are peculiar in that they support replication of

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avian, human, and porcine influenza A viruses, and therefore they are regarded as "mixing vessels" for the creation of new pandemic strains (Scholtissek *et al.*, 1985; Schultz *et al.*, 1991). In 1979, an influenza virus with genes all of avian origin was transmitted to pigs in Europe and has continued to spread and cause disease in pigs (Pensaert *et al.*, 1981; Donatelli *et al.*, 1991). Viruses whose genome consisted of a mixture of influenza segments of human and avian origins were isolated from pigs in Italy (Castrucci *et al.*, 1993). In addition, direct transfer and replication of a virus characteristic of human influenza virus has been reported in pigs (Kundin, 1970), and interspecies transmission of influenza viruses from pigs to humans has occurred (Hinshaw *et al.*, 1978; Rota *et al.*, 1989; Wells *et al.*, 1991). While interspecies transmission and reassortment of influenza viruses have been documented, little information is available on how often these events occur in porcine populations.

Since Southern China has been proposed as an influenza epicenter (Shortridge and Stuart-Harris, 1982) from where the Asian/57 and Hong Kong/68 pandemics first emerged and adjacent to the region in China where the Russian/77 reemerged, we examined porcine influenza viruses from this region. Previous studies had extensively analyzed the surface glycoproteins of porcine influenza viruses from Southern China (Shortridge *et al.*, 1977, 1979, 1987). The present study was designed to determine the host of origin of the six internal genes of porcine influenza viruses isolated from pigs in Southern China between 1976 and 1982, to determine if single or multiple introductions of human influenza viruses occurred in pigs there and to determine what proportion of influenza viruses in them are reassortants with influenza virus genes from different hosts.

MATERIALS AND METHODS

Virus strains

All influenza viruses were isolated from samples collected in Hong Kong during an epidemiological survey from 1976–1982; the pigs originated from Southern China, Taiwan, Singapore, and Hong Kong (Shortridge *et al.*, 1979, 1987). Pigs were randomly sampled as follows: 1976, March–May; 1977, February–May, December; 1978, January–February; 1979, November–December; 1980, February–May; 1981, none sampled; 1982, June–July. Sampling was uncoordinated with pattern of occurrence of human influenza. In Hong Kong and adjacent Guangdong Province, China, human influenza occurs year-round with peaks in February, March, and May–September (Reichelderfer *et al.*, 1989). In all, 94 H1N1 and 32 H3N2 viruses were isolated. Overall, tracheal swabs were collected from 2963 healthy pigs that were 6 to 9 months of age (Table 1). All of the influenza viruses used in the present study were grown in embryonated

chicken eggs in the laboratory in Hong Kong and RNA was extracted and used for the present dot blot, sequence, and phylogenetic analysis.

Hybridization assay

We focused our study on the six viral genes coding for internal proteins. Details of the hybridization assay have been described previously (Wright *et al.*, 1992). Briefly, viral RNA was extracted from allantoic fluid containing influenza viruses. cDNAs of all RNA segments were synthesized using reverse transcriptase (Life Sciences, St. Petersburg, FL) and a 12-base oligodeoxynucleotide primer (5'AGCAAAGCAGG) which is common to all segments of influenza viruses. The cDNAs were amplified by the polymerase chain reaction using pairs of primers specific for the gene under study. The polymerase chain reaction products were cross-linked by ultraviolet light (Stratalinker, Stratagene, La Jolla, CA) to nylon membrane (Zeta Probe, Bio-Rad, Richmond, CA) for dot blot hybridization. The sequences and nucleotide locations for the primers and probes have been reported previously (Wright *et al.*, 1992). Oligonucleotide probes approximately 20 nucleotides in length were prepared for each of the six genes coding for the internal proteins of influenza viruses, namely, nonstructural (NS), matrix (M), nucleoprotein (NP), polymerase (PA), PB1, and polymerase B2 (PB2). Four probes were prepared for each gene: (1) a control probe that would bind to regions conserved by all influenza viruses, (2) a probe specific for viruses from humans, (3) a probe specific for viruses from pigs, and (4) a probe specific for viruses from avian hosts. Probes were tailed with digoxigenin (Boehringer-Mannheim, Indianapolis, IN). Probe binding was detected by a colorimetric reaction with nitroblue tetrazolium and X-phosphate (Boehringer-Mannheim).

Sequence analysis

When clear-cut results were not found by dot blot hybridization, the genes were partially sequenced using direct sequencing of the polymerase chain reaction products or fMol sequencing methods (Promega, Madison, WI). The polymerase chain reaction products were purified by GeneClean (Bio 101, La Jolla, CA). Partial sequences of 563 nucleotides (from 366–929) of PB1 genes were sequenced directly following GeneClean treatment by dideoxynucleotide chain termination (Sanger *et al.*, 1977). The other genes were sequenced by the fMol sequencing method (Krisnan *et al.*, 1991). Briefly, the primers were end-labeled with [γ - 32 P]ATP by incubating at 37° for 10 min and then at 100° for 2 min. The extension/termination reactions were performed in the Programmable Thermal Controller by incubating at 95° for 2 min and then denaturing at 95° for 30 sec, annealing at 42° for 30 sec, and extending at 70° for 1 min, 30

TABLE 1

PORCINE INFLUENZA A VIRUSES ANALYZED IN THIS STUDY

Year of isolation	Origin of pigs	Samples collected	H3N2 isolates	H1N1 isolates
1976	China	478	11	0
1977	China	349	6	10
1977	Hong Kong	282	10	3
1977	Taiwan	85	0	24
1977	Singapore	23	0	13
1978	China	200	2	2
1978	Hong Kong	177	0	4
1979	China	111	0	13
1979	Hong Kong	164	0	3
1979	Taiwan	10	0	1
1980	China	401	0	19
1980	Hong Kong	98	0	2
1980	Taiwan	70	0	0
1982	China	453	0	0
1982	Hong Kong	31	0	0
1982	Taiwan	31	3	0
Total		2963	32	94

cycles in total. After adding the stop solution and heating at 100° for 2 min, 3 µl of each reaction mixture was loaded on a polyacrylamide sequencing gel.

Phylogenetic analysis

All the sequences were analyzed by computer with the FastDB program to find the best matched sequences from GenBank. Phylogenetic analyses of both NP and PB1 sequences were performed with PAUP (Phylogenetic Analysis Using Parsimony) software package (version 2.4, David Swofford, Illinois Natural History Survey, Champaign, IL), which relies on maximum parsimony to generate the phylogeny.

RESULTS

Background of viruses isolated from pigs in Southern China

A total of 2963 tracheal swabs was collected from pigs, and 126 influenza isolates were identified (prevalence rate = 4.3%) (Table 1); comprising 32 H3N2 (25.4%) and 94 H1N1 (74.6%) isolates (Shortridge *et al.*, 1987). All 32 H3N2 viruses and 72 of the H1N1 viruses were used in this study. The H3N2 isolates comprised 19 from China, 10 from Hong Kong, and 3 from Taiwan, while the H1N1 isolates comprised 24 from China, 25 from Taiwan, 10 from Hong Kong, and 13 from Singapore. As the bulk of the isolates studied (82%) were from the geographical area of Southern China (19% were from Singapore) the isolate studied will be referred to as being from Southern China.

From Table 1 it is apparent that H3N2 influenza viruses

were more prevalent in 1976–1978 than in later years. This subtype either disappeared between 1979 and 1980 or was circulating at a very low level. In 1982, H3N2 viruses were again detected. The H1N1 influenza viruses in pigs also showed variation in prevalence from year to year; no H1N1 viruses were detected in 1976 or 1982 but were detected from 1977 to 1980.

Genetic analysis of porcine H3N2 viruses

The genes coding for the internal proteins (192 genes) of the 32 H3N2 isolates were identified by dot blot hybridization. The PA, PB2, NP, NS, and M genes of 29 H3N2 isolates bound to the human virus probe only. The PB1 gene of these 29 H3N2 isolates bound strongly to the human virus probe, while 19 of them had weak reactions with the avian virus probe. Sequence analysis of the PB1 genes showed that they were most homologous with the PB1 genes of human viruses, although they bound weakly to the avian virus probe. Thus, the overall findings for 29 of the 32 H3N2 porcine isolates were that all genes of 29 of these viruses were of human origin (Table 2).

The three other H3N2 isolates (A/Swine/Hong Kong/125/82, A/Swine/Hong Kong/126/82, and A/Swine/Hong Kong/127/82) were different. In dot blot assays, none of the genes encoding the internal proteins of these three

TABLE 2

IDENTIFICATION OF HOST OF ORIGIN OF GENES IN H3N2 INFLUENZA VIRUSES FROM PIGS FROM CHINA

Antigenic analysis of surface proteins	Gene	Number of viruses analyzed	Host of origin of gene ^a		
			Human	Porcine	Avian
H3N2	PB2	29	29	0	0
	PB1	29	29	0	0
	PA	29	29	0	0
	NP	29	29	0	0
	M	29	29	0	0
	NS	29	29	0	0
	Total		174	174	0
H3N2 ^b	PB2	3	0	3	0
	PB1	3	0	3	0
	PA	3	0	3	0
	HA	3	3	0	0
	NP	3	0	3	0
	NA	3	3	0	0
	M	3	0	3	0
	NS	3	0	3	0
Total		24	6	18	0

^a The host of origin of the internal genes was determined by dot blot hybridization; the surface glycoproteins had previously been characterized antigenically with monoclonal antibodies (Shortridge *et al.*, 1987).

^b Viruses included A/Sw/HK/125/82 (H3N2), A/Sw/HK/126/82 (H3N2), A/Sw/HK/127/82 (H3N2). The host of origin of all eight genes was confirmed by partial sequence analysis.

isolates reacted with the probe for avian viruses. Their internal genes cross-hybridized with both human and porcine virus probes. To confirm the dot blot assay, partial sequencing was done on all eight gene segments of these three isolates. The results showed that the six internal genes of A/Swine/Hong Kong/125/82 (H3N2), A/

Swine/Hong Kong/126/82 (H3N2), and A/Swine/Hong Kong/127/82 (H3N2) had highest homology with H1N1 porcine viruses, but the genes coding for the surface proteins had highest homology with the HA and NA of early H3N2 strains of human influenza viruses (Table 3). The HA of A/Swine/Hong Kong/125/82 and A/Swine/

TABLE 3
GENOTYPING OF REASSORTANT INFLUENZA VIRUSES FROM PIGS IN CHINA

Viruses analyzed	Gene	Host of gene origin	Method	Residues sequenced	Homology (%)	References
A/Sw/HK/125/82 (H3N2)	PB1	Porcine	Partial sequence	385-778	Sw/Ontario/2/81 (94.9)	This report
	PB2	Porcine	Partial sequence	1004-1357	Sw/TN/24/77 (94.4)	This report
	PA	Porcine	Partial sequence	54-434	Sw/TN/24/77 (94.5)	This report
	HA	Avian	Majority of sequence ^a			Kida <i>et al.</i> (1988)
	HA	Human	Partial sequence	98-677	Aichi/68 (93.6)	This report
	HA	Avian/human ^b	HI			Shortridge <i>et al.</i> (1987)
	NP	Porcine	Partial sequence	1100-1458	Sw/TN/24/77 (96.0)	This report
	NP	Avian	ELISA			Shortridge <i>et al.</i> (1987)
	NA	Human	NI test ^c			Shortridge <i>et al.</i> (1987)
	NA	Human	Partial sequence	767-1171	Udorn/72 (94.3)	This report
	M	Porcine	Partial sequence	152-509	Sw/Wis/3523/88 (96.4)	This report
	NS	Porcine	Partial sequence	55-440	Sw/TN/26/77 (96.6)	This report
	A/Sw/HK/126/82 (H3N2)	PB1	Avian	Whole sequence		
PB1		Porcine	Partial sequence	385-778	Sw/Ontario/2/81 (94.9)	This report
PB2		Porcine	Partial sequence	1004-1357	Sw/Tn/24/77 (94.4)	This report
PA		Avian	Whole sequence	54-434		Okazaki <i>et al.</i> (1989)
PA		Porcine	Partial sequence		Sw/TN/24/77 (95.5)	This report
HA		Avian	Majority of sequence			Kida <i>et al.</i> (1988)
HA		Human	Partial sequence	98-619	Aichi/68 (94.0)	This report
HA		Avian/human ^b	HI			Shortridge <i>et al.</i> (1987)
NP		Avian	RNA hybridization rescue			Scholtissek <i>et al.</i> (1985)
NP		Porcine	Partial sequence	1100-1458	Sw/TN/24/77 (96.1)	This report
NP		Avian	Whole sequence			Gorman <i>et al.</i> (1991)
NP		Avian	ELISA			Shortridge <i>et al.</i> (1987)
NA		Human	NI test			Shortridge <i>et al.</i> (1987)
NA		Human	Partial sequence	767-1192	Udorn/72 (94.1)	This report
M		Porcine	Whole sequence			Schultz <i>et al.</i> (1991)
M		Porcine	Partial sequence	153-509	Sw/Wis/3523/88 (96.4)	This report
NS		Porcine	Majority of sequence			Schultz <i>et al.</i> (1991)
NS	Porcine	Partial sequence	55-440	Sw/TN/26/77 (96.6)	This report	
A/Sw/HK/127/82 (H3N2)	PB1	Porcine	Partial sequence	1043-1412	Sw/Ontario/2/81 (97.8)	This report
	PB2	Porcine	Partial sequence	984-1252	Sw/TN/24/77 (95.1)	This report
	PA	Porcine	Partial sequence	59-193	Sw/TN/24/77 (94.8)	This report
	HA	Avian	Majority of sequence			Kida <i>et al.</i> (1988)
	HA	Human	Partial sequence	194-991	NT/60/88 (95.3)	This report
	NP	Avian	RNA hybridization rescue			Scholtissek <i>et al.</i> (1985)
	NP	Porcine	Whole sequence			Gammelin <i>et al.</i> (1989)
	NP	Porcine	Partial sequence	1099-1413	Sw/Italy/76 (96.4)	This report
	NA	Human	Partial sequence	765-1159	Bangkok/1/79 (91.6)	This report
	M	Avian	Whole sequence			Ito <i>et al.</i> (1991)
	M	Porcine	Partial sequence	71-435	Sw/Wis/3523/88 (92.3)	This report
	NS	Porcine	Partial sequence	32-445	Sw/Hok/2/81 (95.6)	This report

Note. ELISA using monoclonal antibodies (Shortridge *et al.*, 1987). Abbreviations used: Sw, swine; TN Tennessee; Wis, Wisconsin; Hok, Hokkaido.

^a Greater than 90% of bases were analyzed.

^b Antigenic analysis could not determine the origin of the HA of these viruses.

^c Neuraminidase inhibition test using monoclonal antibodies (Shortridge *et al.*, 1987).

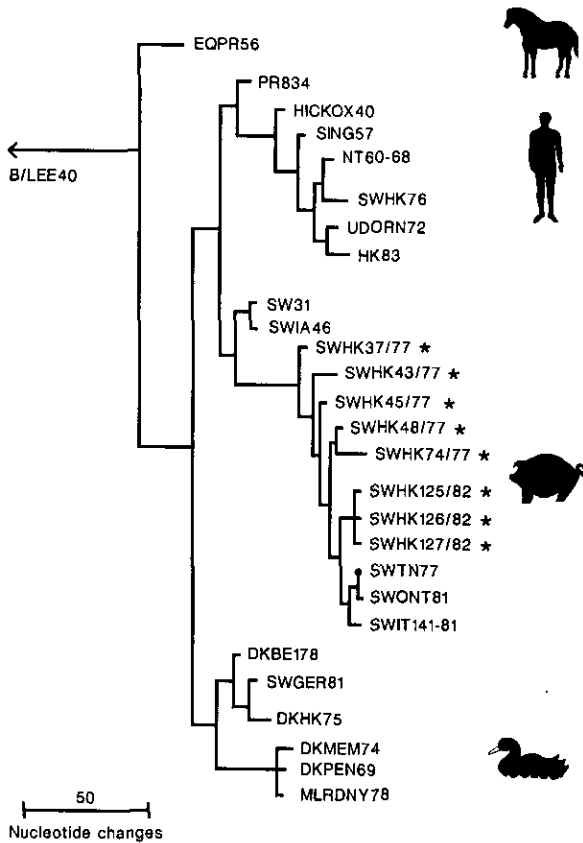


Fig. 1. Evolutionary tree of NP genes of influenza A viruses. The genes from isolates marked by asterisks (*) were sequenced as part of the current study. Others were described previously by Gorman *et al.* (1991). NP genes of five H1N1 porcine influenza isolates (A/Swine/Hong Kong/37/77, A/Swine/Hong Kong/43/77, A/Swine/Hong Kong/45/77, A/Swine/Hong Kong 48/77, A/Swine/Hong Kong/74/77), and three NP genes of H3N2 porcine isolates (A/Swine/Hong Kong/125/82, A/Swine/Hong Kong/126/82, A/Swine/Hong Kong/127/82) were in the classical swine lineage. The tree is rooted in the NP gene of B/Lee/40.

Hong Kong/126/82 showed greatest homology with A/Aichi/2/68 (H3N2) (93.6 and 94.0%) and the HA of A/Swine/Hong Kong/127/82 was most homologous with A/Northern Territory (NT)/60/68 (H3N2) (95.3%).

Since the most extensive phylogenetic analysis of influenza viruses has been done with the NP gene (Gorman *et al.*, 1991), we analyzed this gene of A/Swine/Hong Kong/125/82 (H3N2), A/Swine/Hong Kong/126/82 (H3N2), and A/Swine/Hong Kong/127/82 (H3N2) to establish their phylogenetic relationships. Phylogenetic analyses of the NP genes from these three isolates showed that they are all located on the H1N1 classical porcine virus lineage (Fig. 1). These three H3N2 viruses (A/Swine/Hong Kong/125/82, A/Swine/Hong Kong/126/82, and A/Swine/Hong Kong/127/82) have been partially genotyped previously by a number of investigators including ourselves and the results conflicted with those obtained here (Table 3). For example, the NP gene of A/

Swine/Hong Kong/126/82 (H3N2) was characterized biologically to be of avian origin (Scholtissek *et al.*, 1985); the M gene of this virus was characterized to be of porcine origin in another series of studies (Schultz *et al.*, 1991). Similarly, genetic and antigenic studies on the NP gene of A/Swine/Hong Kong/126/82 (H3N2) in our laboratories showed that it was of avian origin (Shortridge *et al.*, 1987; Gorman *et al.*, 1991), whereas our present studies show that it is of porcine origin. On the other hand, antigenic studies on the HA of three of the isolates, two of which were reported, showed that it was most closely related to the earliest human H3N2 variants or to an avian H3 virus (Shortridge *et al.*, 1987). Comment was made then that antigenic analysis alone would not determine the origin of the HA of these three viruses.

To resolve these conflicting results, RNA was prepared from seed stock in the laboratory in Hong Kong avoiding any possibility of mixed infection. The RNAs of each virus were analyzed independently by two investigators; identical sequences were obtained and the six internal genes were most homologous with porcine influenza viruses (Table 3). The results obtained agree with those of Schultz *et al.*, (1991) and Gammelin *et al.* (1989) that the internal genes of A/Swine/Hong Kong/125/82, A/Swine/Hong Kong/126/82, and A/Swine/Hong Kong/127/82 are of classical porcine origin. The source of the avian H3N2 viruses obtained independently in two laboratories (Scholtissek *et al.*, 1985; Gorman *et al.*, 1991) is impossible to establish but they do not represent the virus in the Hong Kong repository which are viruses processing internal genes of porcine origin.

Were there multiple introductions of human influenza viruses into pigs?

The results reported above indicated that human H3N2 influenza viruses were transmitted to pigs during the period from 1976 to 1982. Phylogenetic analyses were done to determine whether there was a single introduction of H3N2 viruses from humans which then spread in pigs or there were multiple introductions. Dot blot assays of the PB1 genes from the 29 H3N2 isolates differentiated two groups. The probe specific for human influenza viruses bound strongly to 10 PB1 genes, and the remaining 19 PB1 genes bound strongly to the human virus probe and weakly to the avian virus probe. To determine their host(s) of origin, 563 (366–929) bases of the PB1 genes from three randomly chosen isolates were sequenced directly. The PB1 gene from A/Swine/Hong Kong/5/76, isolated from a pig from China in April, 1976, was representative of the group that bound specifically to the human virus probe (Fig. 2, row 1). Its partial sequence was characteristic of human influenza viruses. A/Swine/Hong Kong/9/76 and A/Swine/Hong Kong/21/77 were repre-

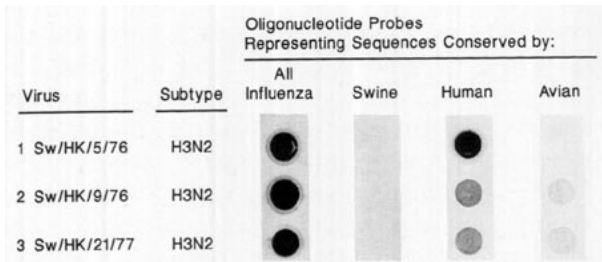


FIG. 2. Determination of host of origin for PB1 genes by dot blot hybridization. Four filters represent the probes used for the assay. A control probe binds to a region conserved by all influenza viruses: porcine, human, and avian probes bind to sequences conserved by each host species. A/Swine/Hong Kong/5/76 (row 1) is an example of strong binding by the probe specific for human influenza viruses. A/Swine/Hong Kong/9/76 and A/Swine/Hong Kong/21/77 (rows 2, 3) are examples of isolates that bind strongly to humans and weakly to avian virus probes.

representative of the isolates that bound strongly to human and weakly to avian virus probes (Fig. 2, rows 2 and 3). A/Swine/Hong Kong/9/76 was isolated in April, 1976, from a pig originating from China, while A/Swine/Hong Kong/21/77 was isolated in March, 1977, from a pig in Hong Kong. The partial sequencing results showed that both of these isolates were of human origin. Only two nucleotide differences were noted between the 563 bases of A/Swine/Hong Kong/9/76 and A/Swine/Hong Kong/21/77. However, there were 13 nucleotide differences between the PB1 genes of these two viruses and A/Swine/Hong Kong/5/76. From these results we could not determine if these viruses represented a single introduction into pigs.

Phylogenetic analysis was done to determine the genetic relationship of these three viruses and to determine if there were multiple introductions into pigs (Fig. 3). The H3N2 viruses (NT/68, Mem/88, and the three porcine influenza viruses from Hong Kong) make up a separate lineage, representing a new introduction of the PB1 gene from an avian source. Within the H3N2 lineage, A/Swine/Hong Kong/9/76 and A/Swine/Hong Kong/21/77 are evolutionarily closely related. However, A/Swine/Hong Kong/5/76 represents another branch, more closely related to the prototype 1968 H3N2 strain than to the other isolates. Thus, the phylogenetic evidence suggests more than one transmission of human H3N2 viruses to pigs in Southern China.

Genetic analysis of porcine H1N1 viruses

The 72 H1N1 influenza viruses used in the study were isolated from pigs from 1977 to 1980. The 432 genes coding for the internal proteins of these isolates were examined in the dot blot assay. All but 5 bound only to the porcine virus probe (Table 4). The NP genes from these isolates — namely, A/Swine/Hong Kong/37/77, A/Swine/Hong Kong/43/77, A/Swine/Hong Kong/45/77, A/Swine/Hong Kong/48/77, and A/Swine/Hong Kong/74/77 bound to both human and porcine virus probes. Partial

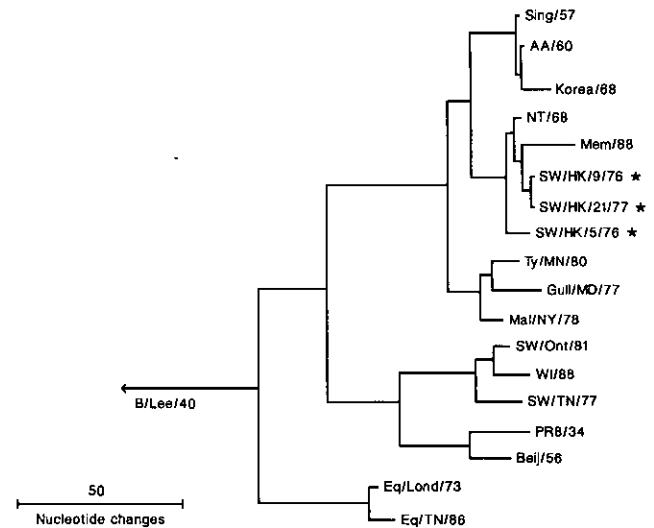


FIG. 3. Evolutionary tree of PB1 genes of influenza viruses. The tree was constructed using PAUP software which relies on maximum parsimony to generate the phylogeny. The lengths of the horizontal branches are proportional to the number of nucleotide changes between viruses. The tree is rooted in the PB1 gene of B/Lee/40. Marked genes (*) were partially sequenced in the present study. Others were described by Kawacka *et al.* (1989).

sequence analysis indicated that these 5 NP genes were also of porcine influenza virus origin (Table 4). They had highest homology with the NP gene of A/Swine/Tennessee/24/77 (H1N1). The above studies show that all of the genes coding for the internal proteins in the 72 porcine H1N1 influenza virus isolates from 1977 to 1980 were of classical porcine origin. Since the nucleotide sequences of a large number of NPs of porcine influenza

TABLE 4

DETERMINATION OF HOST OF ORIGIN FOR H1N1 GENES FROM PORCINE INFLUENZA VIRUSES FROM SOUTHERN CHINA

Gene	Number of genes of porcine origin by dot blot analysis	Number of genes binding to both human and porcine probes by dot blot analysis	Host of origin of genes by sequence analysis
NP	67	5 ^a	5 pig ^b
PB1	72	0	
PB2	72	0	
PA	72	0	
NS	72	0	
M	72	0	
Total	427	5	

^a The isolates were: A/Swine/Hong Kong/37/77, A/Swine/Hong Kong/43/77, A/Swine/Hong Kong/45/77, A/Swine/Hong Kong/48/77, and A/Swine/Hong Kong/74/77.

^b Partial sequencing showed that all of the five A/Swine/Hong Kong/77 isolates had highest homology with A/Swine/Tennessee/24/77 (H1N1).

viruses have been analyzed phylogenetically (Gorman *et al.*, 1991), we placed the NP sequence of these 5 isolates into the evolutionary tree of NP genes. This showed that the NP genes of these isolates are located on the H1N1 classical porcine virus lineage (Fig. 1).

DISCUSSION

Genetic analyses of influenza viruses from pigs from Southern China between 1976 and 1982 provided evidence for both interspecies transmission of human virus and reassortment between human and porcine influenza viruses, but no evidence for the participation of avian influenza virus genes. The failure to detect avian influenza virus genes in pigs indicates that avian influenza virus transmission to pigs is a relatively rare event, whereas the transmission of human influenza viruses of the H3N2 subtype is relatively frequent. The fact that avian influenza virus can transmit to pigs in nature is documented by the fact that the majority of H1N1 influenza viruses currently circulating in pigs in Europe are of recent avian origin (Schultz *et al.*, 1991).

Transmission of human H3N2 influenza viruses to pigs

The isolation of A/Hong Kong/68-like (H3N2) influenza virus from pigs was first recorded in Taiwan in 1969 (Kundin, 1970). The subsequent isolation of a number of H3N2 strains of influenza and indirect evidence from serological surveillance indicated that H3N2 influenza virus variants had spread to the porcine population in other parts of the world (Nerome *et al.*, 1981; McFerran *et al.*, 1972). The H3N2 influenza viruses isolated during the prevalence survey in Southern China from 1976 to 1982 represented 25% of all viruses isolated from pigs during that surveillance. In over 90% of these H3N2 isolates, the genes encoding the internal viral proteins were characteristic of human H3N2 viruses. Our results provide genetic evidence that the internal genes of most porcine H3N2 viruses in Southern China have retained the characteristics of human viruses—at least until 1982. This means that human influenza viruses can transfer to pigs, where they can replicate and have the opportunity for genetic mixing with H1N1 influenza viruses that are circulating in them.

The human influenza viruses that transferred to pigs appear to be genetically more stable than their counterparts in humans (Gorman *et al.*, 1991). Once the human H3N2 viruses were introduced into the porcine population, they appear to have been under less immune selection pressure; the internal genes of the transmitted viruses also evolved more slowly than in humans (Gorman *et al.*, 1992). This may be the reason why most of the internal genes of H3N2 porcine viruses in our study still

maintain the characteristics of early human H3N2 viruses.

Previous evidence has indicated that there have been multiple introductions of H3N2 influenza virus into pigs in Southern China (Shortridge *et al.*, 1987). The H3N2 viruses isolated in 1976 and 1977, including A/Swine/Hong Kong/3/76 and A/Swine/Hong Kong/6/76, were most closely related antigenically to A/Hong Kong/68, while A/Swine/Hong Kong/4/76 was most reactive with H3N2 viruses such as A/Victoria/3/75 and A/Texas/1/77. This suggests that pigs were infected with A/Victoria/3/75-like viruses as well as the prototype A/Hong Kong/68 strains (Shortridge *et al.*, 1979). Additional evidence of multiple interspecies transmission of H3N2 influenza in China is provided by the phylogenetic analysis of the PB1 gene (Fig. 3). Dot blot hybridization coupled with sequence analysis demonstrated that there were at least two separate introductions of H3N2 viruses into pigs from humans.

Notwithstanding that surveillance of pigs was random, it is interesting that viruses resembling contemporary human H3N2 variants were not found in pigs after 1978. This raises the possibility that the HA antigens of viruses arising after A/Victoria/75 and A/Texas/77 may not have the propensity to allow such H3 viruses to become established in the porcine population of China (and elsewhere).

H1N1 influenza viruses in pigs

Classical H1N1 porcine viruses were first isolated from pigs in 1930 (Shope, 1931). Our results showed that all the internal genes of 72 of the 94 H1N1 isolates from Southern China examined were of porcine influenza virus origin; that is, typical of classical porcine H1N1 viruses. The phylogenetic tree showed that the NP genes of 5 of the H1N1 isolates from China are located on the classical porcine H1N1 virus lineage. These results also show that the classical porcine H1N1 viruses circulated continuously in Southern China from 1977 to 1980. Indeed, surveillance studies in 1991 in pigs in Northern China indicated that classical porcine influenza viruses have continued to circulate in pigs (Guo *et al.*, 1992).

In contrast to the situation in Europe, where avian-like H1N1 viruses have circulated continuously in pigs from 1979 (Pensaert *et al.*, 1981; Donatelli *et al.*, 1991; Scholtissek *et al.*, 1983), avian-like H1N1 viruses were not recognized among 72 of the 94 porcine isolates examined from Southern China in this study. It cannot be stated with confidence that avian-like H1N1 viruses do not exist in the pig population of Southern China, but if present they are relatively infrequent. Further surveillance is in order, given that the European avian H1N1 viruses in pigs may spread to Asia or an avian H1N1 currently circulating at lower levels in pigs in China may become dominant. The

transmission of avian H1N1 viruses to pigs, as has been recognized in Europe, offers the possibility of reassortment with resident H3N2 viruses (Castrucci *et al.*, 1993).

Reassortant influenza viruses in pigs

The cocirculation of two subtypes (H3N2 and H1N1) of influenza viruses in the porcine population in Southern China provides an opportunity for genetic exchange. In this study, we found three viruses in which the genes of human-like H3N2 and porcine H1N1 viruses were mixed; in Swine/Hong Kong/125/82, Swine/Hong Kong/126/82, and Swine/Hong Kong/127/82 the surface genes were of human virus origin and the internal genes were of porcine virus origin. These reassortants comprised about 3% of the total number of viruses analyzed. Whether this percentage represents the true percentage of pigs mixedly infected with H3N2 and H1N1 influenza viruses is unclear since they were isolated on only 1 of the 34 sampling occasions over the 7-year surveillance period.

Previous studies on the origin of some of the gene segments in the three viruses designated A/Swine/Hong Kong/125/82 (H3N2), A/Swine/Hong Kong/126/82 (H3N2), and A/Swine/Hong Kong/127/82 (H3N2) have given different results in different laboratories (Table 3). We therefore obtained RNA prepared in Hong Kong and two investigators independently determined by dot blot analysis, partial sequence analysis, and phylogenetic analysis the host of origin of each gene segment. The results establish that the viruses currently in the Hong Kong repository are reassortants possessing the HA and NA genes from human influenza viruses and the internal genes from porcine influenza viruses. The reason two independent laboratories (Scholtissek *et al.*, 1985; Gorman *et al.*, 1991) previously characterized internal gene segments from these viruses as being of avian origin (Table 3) could be due to mixtures of viruses that can no longer be resolved.

Genetic reassortment of influenza viruses in pigs has been reported previously by Sugimura *et al.* (1980) and Nerome *et al.* (1991), who found that the NA gene of A/Swine/Kanagawa/2/78 (H1N2) was derived from a human H3N2 virus, while the seven other genes were from a porcine H1N1 virus.

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