

# AVIAN INFLUENZA VIRUS AS THE ORIGIN OF HUMAN PANDEMIC STRAINS

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## SUMMARY

In Japan, no influenza epidemics have been recognized in domestic fowls since the last fowl plague outbreak caused by H7N7 influenza virus in chickens in 1924-1926. On the other hand, influenza viruses of different subtypes have been isolated from wild ducks, geese, and swans.

Antigenic and genetic analyses of H3 influenza viruses isolated from migratory ducks captured on the Pacific flyway in Japan have shown that the hemagglutinins (HAs) of some of these viruses are closely related to those of the earliest human H3N2 viruses and are highly conserved in nature. It has also been shown that the HAs of some H3N2 influenza viruses isolated from pigs in southern China are closely related not only to those of the earliest human H3N2 influenza viruses but also to those of the strains from migratory ducks. The HAs of some H3 influenza viruses isolated from domestic ducks in southern China were closely related to those of isolates from migratory ducks and pigs antigenically and genetically. The antigenic and genetic similarity between these H3HAs indicate that in southern China, the hypothetical influenza epicenter, domestic ducks may have played a role in the introduction of avian influenza viruses to pigs from migratory ducks.

Experimental infection of pigs with H1 and H3 influenza viruses of different origin showed that the upper respiratory tract of pigs was sensitive not only to the HA of viruses of porcine and human origin but also to that of duck origin. Pigs infected concurrently with avian and porcine virus strains produced genetic reassortants between those. The results indicate that domestic pigs have played a role as a 'mixing vessel', producing a new human pandemic strain by exchange of gene segments between viruses which infect birds and those which infect man.

Experimental infection studies of pigs with influenza viruses of avian

origin revealed that some of virus strains of each HA subtype of H1-13 replicated in the upper respiratory tract of pigs. It is noted that pigs were susceptible to viruses of H4-13 which have been recognized only in avian host species. In the sera of the pigs infected with these avian influenza viruses, antibody response was evident by ELISA although hemagglutination-inhibiting antibodies with the homologous viruses were not detected. These findings indicate that influenza viruses of subtypes other than H1 and H3 HA could also be introduced into pigs and that seroepidemiological survey by HI tests may not provide exact information of infection of pigs with avian influenza viruses.

## RESULTS AND DISCUSSION

### Antigenic and genetic conservation of the HAs of H3 influenza viruses in nature

For antigenic analysis of the HA a panel of monoclonal antibodies to 10 different epitopes on the H3HA molecule was established (Table 1).

Table 1

REACTIVITY PATTERNS AND AMINO ACID CHANGE IN THE HEMAGGLUTININS OF ANTIGENIC VARIANTS OF AICHI/68 AND DK/8/80 VIRUSES SELECTED WITH MONOCLONAL ANTIBODIES

Monoclonal antibodies	Reactivity of antigenic variants selected with the following monoclonal antibodies 1)											Amino acid change in the HA of variants
	13/1	110/2	D12/2	D59/2	D17/4	48/2	22/1	73/2	D11/3	D13/1	D58/2	
* 13/1	-											130 Val→Ile
*110/2		-										158 Gly→Glu
D12/2		-	-	-	-							158 Gly→Glu
D59/2		-	-	-	-							158 Gly→Glu
*D17/4		-	-	-	-							156 Lys→Asn
* 32/2												ND1 <sup>3)</sup>
52/1												ND1
* 48/2						-						162 Pro→Leu
* 22/1							-	-	-			198 Ala→Glu
73/2							-	-	-			198 Ala→Glu
*D11/3 2)	(-)	(-)				(-)	(-)	(-)	-	-		189 Gln→His
*D13/1									-	-		192 Thr→Asn
*D58/2											-	172 Asp→Asn
*D22/3												81 Asp→Asn
D47/4												ND2 <sup>4)</sup>
												ND2

1) Each of the monoclonal antibodies used in variant selection was titrated in ELISA with each antigenic variant; - indicates no binding, no entry indicates significant binding to the variant virus antigen.

2) Aichi/68 and its variants lack the epitope recognized by D11/3 antibody.

3) No antigenic variant was obtained in the presence of these antibodies.

4) Non-neutralizing antibodies.

Using the established panel of monoclonal antibodies, H3 influenza viruses isolated from migratory ducks captured on the Pacific flyway in Japan and human H3N2 vaccine strains were analyzed by ELISA (Table 2).

Table 2

REACTIVITY OF DUCK AND HUMAN H3 INFLUENZA VIRUSES WITH MONOCLONAL ANTIBODIES TO DIFFERENT EPITOPES ON THE HEMAGGLUTININ OF AICHI/68 AND DK/8/80

Viruses	Monoclonal Antibodies									
	13/1	110/2	48/2	22/1	32/2	D11/3	D13/1	D17/4	D58/2	D22/3
Duck Strains										
Dk/Ukraine/63	+	-	+	+	-	+	+	-	-	+
Dk/Hok/5/77	+	+	+	-	+	+	+	+	+	+
Dk/Hok/8/80	+	+	+	+	+	+	+	+	+	+
Dk/Hok/33/80	+	+	+	-	+	+	+	+	+	+
Dk/Hok/7/82	+	+	+	-	+	-	-	+	+	+
Dk/Hok/21/82	+	+	+	-	+	-	-	+	+	+
Dk/Hok/9/85	+	+	+	-	+	+	+	+	+	+
Dk/Hok/10/85	+	+	+	-	+	+	+	+	+	+
Human Strains										
Aichi/2/68	+	+	+	+	+	-	+	+	+	+
Udorn/307/72	+	+	+	+	-	-	+	+	+	+
Tokyo/6/73	-	+	+	+	-	+	+	+	+	-
P. Chalmers/1/73	-	-	-	+	-	+	+	+	+	-
Victoria/3/75	+	-	-	-	-	-	-	+	+	-
Kumamoto/22/76	-	-	-	-	-	-	-	+	+	-
Bangkok/1/79	-	-	-	-	-	-	-	-	+	-
Philippines/2/82	-	-	-	-	-	-	-	-	+	-

Each of the monoclonal antibodies was titrated by ELISA with virus strains; + indicates significant binding, - indicates no binding to the viral antigen.

Human strains showed progressive loss of reactivity with time of isolation, while duck strains did not. Antigenic analysis thus revealed that antigenic drift occurred extensively in human influenza viruses, whereas in duck viruses the antigenicity of the HA was highly conserved. The HAs of most H3 influenza viruses tested, which were isolated from ducks in Southeast Asia and North America were antigenically closely related to each other and to the 1968 human Hong Kong/68 (H3N2) prototype strains.

To provide information on genetic variation and on the possible precursors of human H3 influenza viruses, the HA genes of Aichi/68 and seven H3 influenza viruses isolated from migratory ducks were sequenced. The identities in nucleotide sequences of the HA gene and the deduced amino acid sequences of these viruses are summarized in Table 3. The nucleotide

sequence identities of six isolates from wild ducks to Aichi/68 were high (95.4–97.5%). The HA gene sequence of Dk/21/82 virus showed a considerable nucleotide divergence from the other six isolates from migratory ducks, Dk/Ukr/63, and Aichi/68 viruses (approximately 15%). The HA gene of Dk/21/82 virus probably belongs to a lineage different from those of the other viruses of migratory duck origin. On the other hand, the amino acid sequence identity between the HA of Dk/21/82 and the other viruses were high (95.1–96.4%); most of the differences in nucleotide sequence were silent.

Table 3

NUCLEOTIDE AND AMINO ACID SEQUENCE IDENTITY BETWEEN THE HA GENES OF H3 INFLUENZA VIRUS ISOLATES FROM WILD DUCKS, DK/UKRAINE/63 AND AICHI/68

	Nucleotide Sequence Identity (%)								
	Dk/5 /77	Dk/8 /80	Dk/33 /80	Dk/7 /82	Dk/9 /85	Dk/10 /85	Dk/21 /82	Dk/Ukr /63	Aichi /68
Dk/Hok/5/77		94.6	97.1	94.2	96.4	96.7	84.8	90.5	96.9
Dk/Hok/8/80	97.5		95.8	98.2	94.6	94.9	85.0	91.0	95.9
Dk/Hok/33/80	98.2	98.5		95.3	98.5	98.5	85.6	91.4	97.5
Dk/Hok/7/82	97.6	98.9	98.7		94.4	94.7	85.2	91.4	95.4
Dk/Hok/9/85	97.8	97.8	99.3	98.0		99.4	85.0	91.0	96.7
Dk/Hok/10/85	98.0	98.0	99.1	98.2	99.8		85.5	90.8	97.1
Dk/Hok/21/82	95.3	96.0	96.4	96.2	95.6	95.8		85.6	85.0
Dk/Ukr/63	95.8	96.7	96.9	96.7	96.2	96.4	95.1		91.2
Aichi/2/68	97.1	97.3	97.8	97.6	97.6	97.8	95.3	95.8	

Amino Acid Sequence Identity (%)

Antigenic and genetic analyses of influenza viruses from wild ducks have thus shown that the H3HA genes of the viruses are conserved in nature and that viruses of different lineages cocirculate. Why then is the HA genes of duck influenza viruses so highly conserved in nature? Laboratory studies have shown that the antibody response of ducks to avian influenza viruses is weak and shortlived and ducks appear to be readily reinfected with the same virus (Kida et al., 1980). Even if they produce neutralizing antibodies, the serum antibodies may not be effective at inhibiting viral replication in the intestinal tract, the site where these viruses preferentially replicate in ducks (Slemons and Easterday, 1978; Webster et al., 1978). Every year large numbers of juvenile ducks are added to the population; 40–50% of the duck population each year consists of juvenile birds that hatched that year. Such birds are susceptible to influenza virus infection (Hinshaw et al., 1980).

These features may explain why there is apparently little selection pressure for antigenic variation of influenza viruses in wild ducks.

Antigenic and genetic analyses also revealed that six of seven duck influenza viruses from Japan possess HAs that are more closely related to Aichi/68 than the Dk/Ukr/63 virus. The human H3 influenza virus HA gene that appeared in China in 1968 could have been derived from a member of the duck H3 virus lineage, such as Dk/33/80, Dk/9/85, or Dk/10/85. Each of these viruses is closer in sequence identity to the A/Aichi/68 (H3N2) influenza virus than is the Dk/Ukr/63 virus.

#### Origin of the HA gene of H3N2 influenza viruses from pigs in China

Influenza viruses of the H3N2 subtype similar to Aichi/68 persist in pigs many years after their antigenic counterparts disappeared from humans. To provide information on the mechanisms of conservation of these influenza viruses in pigs, the HAs of four isolates from pigs derived from Taiwan and southern China were analyzed antigenically and genetically.

Table 4 shows the reactivity of swine, wild duck, and human H3 influenza viruses with the panel of monoclonal antibodies to different epitopes on the H3HA. All four swine H3N2 viruses reacted with the 10 monoclonal antibodies in ELISA, indicating that they are closely related not only to the earliest human H3N2 influenza virus but also to virus strains of wild duck origin.

Table 4

#### REACTIVITY OF SWINE, WILD DUCK, AND HUMAN H3 INFLUENZA VIRUSES WITH A PANEL OF MONOCLONAL ANTIBODIES

Viruses	Monoclonal Antibodies									
	13/1	110/2	48/2	22/1	32/2	D11/3	D13/1	D17/4	D58/2	D22/3
Sw/HK/125/82	+	+	+	+	+	+	+	+	+	+
Sw/HK/126/82	+	+	+	+	+	+	+	+	+	+
Sw/HK/127/82	+	+	+	+	+	+	+	+	+	+
Sw/HK/81/78	+	+	+	+	+	+	+	+	+	+
Dk/Hok/8/80	+	+	+	+	+	+	+	+	+	+
Dk/Hok/7/82	+	+	+	-	+	-	-	+	+	+
Aichi/2/68	+	+	+	+	+	-	+	+	+	+
Victoria/3/75	+	-	-	-	-	-	-	+	+	-

Nucleotide sequences of the HA genes of the four influenza virus isolates from pigs in Taiwan and southern China were compared with those of wild duck and human viruses. Table 5 shows the identities in nucleotide and deduced amino acid sequences among the HA genes of the swine H3N2 viruses, Dk/8/80, Aichi/68, and Victoria/75. Since the three H3N2 isolates from pigs in Taiwan had almost identical sequences, the Sw/HK/126/82 is shown as a representative of the Taiwan strains. Sequence analysis thus revealed that the HA genes of the swine strains from Taiwan are more closely related to those of the wild duck viruses than the earliest human H3 virus. The HA gene of the Sw/81/78 isolate from southern China was equally closely related to the avian and human H3 influenza virus strains.

Table 5

NUCLEOTIDE AND AMINO ACID SEQUENCE IDENTITY BETWEEN  
THE HA GENES OF H3 INFLUENZA VIRUSES OF  
SWINE, DUCK AND HUMAN ORIGIN

	Nucleotide Sequence Identity (%)				
	Sw/HK/ 126/82	Sw/HK/ 81/78	Dk/Hok /8/80	Aichi/ 2/68	Vic/3/ 75
Sw/HK/126/82		96.5	<u>98.3</u>	96.5	93.3
Sw/HK/81/78	97.1		95.8	96.3	95.2
Dk/Hok/8/80	<u>98.7</u>	97.1		95.9	93.2
Aichi/2/68	97.1	96.5	97.5		
Victoria/3/75	94.5	95.5	94.4	95.6	
	Amino Acid Sequence Identity (%)				

The number of amino acid differences between the HAs of the swine isolates and Dk/8/80 were very small, especially among the three viruses from Taiwan and Dk/8/80 virus (7 amino acids). In comparison, the HA genes of the swine isolates were more divergent from Victoria/75 (30 amino acids) and from Bangkok/1/79 that was circulating in humans at the time of isolation from pigs in Taiwan. These results indicate that the HA genes of the viruses isolated from pigs in Taiwan have come from viruses circulating in wild ducks.

The deduced amino acid sequence of the HAs of the three swine viruses at residues 226 to 228 in the proposed receptor-binding site is Gln-Ser-Gly which has been found only in the HAs of influenza viruses of avian origin (Table 6). The southern China swine strain showed the same sequence as human strains.

These results provide an explanation for the persistence of H3N2 viruses in pigs after their counterparts have disappeared from humans. The HA gene of duck H3 viruses antigenically similar to the earliest human H3 viruses

which have been maintained in wild ducks was probably transmitted to pigs after these viruses have disappeared from humans.

Table 6

AMINO ACID SEQUENCE IN THE VICINITY  
OF THE RECEPTOR-BINDING SITE  
ON THE H3 HEMAGGLUTININ MOLECULE

Viruses	Amino Acid of Position		
	226	227	228
Aichi/68 and Human Isolates	Leu	Ser	Ser
Duck Isolates	Gln	Ser	Gly
Sw/Taiwan/82	Gln	Ser	Gly
Sw/China/78	Leu	Ser	Ser

A role of domestic ducks in the introduction of avian H3 influenza viruses to pigs in southern China, where the A/Hong Kong/68 (H3N2) strain emerged

Antigenic and genetic analyses of the H3HAs of influenza viruses isolated from wild ducks and pigs indicate that the HA gene of the A/Hong Kong/68(H3N2) influenza virus was introduced into the preceding human H2N2 virus from an H3 influenza virus maintained among wild ducks via pigs. However, it is less likely that the avian H3 influenza viruses are transmitted directly to pigs from wild ducks. In southern China, the predicted epicenter for new pandemic influenza viruses (Shortridge and Stuart-Harris, 1982), domestic ducks and geese are raised in close association with pigs and humans. It is, therefore, reasonable to suggest that the domestic duck might introduce avian influenza viruses transmitted from wild ducks into pigs. To obtain information on the role of domestic ducks in the emergence of new human pandemic influenza viruses, we have analyzed antigenically and genetically the HAs of H3 influenza virus isolates from domestic ducks and a goose in China.

The HAs of six H3 influenza viruses isolated from five domestic ducks and a goose in China were analyzed antigenically by ELISA using the panel of monoclonal antibodies (Table 7). The patterns of reactivity of three strains (Dk/HK/7/75, Gs/HK/10/76, and Dk/HK/64/76) with the panel of monoclonal antibodies were similar to each other and to those of A/swine/HK/126/82(H3N2),

Dk/Hok/8/80, and Aichi/2/68. This finding indicates that the HAs of these three strains are closely related antigenically to those of the swine, wild duck, and the earliest human H3 influenza viruses. Two strains (Dk/HK/231/77 and Dk/HK/526/79) did not react with some of the monoclonal antibodies, and the remaining strain, Dk/HK/24/76, lacked reactivity with almost all the monoclonal antibodies. These findings indicate that H3 influenza viruses possessing antigenically different HAs are present in domestic ducks in China.

Table 7

REACTIVITY OF DOMESTIC AND WILD DUCK, SWINE AND HUMAN H3 INFLUENZA VIRUSES WITH A PANEL OF MONOCLONAL ANTIBODIES

Viruses	Monoclonal Antibodies									
	13/1	110/2	48/2	22/1	32/2	D11/3	D13/1	D17/4	D58/2	D22/3
Dk/HK/7/75	+	+	+	-	+	+	+	+	+	+
Gs/HK/10/76	+	+	+	-	-	+	+	+	+	+
Dk/HK/64/76	+	+	+	-	-	+	+	+	+	+
Dk/HK/231/77	+	-	+	+	+	-	-	+	+	+
Dk/HK/526/79	+	-	-	-	-	+	+	+	+	+
Dk/HK/24/76	+	-	-	-	-	-	-	-	+	-
Dk/Hok/8/80	+	+	+	+	+	+	+	+	+	+
Sw/HK/126/82	+	+	+	+	+	+	+	+	+	+
Aichi/2/68	+	+	+	+	+	-	+	+	+	+

Table 8

NUCLEOTIDE AND AMINO ACID SEQUENCE IDENTITY BETWEEN THE HA GENES OF H3 INFLUENZA VIRUSES OF DOMESTIC AND WILD DUCK, SWINE AND HUMAN ORIGIN

	Nucleotide Sequence Identity (%)							
	Dk/HK/7/75	Gs/HK/10/76	Dk/HK/64/76	Dk/HK/231/77	Sw/HK/126/82	Dk/Hok/8/80	Aichi/2/68	Vic/3/75
Dk/HK/7/75		96.6	97.1	92.5	99.0	98.0	95.6	93.0
Gs/HK/10/76	97.5		99.0	93.5	96.7	96.2	95.2	92.9
Dk/HK/64/76	97.8	98.9		93.2	97.3	96.7	95.4	93.2
Dk/HK/231/77	96.6	97.3	96.9		93.2	92.7	94.0	91.5
Sw/HK/126/82	99.5	97.6	98.0	96.9		98.3	96.5	93.3
Dk/Hok/8/80	97.8	97.5	97.8	96.7	98.7		95.9	93.2
Aichi/2/68	96.9	96.9	96.9	96.9	97.1	97.3		96.5
Vic/3/75	94.0	93.6	93.6	93.1	94.6	94.4	95.6	

Amino Acid Sequence Identity (%)

Sequence analysis revealed that the HA genes of the two duck viruses and the goose virus were closely related to those of isolates from wild ducks and pigs; the identities between the deduced amino acid sequence of the HA of Dk/HK/7/75 and those of isolates from a wild duck and a pig were 98.7% and 99.5%, respectively (Table 8).

The antigenic and genetic similarity between these H3HAs suggests that in southern China, the hypothetical epicenter, domestic ducks may have played a role in the introduction of avian influenza viruses to pigs from migrating ducks. We thus propose the route of introduction of the H3 HA gene, that is, from H3 influenza virus circulating in migratory duck population to domestic duck in southern China, then to pigs probably by water-borne transmission (Figs.1 and 2). The findings also support the hypothesis that the pig was a 'mixing vessel', producing a new human pandemic strain, A/Hong Kong/68 (H3N2), by genetic reassortment (Scholtissek et al., 1985). In relation to this hypothesis, it is important to examine susceptibility of pigs to influenza viruses originated from ducks and geese.

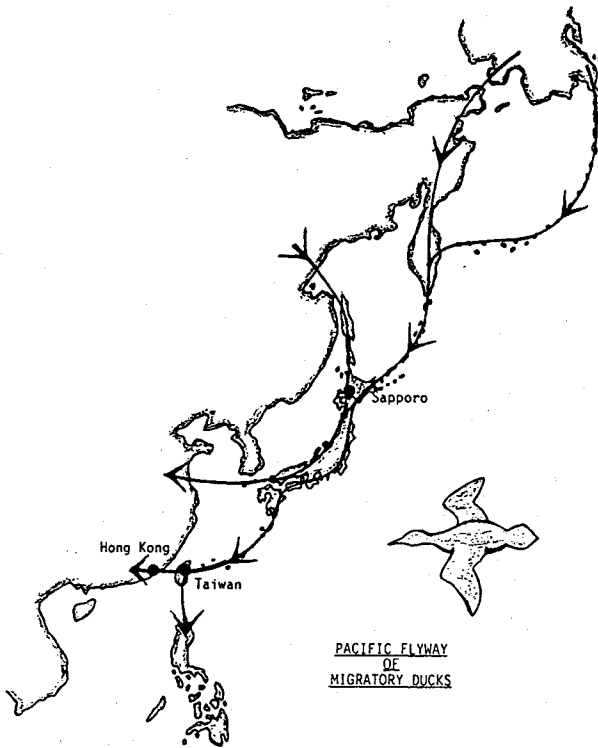


Fig. 1. PACIFIC FLYWAY OF MIGRATORY DUCKS

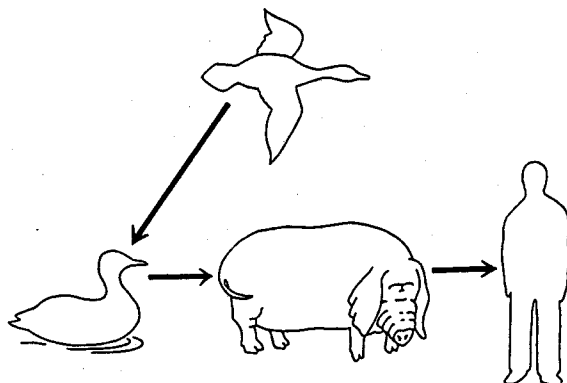


Fig. 2. ROUTE OF INTRODUCTION OF THE H3 HA GENE

### Susceptibility of pigs to the H3 HA of influenza viruses isolated from migratory ducks

The results of experimental infection of pigs with H1 and H3 influenza viruses of different origin are summarized in Table 9. After dropping virus by nasal route, in the case of viral replication positive, virus was recovered most effectively from the nasal swabs for 5 to 7 days and less in the tracheal swabs but never detected from the fecal materials, intestinal tract, or the blood. In the case of viral replication negative, virus was not detected in the nasal swabs on the day 2 post inoculation nor thereafter. None of the pigs showed any clinical signs or pathological lesions upon necropsy.

Table 9

#### Susceptibility of Pigs to Influenza Viruses Isolated from Different Host Species

Virus Tested	Virus Replication	Antibody Response
A/Aichi/2/68 (H3N2)	+	+/+*
V/IR/Aichi/2/68 (H3N2)	+	+/+
A/sw/Hok/2/81 (H1N1)	+	+/+
A/sw/Hok/10/85 (H3N2)	+	+/+
A/dk/Hok/8/80 (H3N8)	-	-/-
V-58/2/dk/Hok/8/80 (H3N8)	-	-/-
A/dk/Hok/7/82 (H3N8)	-	-/-
Dk/Hok/8/80 + Sw/Hok/2/81	+++	+/+ +/+

\* Hemagglutination-inhibition / ELISA

Human and swine influenza viruses replicated in the upper respiratory tract in pigs while those of migratory duck origin did not. However, when the duck virus and swine virus were concurrently inoculated by nasal route, both viruses and reassortant viruses were recovered from the nasal swabs. Genome derivation of recovered virus clones from a pig inoculated concurrently with viruses of duck and swine origin was determined (Table 10).

Table 10

Genome Derivation of Recovered Virus Clones from a Pig Mixedly Infected with Dk/8/80(H3N2) and Sw/2/81(H1N1) and Their Transmissibility in Pigs

Virus Clone	Genome Derivation*								Replication in Pig
	PB2	PB1	PA	HA	NP	NA	M	NS	
3 (H3N8)	D	D	D	D	D	D	D	D	-
6 (H3N1)	D	D	D	D	S	S	S	D	+
7 (H3N1)	D	D	D	D	S	S	D	S	+

\* D : A/duck/Hokkaido/8/80 (H3N8) gene  
 S : A/swine/Hokkaido/2/81 (H1N1) gene

H3N8 viruses were recovered from the pig until 6 post inoculation days. All of the 8 genome segments of H3N8 clones examined were derived from the duck virus. These clones, however, could not be passaged in pigs unless swine virus was coinfecting. Reassortant H3N1 virus clones were also recovered and were transmissible from pig to pig. NP and NA, and M or NS gene segments of these clones were derived from the swine virus.

Experimental infection of pigs with H1 and H3 influenza viruses of different origin thus showed that the upper respiratory tract of pigs was sensitive not only to the HA of viruses of swine and human origin but also to that of wild duck origin. Pigs infected concurrently with avian and swine virus strains produced genetic reassortants between those. The results indicate that domestic pigs have played a role as a 'mixing vessel' producing a new human pandemic strain by exchange of gene segments between viruses which infect birds and those which infect man.

Then we tested H3 influenza viruses isolated from domestic ducks in China for their ability to replicate in the upper respiratory tract of pigs. Out of 6 virus strains tested, 3 replicated (data not shown). The sera of the

pigs infected with and shed these influenza viruses did not inhibit hemagglutination of the homologous viruses, but antibody response was clearly demonstrated by ELISA.

### **Susceptibility of pigs to avian influenza viruses**

As far as we know, three subtypes of influenza A viruses have appeared in humans as new pandemic strains. They are H1N1, H2N2, and H3N2. Then what is possible subtype of the new pandemic influenza virus strain? In order to provide information for prediction of new pandemic influenza viruses, H1-13 influenza viruses of avian origin were tested for their ability to replicate in the respiratory tract of pigs.

Experimental infection of pigs with avian influenza viruses revealed that some of virus strains of each HA subtype of H1-13 replicated in the upper respiratory tract of pigs. It is noted that pigs were susceptible to H4-13 viruses which have been recognized only in avian host species. These animals infected with avian influenza viruses shed those in the nasal discharge for 5 to 7 days without showing any disease signs. In the sera of the infected pigs antibody response was evident by ELISA although hemagglutination-inhibiting antibodies with the homologous viruses were not detected. These findings indicate that influenza viruses of other subtypes than H1-3 HAs could also be introduced into pigs from avian species and that seroepidemiological survey by HI tests may not provide exact information of infection of pigs with avian influenza viruses.

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