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## Emergence of H3N2 reassortant influenza A viruses in North American pigs

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### Abstract

In late summer through early winter of 1998, there were several outbreaks of respiratory disease in the swine herds of North Carolina, Texas, Minnesota and Iowa. Four viral isolates from outbreaks in different states were analyzed, both antigenically and genetically. All of the isolates were identified as H3N2 influenza viruses with antigenic profiles similar to those of recent human H3 strains. Genotyping and phylogenetic analysis demonstrated that the four swine viruses had emerged through two different pathways. The North Carolina isolate is the product of genetic reassortment between human and swine influenza viruses, while the others arose from reassortment of human, swine and avian viral genes. The hemagglutinin genes of the four isolates were all derived from the human H3N2 virus circulating in 1995. It remains to be determined if either of these recently emerged viruses will become established in the pigs in North America and whether they will become an economic burden. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Swine; H3N2; Influenza viruses; Reassortants; Human–avian–swine

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## 1. Introduction

All known subtypes of influenza A virus are found among wild avian species that serve as primary reservoirs for these agents (Hinshaw and Webster, 1982). In general, an influenza virus infects only a single species; however, whole viruses may occasionally be transmitted from one species to another, and genetic reassortment between viruses from two different hosts can produce a new virus capable of infecting a third host. Avian influenza viruses are not readily introduced into humans (Beare and Webster, 1991), possibly because humans do not possess the  $\alpha(2,3)$  sialyllactose (NeuAc 2,3Gal) receptors required for attachment of the viruses to epithelial cells. However, individual viral genes can be transmitted between humans and avian species, as demonstrated by avian–human reassortant viruses that caused the 1957 and 1968 influenza pandemics (Scholtissek et al., 1978; Kawaoka et al., 1989). This finding suggested that an intermediate host may be needed for genetic reassortment of human and avian viruses. Pigs are considered a logical candidate for this role because they can be infected by both, avian and human viruses (Kundin, 1970; Schultz et al., 1991; Kida et al., 1994) and because they possess both, NeuAc 2,3Gal and NeuAc 2,6Gal receptors (Ito et al., 1996). In addition, there is good evidence that pigs are more frequently involved in interspecies transmission of influenza A viruses than are other animals (Scholtissek et al., 1983; Mancini et al., 1985; Rota et al., 1989).

Two subtypes of influenza A viruses have been isolated from pigs: H1N1, represented by the classical H1N1 and avian-like H1N1 viruses; H3N2, represented by human-like H3N2 viruses (Webster et al., 1992). The term avian- or human-like means that these viruses were originally detected in birds or humans and later transmitted to pigs. The classical H1N1 swine viruses were derived from A/Swine/Iowa/15/30 and have circulated continuously among pigs in the United States (Hinshaw et al., 1984a). Avian-like H1N1 viruses were first introduced into European pigs in 1979 and have been maintained exclusively in this swine population since then (Campitelli et al., 1997). Human-like H3N2 viruses have been isolated repeatedly from pigs in Europe and sporadically from those in eastern Asia (Katsuda et al., 1995; Nerome et al., 1995).

The first cases of influenza in pigs were seen in the United States during the catastrophic human influenza pandemic of 1918–1919 and the virus was first isolated by Shope (1931). Classical H1N1 swine influenza viruses are genetically and antigenically similar to the type A influenza viruses implicated in the human pandemic (Taubenberger et al., 1997; Reid et al., 1999), and have become one of the most common causes of respiratory disease in North American pigs. Serologic studies of pigs in the United States in the late 1980s indicated a high (51%) prevalence of classical H1 influenza viruses, contrasted with a very low (1.1%) prevalence of H3 viruses antigenically similar to human H3 strains (Chambers et al., 1991). The most recent isolation of H3N2 swine viruses in North America was in Quebec, Canada, in 1991 (Bikour et al., 1995). The virus isolated is antigenically conserved and is similar to 1975 human strains.

In late August of 1998, a severe outbreak of influenza-like disease occurred in the swine population of North Carolina, causing abortions and deaths among the breeding females. Subsequent outbreaks, in November and December of 1998, affected pig populations in Texas, Minnesota and Iowa. Although less severe than the previous

outbreak, these episodes encompassed wider geographic regions. Influenza A viruses of H3N2 subtype were isolated from pigs in all four states, providing an explanation for the lack of efficacy of the H1N1 vaccine previously given to some of the herds.

In this study, we examined the antigenic characteristics of hemagglutinin (HA) molecule of four swine H3N2 viruses isolated during the outbreaks. The origin of the viruses was determined by partially sequencing all eight gene segments and comparing the results with published data to establish genetic relatedness. We demonstrate that one of the viruses is a human–swine genetic reassortant, while the remaining isolates had genes from avian as well as human and swine viruses.

## 2. Material and methods

### 2.1. Viruses

Four swine viruses isolated between September and December of 1998 during swine influenza outbreaks in different states were used in this study (see Table 1 for swine virus abbreviations). The viruses were isolated from nasal swabs and tissues of sick or dead pigs and propagated in chicken embryos.

### 2.2. Antigenic analysis

Antigenic analysis was performed according to instructions provided with a WHO influenza reagent kit.

### 2.3. Experimental infection of pigs

Two one-month-old white Hanford mini-pigs were infected intranasally and orally with 1 ml of A/swine/North Carolina/34948/99 (H3N2) influenza virus. One additional pig was kept in contact throughout the experiment. Temperatures were recorded daily for three days pre-infection and 10 days post-infection. Nasal swabs were collected daily and titrated for infectious virus.

Table 1  
Antigenic analysis of SW98 viruses

Isolate	Abbreviation	HI titers				
		H1N1 A/Beijing/ 262/95	H3N2 A/Nanchang/ 933/95	H3N2 A/Sydney/ 5/97	B B/Beijing/ 184/93	B B/Beijing/ 243/97
A/Swine/NC/35922/98	SWNC98	<10	320	320	20	<10
A/Swine/TX/4199-2/98	SWTX98	<10	1280	1280	40	20
A/Swine/MN/9088-2/98	SWMN98	<10	640	320	40	40
A/Swine/IA/8548-1/98	SWIA98	<10	1280	640	40	20

## 2.4. Sequencing and analysis

Viral RNA was extracted from infected allantoic fluid with the RNeasy Extraction Kit (Qiagen, Santa Clara, CA) and amplified by reverse transcription-PCR (RT-PCR) (Shu et al., 1993). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), sequenced with synthetic oligonucleotides using rhodamine Dye-Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq<sup>®</sup> DNA polymerase FS (Perkin-Elmer, Applied Biosystems [PE/ABI], Foster City, CA) and electrophoresed on PE/ABI 377 DNA sequencers by the Center for Biotechnology at St. Jude Children's Research Hospital.

The sequence data were analyzed with the Wisconsin Package Version 9.1-Unix (Genetics Computer Group, Madison, WI) and GeneDoc Version 2.3.000 software (Nicholas and Nicholas, 1997). Phylogenetic analysis was done by the maximum parsimony method in PAUPSEARCH and PAUPDISPLAY programs of Wisconsin Package Version 9.1-Unix.

## 3. Results

### 3.1. Antigenic analysis of swine influenza isolates

The hemagglutination inhibition assay, performed with the WHO influenza reagents, was used to analyze the swine viruses isolated in 1998 (abbreviated as SW98 viruses). All isolates are antigenically similar to recent H3N2 human viruses, and their reactivity profiles showed no appreciable differences (Table 1).

### 3.2. Genotyping of the swine H3N2 influenza viruses

In order to address the question of the genetic composition and origin of the causative viruses in the four influenza outbreaks, we partially sequenced each of the eight viral gene segments. Nucleotide sequences ranging from 595 to 1557 bases in each gene were compared with sequences available in GenBank to identify any close homologies (Table 2). The four SW98 viruses could be separated on the basis of closest genetic relatedness. For the earlier isolate, SWNC98, the two genes encoding the HA and NA surface glycoproteins and one internal gene encoding PB1, are most closely related to the corresponding genes from recent human H3N2 influenza viruses (98–99% homology). The other five internal genes of SWNC98 have the highest homology with genes of the classical swine lineage (93–98%). For the remaining three viruses, the HA, NA and PB1 genes belong to the same lineage as those from SWNC98 (98–99% homology), while the NP, M and NS genes have a classical swine lineage origin (98% homology). Interestingly, two internal genes from these isolates, PB2 and PA, are closely related to the genes of avian influenza A viruses (93–97% homology). Since only a few PB2 and PA gene sequences from recently isolated avian viruses were available for comparison using GenBank data, the actual homologies between swine and avian PB2 and PA genes may be closer than the estimates reported here.

Table 2  
Genetic homology of the swine viruses isolated in the United States

Gene segment	Region compared	SWNC98			SWTX98, SWMN98 and SWIA98			Average (%) homology overall <sup>a</sup>
		Lineage	Virus with the greatest homology	Homology (%)	Lineage	Virus with the greatest homology	Average (%) homology	
PB2	44–1600	Swine	A/Swine/TN/24/77 (H1N1)	93	Avian	A/Seal/MA/133/82 (H4N5)	93	82
PB1	1088–2236	Human	A/Miyagi/29/95 (H3N2)	98	Human	A/Miyagi/29/95 (H3N2)	99	98
PA	25–620	Swine	A/WI/4755/94 (H1N1)	94	Avian	A/Swine/HK/81/78 (H3N2)	97	81
HA	78–1061	Human	A/Finland/363/95 (H3N2)	98	Human	A/Finland/339/95 (H3N2)	98	96
NP	34–1024	Swine	A/MD/12/91 (H1N1)	98	Swine	A/Swine/IA/17672/88 (H1N1)	98	96
NA	26–1411	Human	A/Shiga/20/95 (H3N2)	99	Human	A/Shiga/25/97 (H3N2)	99	97
M	52–964	Swine	A/WI/4755/94 (H1N1)	95	Swine	A/WI/4755/94 (H1N1)	98	93
NS	22–866	Swine	A/Swine/IA/17672/88 (H1N1)	97	Swine	A/Swine/IA/17672/88 (H1N1)	98	98

<sup>a</sup> SWNC98 vs. SWTX98, SWMN98 and SWIA98.

Thus, the swine H3N2 viruses isolated during the 1998 outbreaks are genetic reassortants incorporating gene segments of different origins. SWNC98 was generated by reassortment between a recent human virus and a classical swine virus, whereas each of the remaining three isolates arose from reassortment events among recent human, classical swine and avian influenza A viruses.

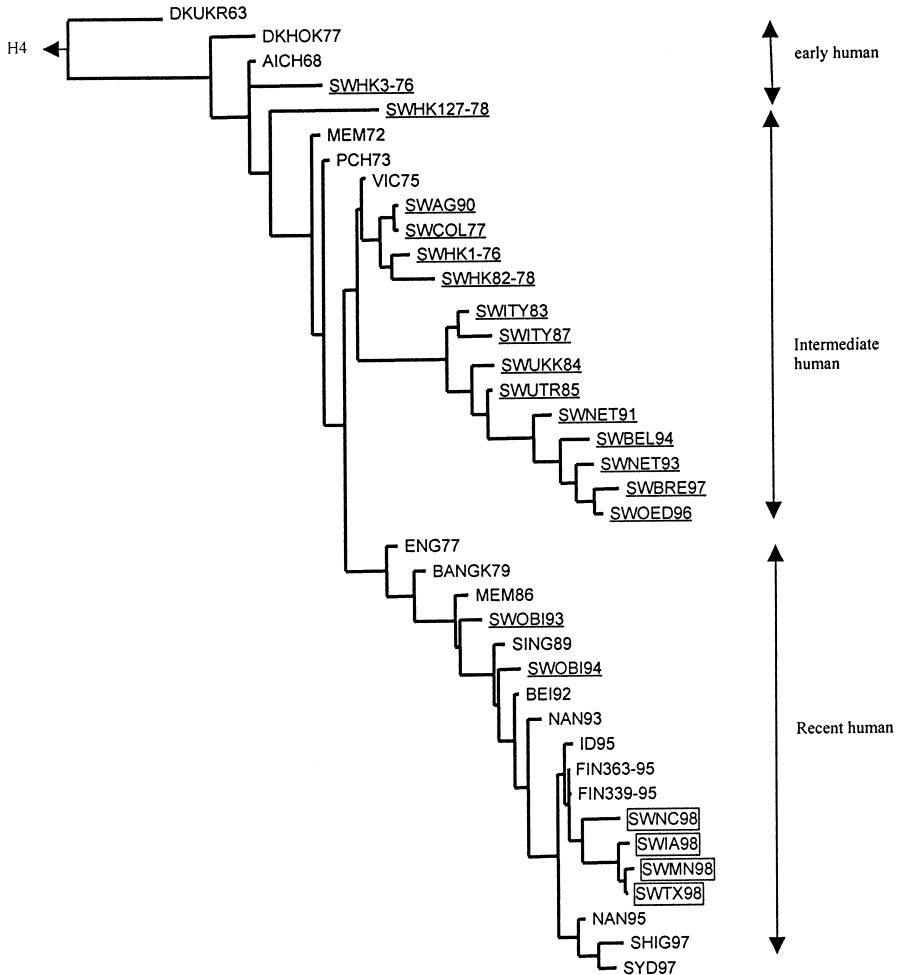


Fig. 1. Phylogenetic tree of HA1 nucleotide sequences of the H3 hemagglutinin. The tree was generated with the maximum parsimony method in PAUPSEARCH and PAUPDISPLAY programs of the Wisconsin Package Version 9.1-Unix (Genetics Computer Group, Madison, WI), and is rooted to the H4 sequence of A/Duck/Czechoslovakia/56 (H4N6). Horizontal lines are proportional to the numbers of nucleotide substitutions between branch points. Swine viruses are underlined, and the SW98 viruses are enclosed. Abbreviations can be found in the publication by Zhou et al., 1999.

### 3.3. Phylogenetic analysis of the swine H3N2 viruses

In order to identify more precisely the host or hosts in which the SW98 viruses originated, we constructed phylogenetic trees, using the HA1 region of the HA gene and partial sequences of the PB2 and NP genes.

In order to establish whether or not the swine H3N2 viruses isolated in the United States are related to the H3 swine virus isolated previously, we used nucleotide sequences of representative swine viruses isolated at different times and in different regions of the world to construct HA1 tree. As shown in Fig. 1, the swine H3 HA genes can be segregated into three lineages, including early human strains, intermediate human strains and recent human strains. This grouping differs somewhat from the previous report by Nerome et al., 1995. The swine viruses of the early human lineage seem to be derived from the human strain A/Aichi/2/68. The intermediate human swine viruses, which have been isolated continuously in Europe since 1983 (Campitelli et al., 1997), were closely related to A/Victoria/3/75-like viruses, and appeared to have established a special lineage.

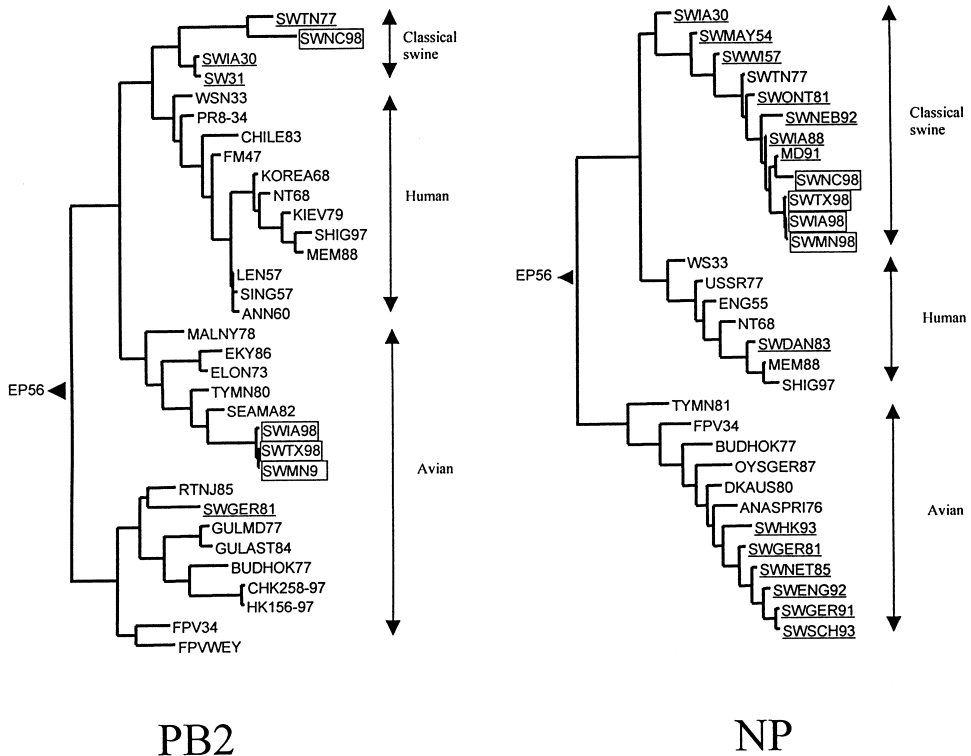


Fig. 2. Phylogenetic trees for partial PB2 and NP sequences of influenza A viruses. Nucleotide residues 44–1600 of all PB2 genes and residues 34–1024 of all NP genes were analyzed. The trees were generated by the same method as described in Fig. 1. Abbreviations can be found in the publication by Zhou et al. (1999).

The recent human lineage includes two swine viruses isolated in 1993 and 1994 in Japan, A/Swine/Obihiro/1/93 and A/Swine/Obihiro/1/94 (Katsuda et al., 1995), as well as the four SW98 viruses isolated in the United States. It is apparent from the phylogenetic tree that the HAs of the SW98 isolates were derived from neither the recent Japanese swine viruses nor the A/Swine/Ange-Gardien/150/90 (SWAG90) virus isolated in Canada (Bikour et al., 1995). Rather, they are all descendants of human viruses circulating in 1995.

Phylogenetic analysis of the PB2 and NP genes showed a clear division of the SW98 viruses into different lineages (Fig. 2). In the PB2 tree, swine viruses are separated into a classical swine lineage and an avian lineage. SWNC98 belongs to the classical swine lineage, while the remaining three SW98 viruses cluster together on the North American avian branch. In the NP tree, swine viruses are divided into three lineages, including classical swine, human-like and avian-like lineages. All of the NP genes from the SW98 viruses belong to the classical swine lineage. SWNC98 is more related to A/MD/12/91 than to the remaining SW98 isolates.

#### 3.4. Experimental infection of mini-pigs with SWNC98

The SWNC98 virus replicated to high titers in both of the infected pigs and transmitted to the contact pig (Fig. 3). The animals showed no significant disease signs, but they all showed sneezing after collection of nasal swabs and mild diarrhea for two days after infection. The animals remained active and consumed food at the same rate after infection as before.

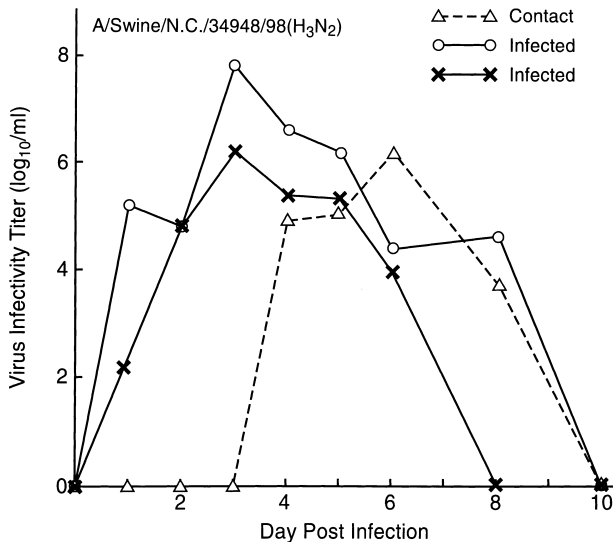


Fig. 3. Replication of A/swine/North Carolina/34948/98 (H3N2) in mini-pigs after experimental infection.

#### 4. Discussion

Pigs appear to have a relatively weak species barrier against infection by avian as well as human influenza A viruses (Scholtissek et al., 1993; Kida et al., 1994). Avian influenza viruses were introduced into pigs in Asia and Europe (Scholtissek et al., 1983; Hinshaw et al., 1984a; Kida et al., 1988; Guan et al., 1996), and genetic reassortment between human-like and avian-like or swine viruses occurred in pigs (Castrucci et al., 1993). Our genetic analysis of the influenza viruses isolated from pigs in 1998 provides compelling evidence for interspecies transmission of human and avian viruses to pigs, and for genetic reassortment among the human, swine and avian influenza A viruses. To our knowledge, this is the first reported case in which the genome of an influenza virus consisted of gene segments from three different viral lineages.

Among the nucleotide sequences most homologous with those of the SW98 viruses, several came from an influenza A virus which had undergone interspecies transmission. A/Seal/MA/133/82 was introduced from aquatic birds to sea mammals (Hinshaw et al., 1984b), the human A/WI/4755/94 strain, was derived from a classical swine virus that infected pigs experimentally (Wentworth et al., 1997), and A/Swine/ Hong Kong/81/78 was transmitted from birds to pigs (Kida et al., 1988). This raises the possibility that certain viral gene segments have a greater propensity to be transmitted from species to species, and therefore could play a pivotal role in genetic reassortment events.

An avian H1N1 influenza virus was transmitted to European pigs in the early 1980s (Scholtissek et al., 1983) and replaced the classical H1N1 strain that had been circulating in these animals since 1976. In 1983–1985, genetic reassortment took place between the avian-like and human-like H3N2 swine virus that had been transmitted to European pigs in the mid-1970s (Castrucci et al., 1993). The reassortant strain, which possessed ‘surface’ genes of human origin and ‘internal’ genes of avian origin, later replaced the human-like H3N2 virus (Campitelli et al., 1997). The H3N2 reassortant with avian internal genes has apparently superseded the antigenically related human-like strain. Successful adaptation of the reassortants with avian-derived internal genes suggests that these viruses might have a growth advantage in pigs compared with classical swine- and human-derived viruses. Our finding also suggests that combination of gene segments from human, avian and classical swine influenza viruses can create a favorable genetic background for transmission and replication of the hybrid virus in pigs. Indeed, the ‘triple’ reassortant viruses described here caused influenza outbreaks in at least three states, in contrast to the human-swine isolate (SWNC98), which appears to have infected pigs in North Carolina only. However, the milder outbreaks in pigs infected by the triple reassortants may indicate that the introduction of two avian polymerase genes into human-swine viruses attenuated their pathogenicity. This possibility is supported by studies in which artificial introduction of avian internal genes into a human virus attenuated the virulence of the latter (Clements et al., 1992). PB2 is an important target gene for the production of attenuated temperature-sensitive influenza viruses through genetic manipulation (Subbarao et al., 1995). Thus, it is not surprising that the gain of two avian polymerase genes by the SWTX98, SWMN98 and SWIA98 viruses resulted in a virulence difference between this group of reassortants and SWNC88.

Isolation of H3N2 influenza A viruses from pigs was first recorded in Taiwan during a human epidemic in 1969 (Kundin, 1970). Subsequent isolation of a number of H3N2

strains, together with indirect evidence from serologic surveillance, indicated that variants of the original H3N2 influenza virus had spread to the swine population in other parts of the world (McFerran et al., 1972). Additional reports indicated multiple introductions of H3N2 influenza viruses into pigs in southern China (Nerome et al., 1995), as well as multiple isolations of H3N2 viruses in European pigs (Castrucci et al., 1994). Most of the H3 HAs from swine viruses resembled the A/Victoria/3/75 virus; only one report before ours, from Obihiro, Japan, described the isolation of swine viruses derived from recent human H3N2 viruses (Katsuda et al., 1995). Infection of pigs by H3N2 viruses is rare in the United States, by comparison with the prevalence of H1N1 viruses (Chambers et al., 1991). Prior to isolation of the SW98 strains, only one H3N2 swine virus, A/swine/Colorado/1/77, had emerged in American pig populations. We speculate that the higher incidence of H3N2 virus isolation in pigs in eastern Asia and Europe is due to denser human and animal populations (e.g. in southern China), resulting in more frequent contacts between humans and pigs, and the establishment of H3N2 viruses in European pigs (Fig. 1). Another possibility is that the properties of the A/Finland/339/95-like HA gene favored its transmission to pigs.

The clear distinctions between SWNC98 and other SW98 strains raise the question of how these viruses arose. Did the latter evolve from SWNC98 or were they generated by independent reassortment events? Although the viruses share the same lineage with regard to their PB1, HA, NP, NA, M and NS genes, they differ in homologies with GenBank sequences for the PB2, PA, HA, NP and NA genes. The differences in nucleotide sequences of all gene segments (except NS) between the SWNC98 and the remaining strains is greater than those between each group and its most homologous sequences in GenBank. The average M gene nucleotide identity between the two groups is as low as 93%, while the difference in HA1 domains is as high as  $\approx 6\%$ . These discrepancies were not likely due to antigenic drift since the viruses were subjected to relatively low immunologic pressure in pigs (Bikour et al., 1995). We conclude that SWNC98 and subsequent SW98 strains were generated by independent genetic reassortment events.

In conclusion, two different H3N2 reassortant influenza viruses appeared in the swine population of the United States in 1998. The North Carolina isolate is a reassortant between a 1995 human H3N2 influenza virus and classical swine influenza virus; the other H3N2 swine viruses arose by reassortant between human, swine and avian influenza viruses. Emergence of the H3N2 reassortant influenza viruses in the pigs of the United States raises concerns about whether the viruses will become established in the pigs in North America and whether they will become an economic burden. It is worth considering the inclusion of H3N2 inactivated virus in swine vaccines. This event also emphasizes the need for surveillance of pig populations in North America for evidence of continuous circulation of the extent of spread of these viruses and whether they establish permanent lineages with the possibility to spread to humans.

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