Serologic and genetic characterization of North American H3N2 swine influenza A viruses

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Abstract

The H3N2 subtype of influenza A viruses isolated from pigs in the United States and Canada has shown both genetic and antigenic diversity. The objective of this study was to determine the serologic and genetic characteristics of contemporary strains of these viruses. Genetic analysis of 18 reference strains and 8 selected strains demonstrated differences in 1% to 9% of the nucleotides of the hemagglutinin (HA) gene. Phylogenetic analysis of the HA gene revealed 3 genetic clusters, as well as divergence of cluster III viruses from a cluster III prototype virus (A/Swine/Illinois/21587/99). By means of 1-way cross-hemagglutination inhibition with antiserum against 5 field isolates and 3 vaccine viruses, most of 97 isolates tested could be placed in 1 of 3 serogroups. The several isolates that did not react with any antiserum were in genetic cluster III, which suggests that continuous antigenic drift in cluster III may have resulted in virus variants. The efficacy of commercial vaccines against these virus variants should be evaluated with vaccination and challenge studies.

Résumé

Le sous-type H3N2 du virus de l'influenza A isolé de porcs aux États-Unis et au Canada montre une diversité génétique et antigénique. Les objectifs de la présente étude étaient de déterminer les caractéristiques sérologiques et génétiques de souches contemporaines de ces virus. Une analyse génétique de 18 souches de référence et de 8 souches sélectionnées a démontré des différences variant de 1 % à 9 % des nucléotides du gène de l'hémagglutinine (HA). Une analyse phylogénétique du gène HA a révélé 3 regroupements génétiques, de même qu'une divergence de virus du regroupement III du prototype du regroupement III (A/Swine/Illinois/21587/99). À l'aide d'une épreuve d'inhibition de l'hémagglutination croisée unidirectionnelle, utilisant des antisérums dirigés contre 5 isolats de champs et 3 souches vaccinales, la plupart des 97 isolats testés ont pu être classés dans 1 des 3 sérogroupes. Les isolats qui n'ont pas réagi avec aucun antisérum se retrouvaient dans le regroupement III, ce qui suggère qu'une dérive antigénique continue dans le regroupement III pourrait avoir entraîné l'apparition de variants. L'efficacité des vaccins commerciaux envers ces virus variants devrait être évaluée par des études de vaccination et d'infections défis.

(Traduit par Docteur Serge Messier)

Introduction

Swine influenzavirus (SIV) causes respiratory disease in pigs that is characterized by high fever, lethargy, nasal discharge, coughing, dyspnea, and weight loss; morbidity rates are high and mortality rates low. The viruses are type A influenza viruses, a category encompassing influenza viruses of birds and other mammals, including humans. In swine, 3 influenza A virus subtypes (H1N1, H3N2, and H1N2) are circulating throughout the world (1–5). In the United States, the classic H1N1 subtype was exclusively prevalent among swine populations before 1998; however, since late August 1998, H3N2 subtypes have been isolated from pigs (6,7). Most H3N2 virus isolates are triple reassortants, containing genes from human (HA, NA, and PB1), swine (NS, NP, and M), and avian (PB2 and PA) lineages (7,8). Soon after the H3N2 subtype emerged, isolations of reassortant H1N2 subtype were also reported (2,9,10).

Gene sequence analysis of the triple-reassortant H3N2 viruses has shown that their hemagglutinin (HA) genes belong to 1 of 3 phylogenetically distinct human-like HA lineages. Therefore, H3N2 viruses are classified into 3 distinct clusters (1,7). Cluster I includes A/Swine/Texas/4199-2/98 triple-reassortant H3N2 and A/Swine/North Carolina/35922/98 double-reassortant H3N2, viruses that appear to be the most closely related to the H3N2 viruses isolated from humans in 1995. Cluster II includes A/Swine/Colorado/23619/99, which is closely related to the human virus A/Sydney/97, which was predominant in the 1997–98 influenza season. Cluster III includes A/Swine/Oklahoma/18089/99 and A/Swine/Illinois/21587/99, viruses that are most similar to the predominant human strains of 1996: A/Wuhan/95-like H3N2 viruses (1). The 3 clusters of viruses have some genetic and antigenic differences that further support their different ancestry (7).

Some antigenic relatedness exists between the H3N2 viruses A/Sw/TX/4199-2/98 in cluster I and A/Sw/OK/18089/99 in cluster III (7). However, it is not known if contemporary H3N2 viruses in different clusters are antigenically distinct. We hypothesize that recent field isolates of H3N2 virus have high diversity in their genetic and

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serologic characteristics. The purpose of this study was to determine if field isolates of SIV H3N2 can be classified into genetic or serologic groups by phylogenetic analysis of the HA gene nucleotide sequence and cross-reactivity in HI tests.

Materials and methods

Isolates of H3N2 SIV

Ninety-seven H3N2 SIV isolates were randomly selected from virus collections at the Minnesota Veterinary Diagnostic Laboratory (MVDL). The viruses had been isolated from swine samples submitted for diagnosis of respiratory disease between 2000 and 2004. All viruses were grown on Madin–Darby canine kidney (MDCK) cells (11). Briefly, the MDCK cell monolayers were washed with phosphate-buffered saline (PBS) containing trypsin treated with tosylphenylalanylchloromethane (TPCK), inoculated with sample supernatant, and incubated at 37°C for 2 h. The samples were then decanted, and Eagle's minimum essential medium containing 0.05% TPCK-treated trypsin and 0.3% bovine serum albumin was added. The inoculated cells were incubated at 37°C and examined for cytopathic effect (CPE) daily for 5 d. The supernatants with CPE were tested for hemagglutination with turkey erythrocytes to confirm the presence of SIV. Samples without CPE were passed again on MDCK cell monolayers and observed for another 5 d. The samples with no CPE after the 2nd passage were considered negative for virus.

Gene sequencing of viral RNA

Viral RNA was extracted by means of a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, California, USA). Then 2 multiplex reverse-transcription polymerase chain reaction (RT-PCR) assays were carried out to detect H1 and H3 genes as previously described (12). Fragments of the expected 663 base pairs for the H3 gene were amplified from each extraction of RNA. The amplified RT-PCR products were purified with the use of a QIAquick PCR purification kit (Qiagen) and cloned into a pGEM T vector for nucleotide sequencing, which was performed at the Advanced Genetic Analysis Center of the University of Minnesota with a DNA sequencer (model 377; Applied Biosystems, Perkin-Elmer, Foster City, California, USA) and a Taq Dye Deoxy terminator cycle sequencing kit (Applied BioSystems).

Sequence and phylogenetic analysis

The nucleotide and amino acid sequences of 18 previously identified swine and human influenzavirus strains were obtained from the GenBank database of the National Center for Biotechnology Information; they were AY035589, AF268128, AF268124, AF251411, AF251419, AF251427, AF268123, AB019356, AF251403, AF153232, AF153233, AF363502, AF251395, AJ311466, AF017270, AF017272, U26830, and AJ289703. The nucleotide sequences and alignments of the 18 reference strains and of 8 field strains of H3N2 SIV isolated between 2000 and 2004 were constructed and edited with use of the Lasergene sequence analysis software package (DNASTAR, Madison, Wisconsin, USA). The HA gene nucleotide sequences were aligned and compared phylogenetically.

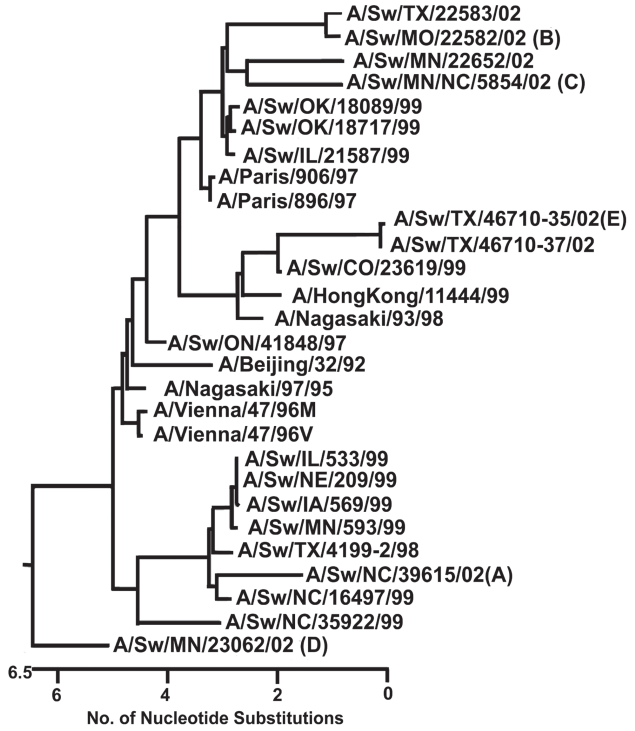

Figure 1. Phylogenetic tree of the hemagglutinin (HA) genes of selected H3N2 influenza A viruses, constructed in Megalign (DNAStar, Madison, Wisconsin, USA). The length of each pair of branches represents the distance between sequence pairs. The units 0 to 6.5 at the bottom of the tree indicate the number of nucleotide substitutions. The 5 isolates used to prepare antiserum for the study were classified as follows: A and D, cluster I (A/Swine/Texas/4199-2/98-like); B and C, cluster III (A/Swine/Illinois/21587/99-like); and E, cluster II (A/Swine/Colorado/23619/99-like). TX — Texas; MO — Missouri; MN — Minnesota; NC — North Carolina; OK — Oklahoma; IL — Illinois; CO — Colorado; ON — Ontario; NE — Nebraska; IA — Iowa.

Antiserum preparation and use in cross-reactivity testing

To examine serologic cross-reactivity, antiserum was prepared in pigs against 5 H3N2 viruses, A/Swine/North Carolina/39615/02 (A), A/Swine/Missouri/22582/02 (B), A/Swine/North Carolina/5854/02 (C), A/Swine/Minnesota/23062/02 (D), and A/Swine/Texas/46710-35/02 (E), selected on the basis of their location in different phylogenetic clusters (Figure 1). All 5 viruses were grown in MDCK cells and had initial HA titers of 1:128 to 1:256 per 0.1 mL. The viruses were inactivated by the addition of formalin (final concentration 0.1%) and then added to an adjuvant mixture of mineral oil (9 parts) and emulsifier (1 part; equal volumes of Span 85 and Tween 85) in a 1:1 ratio. Antiserum against 3 bivalent inactivated SIV commercial vaccines ("X", "Y", and "Z") was produced similarly in pigs.

The pigs were obtained from herds free from infection with *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Mycoplasma hyopneumoniae*, and SIV. They were cared for and handled according to a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee. The pigs were inoculated intramuscularly with inactivated virus at 4 wk of age and again 3 wk later. Blood was collected from each pig 2 to 3 wk after the last injection, and aliquots of serum were stored at −20°C until used. All

Table I. Frequency distribution of 97 H3N2 swine influenzavirus (SIV) isolates according to antibody titer in 1-way cross-hemagglutination inhibition (HI) tests with antiserum produced against 5 field strains of H3N2 SIV and 3 SIV vaccine viruses

	No. of isolates reacting to antiserum							
	Field strain[a]					Vaccine		
Titer	A	B	C	D	E	X	Y	Z
< 1:40	62	5	10	29	6	5	4	5
1:40	24	40	24	19	30	13	14	21
1:80	9	27	19	7	39	37	19	38
1:160	1	15	15	9	10	34	23	17
1:320	2	3	9	11	5	7	22	13
1:640	0	2	0	7	5	2	9	4
1:1280	0	3	9	9	2	0	7	0
≥ 1:2560	0	3	2	7	1	0	0	0

[a] A — A/Swine/North Carolina/39615/02; B — A/Swine/Missouri/22582/02; C — A/Sw/NC/5854/02; D — A/Swine/Minnesota/23062/02; E — A/Sw/TX/46710-35/02.

serum samples were heat-inactivated and treated with 25% kaolin and turkey erythrocytes as previously described (13) and then were tested for SIV antibody titers by 1-way cross-hemagglutination inhibition (HI), as previously described (14); titers of < 1:40 were considered negative. Because there was cross-reactivity between types of antiserum with some isolates, serologic group classification was based on a 4-fold or greater difference in titer.

Results

Phylogenetic and serologic characterization

The divergence in HA gene nucleotide sequences between the 97 H3N2 SIV isolates from swine obtained between 2000 and 2004 and the previously described human and swine H3N2 reference viruses in GenBank was 1% to 9%. Five phylogenetic clusters were evident, and within each cluster the divergence in HA gene sequences was 1% to 6% (data not shown). Figure 1 shows the phylogenetic analysis of the 5 SIV H3N2 viruses (A to E) selected for further characterization on the basis of their location in the phylogenetic tree in comparison with the previously described human and swine H3N2 viruses: A and D belonged to cluster I, E to cluster II, and B and C to cluster III. The similarity of HA gene sequences between strains A and E ranged from 89.8% to 91.7%.

Tested with the antiserum prepared against the 5 selected H3N2 viruses and the 3 commercial vaccine viruses (X, Y, and Z), the 97 H3N2 isolates showed wide variation in SIV HI antibody titer: A, negative to 1:320; B, 1:20 to ≥ 1:2560; C, negative to ≥ 1:2560; D, negative to ≥ 1:2560; E, 1:20 to 1:2560; X, negative to 1:640; Y, 1:20 to 1:1280; and Z, 1:20 to 1:640 (Table I). In cross-HI tests, the highest titers were obtained for homologous reactions; that is, reactions in which the virus was tested against antiserum created with the same virus. Homologous HI titers were 8 times higher than heterologous HI titers. With use of the serologic reactions obtained with this antiserum panel, viruses A to E were classified into 3 serologic groups (vaccine, B/E, and C/D), a 4-fold difference in titer defining the groups (Table II). Of the 97 isolates, 41 (42%) had the highest HI titers with X, Y, or Z antiserum (vaccine group), 14 (14%) with B or E antiserum (B/E group), and 33 (34%) with C or D antiserum (C/D group). Nine (9%) of the 97 isolates had low HI titers (1:80 or less) to all of the antiserum panel (Table III) and were designated the nonreactive group.

In HI testing of a reference H1N1 virus (A/Swine/Iowa/301IDV0103/1973), there was no reaction with the antiserum prepared against the 5 H3N2 viruses, but there were reactions to the antiserum prepared against the 3 commercial vaccines (Table III). This demonstrates that the 5 viruses used to generate H3N2 antiserum were H3N2 viruses and confirms that the 3 vaccines were bivalent, containing both H1N1 and H3N2 strains. In HI testing of a reference H3N2 virus (A/Sw/TX/4199-2/98), results were positive for the entire H3N2 antiserum panel, but the highest titers were with X, Y, and Z antiserum (Table III).

Characteristics of the HA gene of nonreactive isolates

For 6 of the 9 nonreactive strains, HA gene sequences were obtained. After initial sequence analysis and comparison with influenzaviruses in GenBank, only 1 isolate (A/Swine/North Carolina/50270/01) showed high HA gene similarity (98%) to the cluster III H3N2 viruses A/Sw/OK/18089/99 and A/Sw/IL/21587/99 (data not shown). However, 5 of the 9 isolates had approximately 95% to 98% HA nucleotide sequence homology with A/Turkey/Minnesota/764-2/03 H3N2 and A/Turkey/North Carolina/12344/03 H3N2 (Figure 2). A broader comparison revealed that these 5 nonreactive viruses grouped with cluster III-like H3N2 SIV from the United States and Canada. The broader phylogenetic analyses were completed with the use of database management tools in the Influenza Sequence Database (15) and compared more than 250 HA gene sequences from H3N2 SIV isolates submitted to the MVDL between 2003 and 2005. Figure 3 is a phylogenetic tree comparing the HA gene sequences of these 5 nonreactive viruses and 34 of the MVDL H3N2 viruses.

Discussion

Since the original identification of the H3N2 subtype of SIV in North American swine in 1998, SIV has become an increasingly important cause of respiratory disease in swine. In the present study, genetic and serologic characteristics of contemporary H3N2 SIV field isolates were examined to demonstrate their diversity. As shown in Figure 1, these isolates could be classified into genetic clusters I, II, and III, supporting previous reports (1,6).

Serologic diversity was then examined by 1-way cross-HI tests of 97 H3N2 isolates against an antiserum panel derived from 8 selected H3N2 viruses. At least 3 serogroups (vaccine, B/E, and C/D) were identified. The titers were highest with antiserum to the vaccines for 42% of the 97 isolates; 9% of the isolates did not react to the antiserum panel or had a titer of 1:80 or less. There are several limitations with using HI cross-reactivity panels as a means to distinguish the serologic characteristics of influenzaviruses. First, repeatability problems between experiments, technicians, and laboratories sometimes

Table II. Serologic grouping of the 5 field strains of H3N2 SIV, according to HI antibody titers in pigs vaccinated against viruses from different genetic clusters

		Titer[a] in testing with antiserum								
		Field isolate					Vaccine			
Strain	Cluster	A	B	C	D	E	X	Y	Z	Serogroup
A	I	*320*	80	160	—	80	160	**1280**	**640**	Vaccine
B	III	—	***1280***	—	40	**640**	80	40	40	B/E
C	III	80	160	***2560***	**1280**	80	160	320	160	C/D
D	I	—	40	**1280**	***2560***	40	160	160	80	C/D
E	II	—	**2560**	40	40	***2560***	80	80	80	B/E

[a] Italicization indicates a homologous reaction; bolding indicates the 2 highest titers for each H3N2 virus; — indicates a negative result.

Table III. Titers of the 9 nonreactive H3N2 SIV isolates and 2 reference strains (H1N1 and H3N2)

	Titer[a] in testing with antiserum							
	Field isolate					Vaccine		
Isolate	A	B	C	D	E	X	Y	Z
A/Sw/NC/50270/01	—	40	—	—	—	40	80	80
A/Sw/NC/47041/02	—	40	—	—	80	40	—	—
A/Sw/IN/8153/03	—	80	40	—	—	—	40	—
A/Sw/NC/43532/03	—	40	80	40	40	80	80	80
A/Sw/NC/9245/03	—	40	—	—	—	80	40	—
A/Sw/MN/28419/03	—	—	40	—	80	80	80	40
A/Sw/MN/33106/03	—	—	—	—	40	40	40	40
A/Sw/MN/34409/03	—	—	—	—	40	40	80	40
A/Sw/NC/10299/04	—	40	—	—	80	80	40	—
A/Sw/IA/301IDV0103/1973 (H1N1)	—	—	—	—	—	80	320	40
A/Sw/TX/4199-2/98 (H3N2)	20	40	320	160	40	640	640	640

IN — Indiana; IA — Iowa; TX — Texas; — indicates a negative result or a titer $<$ 1:40.

make comparisons of results difficult (16). Additionally, the antiserum used in the HI assays may not be able to distinguish antigenic differences between the viruses (16). Results may also be disparate; for example, a heterologous reaction may have a higher titer than a homologous reaction (16). Finally, there may be a lack of symmetry between reactions; for example, in the present study, antiserum B versus antigen B had a titer of 1:1280, and antiserum B versus antigen C had a titer of 1:160, but antiserum C versus antigen B had no reaction (titer $<$ 1:40). For these reasons, HI should be coupled with sequencing of the HA gene, as in the present study, to correlate antigenic and genetic differences (17).

Further studies correlating antigenic and genetic variation should include inoculation and challenge studies to compare in vivo and in vitro HI results (18) or mathematical and spatial analyses (19). Only then will we be able to understand the relationship between antigenic and genetic variation in the HA gene. Understanding this relationship is important because vulnerability to infection is, for the most part, determined by immunity against HA (20). Studies of human influenzavirus strains have shown that the HA gene evolves rapidly and that mutations, through a process known as antigenic drift, occur throughout the hypervariable region of the gene, gradually changing the antigenic properties (21). In general, the level of cross-reaction decreases as the differences between strains in the HA gene increase (22). However, large genetic differences do not always result in a lack of antigenic cross-reaction. In a European swine study, vaccination with an H1N1 virus from 1976 provided exceptional protection in pigs against challenge with an H1N1 virus from 1998, even though there were 28 amino acid differences between the 2 viruses (18). This suggests that not all amino acid substitutions or genetic mutations are antigenically significant. Exhaustive studies are needed to elucidate the significance of antigenic and genetic changes.

In this study, 5 of the 9 nonreactive viruses were initially shown to be 95% to 98% identical in HA gene nucleotide sequence to 2 H3N2 isolates from turkeys. The 2 turkey isolates, A/Turkey/MN/764-2/03 and A/Turkey/NC/12344/03, were first reported as swine-like H3N2 influenzaviruses that were transmitted from pigs to turkeys in 2 geographically distinct farms (23). On further analysis, only 3 of these 5 isolates were found to be most similar to swine-like turkey H3N2, and the remaining 2 isolates were most similar to other contemporary H3N2 SIV from the United States and Canada. All 9 nonreactive viruses were classified into genetic cluster III, which includes endemic H3N2 SIV that initially emerged in 1998 and are most similar to prototype viruses A/Sw/IL/21587/99 and A/Sw/OK/18089/99 (7).

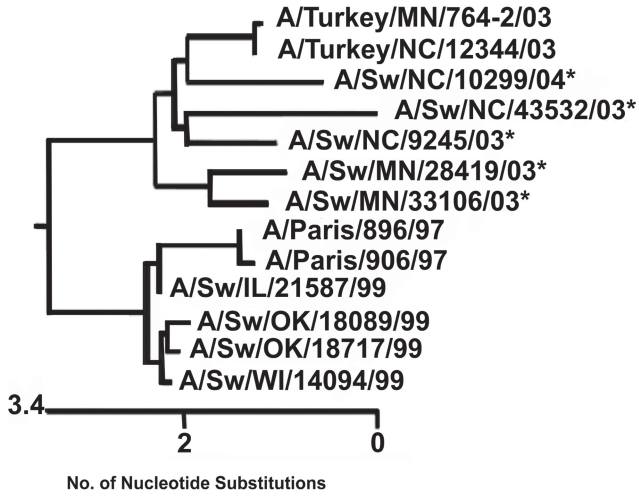

Figure 2. Phylogenetic tree of HA gene nucleotide sequences of 5 nonreactive H3N2 viruses (starred) and 8 H3N2 viruses in GenBank. WI — Wisconsin.

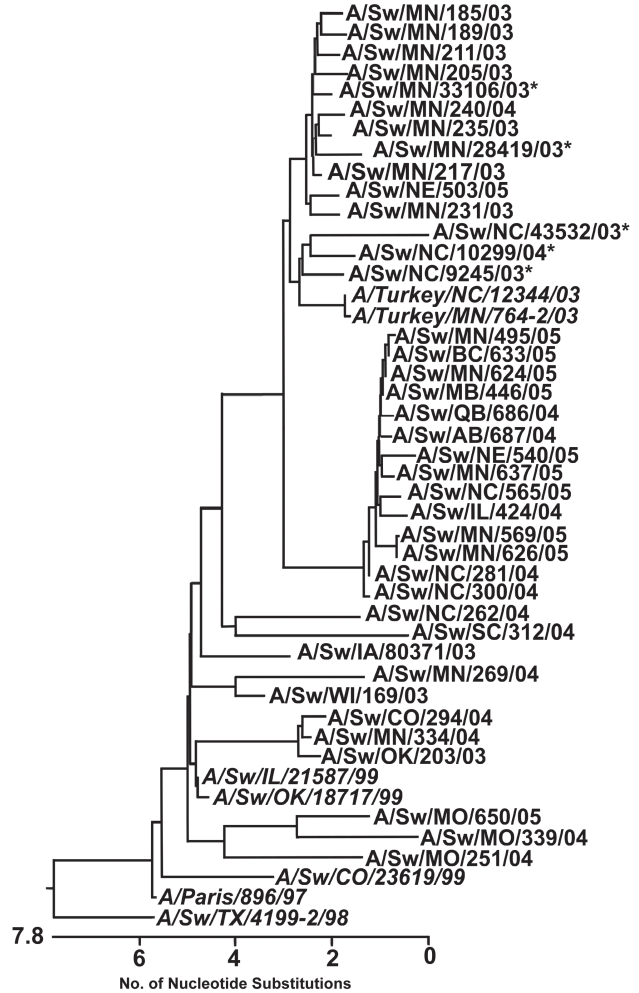

Figure 3. Phylogenetic tree of HA nucleotide sequences of the 5 nonreactive H3N2 viruses (starred), 7 H3N2 viruses in GenBank (italicized and bolded), and 34 contemporary H3N2 viruses from the MVDL database. BC — British Columbia; MB — Manitoba; QC — Quebec; AB — Alberta; SC — South Carolina.

These results indicate that genetic and serologic diversity exists in North American H3N2 SIV. This diversity may pose a serious problem for both animal and human populations for several reasons. These viruses have become well established in pigs since their emergence in 1998 and have demonstrated a propensity for reassortment (1,2,6,23). Furthermore, interspecies transmission of H3N2 viruses, in addition to reassortment and continuing antigenic drift, apparently drives viral evolution (24). As the viruses evolve, variants may emerge that complicate diagnostic efforts and limit the success of vaccination strategies.

Present vaccination strategies for SIV control and prevention in swine farms typically include the use of 1 of several bivalent SIV vaccines commercially available in the United States. Of the 97 recent H3N2 isolates examined, only 41 isolates had strong serologic cross-reactions with antiserum to 3 commercial SIV vaccines. Since the protective ability of influenza vaccines depends primarily on the closeness of the match between the vaccine virus and the epidemic virus (17), the presence of nonreactive H3N2 SIV variants suggests that current commercial vaccines might not effectively protect pigs from infection with a majority of H3N2 viruses. A vaccination and challenge study using genetic and serologic H3N2 variant viruses is necessary to determine the effectiveness of the current SIV vaccines.

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