JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2004, p. 4349–4354 Vol. 42, No. 9
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.9.4349–4354.2004

Characterization of Avian H3N3 and H1N1 Influenza A Viruses Isolated from Pigs in Canada

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Received 27 January 2004/Returned for modification 24 April 2004/Accepted 20 May 2004

H3N3 and H1N1 influenza A viruses were isolated from Canadian pigs in 2001 and 2002. These viruses are phylogenetically related to waterfowl viruses and antigenically distinct from reference swine influenza viruses. The isolation of these viruses reemphasizes the potential for interspecies transmission of influenza viruses from waterfowl to pigs in North America.

Influenza A virus infections in animals are important contributors to the evolution of human influenza viruses (41, 65, 66, 68). In particular, waterfowl and seabirds constitute a global reservoir of all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza A viruses (15, 65, 66, 68). Viruses from waterfowl reassorted with existing human influenza viruses to generate the 1957 (HA, NA, and PB1 polymerase genes were derived from an avian virus) and 1968 (HA and PB1 polymerase genes were derived from an avian virus) pandemic human influenza viruses (24, 54, 66) and may play a similar role in the creation of future pandemic viruses.

The specific host that supported the reassortment events leading to the 1957 and 1968 pandemic viruses remains unclear. Human infections with avian H5N1 and H9N2 influenza viruses in southeast Asia since 1997 (7, 11, 32, 46, 59), avian H7N7 viruses in Europe in 2003 (27a), and previous evidence of localized ocular or subclinical human infections with avian influenza viruses (28, 62, 67) all demonstrate that viruses can be directly transmitted from birds to humans. However, avian influenza viruses typically replicate poorly in humans and nonhuman primates, and vice versa, human influenza viruses generally do not replicate efficiently in birds (2, 14, 37, 58, 66, 68). The basis for species specificity of influenza viruses appears to be polygenic (27, 53, 58, 63, 65, 66, 68), but the HA is hypothesized to be a major factor because of its role as the receptor-binding protein (29, 68). Avian influenza viruses bind preferentially to sialyloligosaccharides with an α2,3 linkage of *N*-acetylneuraminic acid to galactose (NAcα2,3Gal), whereas human influenza viruses prefer NAcα2,6Gal receptors, consistent with the fact that NAcα2,3Gal is the major receptor found on duck intestinal cells and that human tracheal epithelial cells express predominantly NAcα2,6Gal molecules (8, 9, 16–18, 36, 49, 50, 68). Pigs, however, express both NAcα2,3Gal and NAcα2,6Gal receptors as well as α2,3-linked *N*-glycolneuraminic acid (NGc) receptors that are also expressed by duck intestinal cells (16, 17, 19, 60). Thus, pigs have been suggested to be the intermediary host in which avian influenza viruses adapt to replication in mammals and in which avian-human influenza virus reassortment may occur (3, 16, 53, 55, 66). As such, detection and characterization of avian influenza viruses in pigs are important components of influenza virus surveillance.

Kida and colleagues demonstrated that pigs can be infected experimentally with avian viruses of nearly all subtypes (H4 to H13) (25). In addition, avian H1N1, H3N2, and H9N2 viruses have been recovered from pigs in Asia (12, 26, 45), and avian H1N1 viruses spread widely among pigs in northern Europe after their introduction in 1979 (10, 13, 47, 52, 56, 66). In North America, reassortant H3N2, H1N2, and H1N1 viruses containing avian genes have been isolated since 1998 (5, 6, 20, 22, 23, 39, 40, 64, 69), and there has been limited serologic evidence for avian virus infection of pigs (4, 42). However, there has previously been only a single report of isolation of wholly avian viruses from North American pigs. These were H4N6 viruses that we isolated from pigs in Ontario in 1999 (21). We now report the genetic and antigenic characteristics of additional wholly avian viruses of H3N3 and H1N1 subtypes isolated from Canadian pigs.

The first virus, A/Swine/Ontario/42729A/01 (Sw/ON/42729), was isolated in October 2001 from one of a group of approximately 16-week-old pigs in eastern Ontario suffering from weight loss and coughing. (Two additional influenza viruses, 42729B and 42729C, were isolated at the same time from additional pigs involved in this outbreak. However, since complete genome sequencing ultimately demonstrated that the A, B, and C isolates were virtually identical genetically, only isolate 42729A is considered hereafter.) Interestingly, this is the same farm from which the H4N6 viruses were isolated previously (21). The source of the H4N6 viruses for pigs was thought to be raw lake water that was pumped into a barn from an adjacent lake, and this is a practice that continues on the farm. Bronchoalveolar pneumonia was confirmed upon histopathologic examination of the pig from which Sw/ON/42729 was isolated. Both influenza A virus and *Mycoplasma hyopneumoniae* antigens were detected in the lungs by immunohistochemistry, whereas immunohistochemistry assays for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 antigens were negative. Secondary bacterial pathogens (*Pasteurella multocida* and *Streptococcus suis*) were

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TABLE 1. Nucleotide sequences of primers used for RT-PCR and/or sequencing in this study

Primer	5′-3′ nucleotide sequence	Use[a]	Reference or source
SZAHA+	CTCGAGAGCAAAAGCAGGGG	P, S	70
SZAHA−	AGTAGAAACAAGGGTGTTTTT	P, S	70
H1HA1095R[b]	AGGCATGATAGATGGGTGG	S	This study
H3HA1169R	AAGCACTCAAGCCGCCAT	S	This study
SZANA+	AGCAAAAGCAGGAGTTTAAAATG	P, S	70
SZANA−	AGTAGAAACAAGGAGTTTTTT	P, S	70
N1NA408F	AGGGGCCTTGTTGAATGA	S	This study
N3NA522F	GATATGTATTGCTTGGTCT	S	This study
SZANP+	CTCGAGAGCAAAAGCAGGGT	P, S	70
SZANP−	AGTAGAAACAAGGGTATTTTTC	P, S	70
NP484F	AGGATGTGTTCTCTGATGC	S	This study
SZAM+	CTCGAGCAAAAGCAGGTAGAT	P, S	70
SZAM−	AGTAGAAACAAGGTAGTTTTTT	P, S	70
SZANS+	AGCAAAAGCAGGGTGACAAA	P, S	70
SZANS−	AGTAGAAACAAGGGTGTTTTTT	P, S	70
SZAPB1+	AGCAAAAGCAGGCAAACCAT	P, S	70
SZAPB1−	AGTAGAAACAAGGCATTTTTTCAT	P, S	70
PB1589F	AAGAAAATGGTCACACAA	S	This study
PB11548R	AGTTTTGGAGTGTCTGGA	S	This study
SZAPB2+	CTCGAGCAAAAGCAGGTCAA	P, S	70
SZAPB2−	AGTAGAAACAAGGTCGTTTTTAAAC	P, S	70
PB2657F	AGTGGCTGGTGGAACAAG	S	This study
PB21750R	AGCCATTCCAGTCTCTGG	S	This study
SZAPA+	CTCGAGCAAAAGCAGGTACTGAT	P, S	70
SZAPA−	AGTAGAAACAAGGTACTTTTTTGGAC	P, S	70
PA691F	GCCTATGTGGATGGATTC	S	This study
PA1467R	CCAATGATAAGCAAATGT	S	This study

[a] P, PCR; S, sequencing.
[b] R, reverse primer; F, forward primer.

also recovered from the lungs. Influenza A viruses were isolated from lung tissue in both embryonated chicken eggs and Madin-Darby canine kidney (MDCK) cells, but all antigenic and genetic analyses were conducted on the MDCK cell isolate. The Sw/ON/42729 virus was initially subtyped by reverse transcription-PCR (RT-PCR) (20) as an H3 virus, but the NA subtype could not be determined by using N1- or N2-specific primer sets. RT-PCR amplification with global NA primers (70) and subsequent cycle sequencing (ABI Big Dye; PE Applied Biosystems, Foster City, Calif.) and BLAST analyses (1) demonstrated that this was an H3N3 virus.

A second virus, A/Swine/Ontario/K01477/01 (Sw/ON/K01477), was similarly shown to be an H3N3 virus. It was also isolated in October 2001 from pigs in eastern Ontario but on a farm that is located approximately 30 km from the farm of origin of Sw/ON/42729. The second farm uses only well water and stringently prevents pig and pig feed contact with birds. There is also no movement of animals between these two farms.

The third virus described in this report, A/Swine/Saskatchewan/18789/02 (Sw/SK), was isolated from pigs on a 1,200-sow, farrow-to-finish farm in Saskatchewan in May 2002. Influenza-like illness had been occurring on the farm since December 2000 involving pigs of all ages but most severely affecting the nursery pigs. Bacterial pneumonia agents (*S. suis*, *Haemophilus parasuis*, and *Actinobacillus suis*) were also endemic problems on the farm. RT-PCR, cycle sequencing, and BLAST analyses demonstrated that this was an H1N1 virus.

Following initial sequencing of the HA and NA genes of Sw/ON/42729, Sw/ON/K01477, and Sw/SK for subtype determination, the sequences of the full-length protein coding regions of all eight RNA segments for each virus were determined by cycle sequencing with influenza virus universal primers (70) and subsequently designed sequence-specific primers. (The sequences of the primers used for characterization of these viruses are provided in Table 1). BLAST analyses were conducted on each sequence to identify related reference viruses. Sequence comparisons to selected reference viruses were conducted by using DNASTAR software (version 5.0 for Win32), and phylogenetic relationships among Sw/ON/42729, Sw/ON/K01477, Sw/SK, and the reference viruses were estimated by the method of maximum parsimony (PAUP software, version 4.0b6; David Swofford, Smithsonian Institution, and Sinauer Associates, Sunderland, Mass.) using a bootstrap resampling method (500 replications) with a fast heuristic search algorithm. These analyses demonstrated that Sw/ON/42729, Sw/ON/K01477, and Sw/SK are all wholly avian viruses and that each of the RNA segments are phylogenetically related to viruses of the North American lineage of avian influenza viruses. Interestingly, these three viruses are also topologically closely related to one another on the nucleoprotein (NP), the matrix protein, and PB1, PB2, and PA polymerase gene phylograms. However, the nonstructural (NS) gene of Sw/SK was derived from a clade distinct from that of the Sw/ON viruses. Finally, the NA genes of the Sw/SK and Sw/ON viruses, though all clearly of North American avian origin, are phylogenetically distinct from one another since they represent different NA subtypes. By way of example of the phylogenetic analyses conducted, the H1 HA, H3 HA, NP, and NS phylogenetic trees are shown in Fig. 1.

The replication of avian influenza viruses in pigs raises concerns for adaptation to mammalian cells and ultimately enhanced infectivity for humans. Therefore, the deduced amino

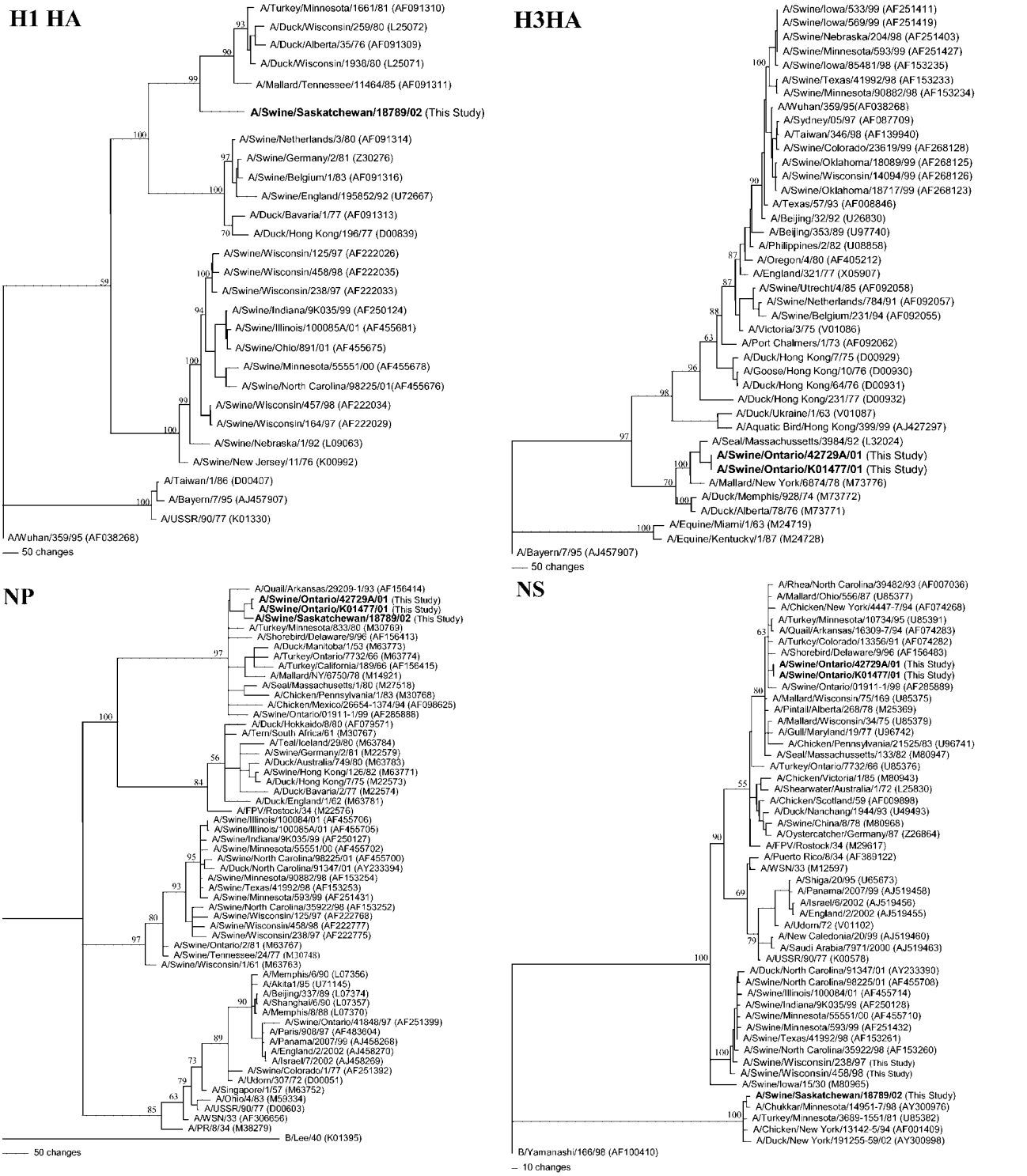

FIG. 1. Nucleotide phylograms for the H3 HA, H1 HA, NP, and NS genes of Sw/ON/42729 (H3N3), Sw/ON/K01477 (H3N3), Sw/SK (H1N1), and related reference viruses. The evolutionary relationships among these viruses were estimated by the method of maximum parsimony (PAUP software version 4.0b6; David Swofford, Smithsonian Institution, and Sinauer Associates) by using a bootstrap resampling method (500 replications) with a fast heuristic search algorithm. The numbers at the nodes of the phylograms indicate the bootstrap confidence levels. Horizontal line distances are proportional to the minimum numbers of nucleotide changes needed to join nodes and gene sequences. The vertical lines are present simply to space the branches and labels. The GenBank accession numbers for the sequences of all of the reference viruses are provided in parentheses following the virus names.

TABLE 2. Analysis of the sequences of Sw/ON/42729 (H3N3), Sw/ON/K01477 (H3N3), and Sw/SK (H1N1) HA proteins at residues that define avian versus mammalian evolutionary lineages and/or receptor preferences

HA analysis and amino acid residue position[a]	Amino acid found in:		Amino acid(s) typical of[b]:		
	Sw/ON/42729 and Sw/ON/K01477	Sw/SK	Avian-lineage viruses and/or NAcα2,3Gal receptor use	Mammalian-lineage viruses and/or NAcα2,6Gal receptor use	NGcα2,3Gal receptor use
H3					
143	P	NA[c]	NA[e]	NA[e]	P/S
155	T	NA[c]	NA[e]	NA[e]	Y
158	G	NA[c]	NA[e]	NA[e]	G
226	Q	NA[c]	Q	L	NA[f]
228	A	NA[c]	G	S	NA[f]
H1					
77	NA[d]	D	D	E	NA[f]
138	NA[d]	A	A	S	NA[f]
145	NA[d]	S	S	L	NA[f]
155	NA[d]	T	T	V/T	I/V
159	NA[d]	T	T	N/G	NA[f]
186	NA[d]	P	P	S	NA[f]
190	NA[d]	D	E	D	NA[f]
194	NA[d]	I	L	I	NA[f]
225	NA[d]	E/G	G	E	NA[f]

[a] All numbers are based on the H3 numbering scheme.
[b] Lineage- and receptor-specific amino acids are based on previously published sequence analyses (8, 17, 34–36, 38, 48, 49, 51, 61).
[c] NA, analysis applicable only to H3 HA analysis.
[d] NA, analysis applicable only to H1 HA analysis.
[e] NA, residue analyzed only in reference to NGc receptor analysis.
[f] NA, residue not applicable to NGc receptor analysis.

acid sequences of the HA genes of Sw/ON/42729, Sw/ON/K01477, and Sw/SK were examined for signatures related to avian versus mammalian (human and swine) receptor preferences and/or evolutionary lineages as defined in previous studies (8, 17, 34–36, 38, 48, 49, 51, 61). As shown in Table 2, the Sw/ON/42729 and Sw/ON/K01477 H3 viruses maintain the glutamine typical of avian viruses and NAcα2,3Gal receptor use at residue 226 (38, 51). At residue 228, these viruses have an alanine; avian viruses that bind to NAcα2,3Gal receptors typically have a glycine at amino acid 228, while human viruses that bind to NAcα2,6Gal receptors typically have a serine (8, 38). The Sw/SK H1 virus maintains amino acids typical of avian viruses at residues 77, 138, 145, 155, 159, 186, 225, and 228 (using the H3 numbering scheme). However, at residues 190 and 194, this virus has amino acids typical of human H1 influenza viruses (17, 35, 36, 48). As regards NGc receptor signature sequences, the Sw/ON/42729 and Sw/ON/K01477 H3 viruses have amino acids typical of NGc receptor use at residues 143 and 158, but they have threonine at residue 155 in place of the tyrosine typical of NGc receptor binding (34). The Sw/SK H1 virus lacks the isoleucine or valine at residue 155 that is typical of H1 swine viruses and postulated to enhance binding to NGc receptors (35). Finally, note that the sequences defined for these viruses are very unlikely to have originated from cross-contamination or laboratory errors. The lab where the RT-PCR and sequencing were conducted had never worked with avian H3 or N3 viruses in the past and had not worked with avian H1 or N1 viruses for many months prior to the arrival of the Sw/ON and Sw/SK isolates. Furthermore, each of the PCR amplifications included negative control reactions containing all reagents except the template.

Beyond these genetic comparisons, the antigenic characteristics of the HA proteins of Sw/ON/42729 and Sw/SK were also assessed in hemagglutination inhibition (HI) assays by using either monoclonal antibodies (MAbs) specific for H1 HA proteins or polyclonal anti-H1 or anti-H3 swine serum. The anti-H1 MAbs (ascites) had been produced against A/Swine/Indiana /1726/88 (H1N1) and A/Swine/Wisconsin/27/86 (H1N1) as previously described (33, 57). The anti-H1 polyclonal swine serum was collected from pigs 14 days following the administration of two doses of classical H1N1 swine influenza virus vaccine (MaxiVac-FLU; SyntroVet, Inc.) (31). The anti-H3 polyclonal swine serum was collected from pigs 7 days following intranasal infection with A/Swine/Minnesota/593/99 (H3N2) (30). The HI assays were conducted as previously described (42, 44) with serial twofold dilutions of MAb ascites (1:100 to 1:6,400) or polyclonal swine serum (1:10 to 1:640). HI titers were defined as the reciprocal of the highest dilution of MAb ascites or serum that completely inhibited virus-induced agglutination of chicken red blood cells. As shown in Table 3, the Sw/SK H1 virus lacked reactivity with two of the four MAbs that recognize classical swine influenza viruses. This pattern is consistent with previous results from a North American waterfowl H1N1 virus, A/Duck/Alberta/35/76 (33, 43, 57). The Sw/SK virus also reacted to an eightfold-lower titer with anti-H1 swine serum compared to that of a reference classical H1N1 swine virus. The Sw/ON/42729 H3 virus was completely nonreactive with serum recovered from a pig previously infected with a triple-reassortant H3N2 virus (30) that is typical of those that emerged among American pigs in 1998.

In summary, this work documents the isolation of wholly avian H3N3 and H1N1 influenza viruses from pigs in Canada during 2001 and 2002. In one case, the likely source of virus for the pigs was lake water contaminated with waterfowl feces, while the source of virus in the other two instances is unclear. However, the phylogenetic data for all three viruses clearly

TABLE 3. Reactivity of Sw/ON/42729 and Sw/SK compared to reference swine viruses in HI assays[b]

Virus[a]	HI titer in:					
	Anti-H1 MAbs				Anti-H1 polyclonal swine serum	Anti-H3 polyclonal swine serum
	1-6B2	3F2C	2-15F1	7B1B		
Sw/ON/42729A (H3N3)	–[b]	–	–	–	–	–
A/Swine/Minnesota/593/99 (H3N2)	–	–	–	–	–	320
Sw/SK (H1N1)	>6,400	–	–	800	10	–
A/Swine/Indiana/1726/88 (H1N1)	>6,400	>6,400	1,600	>6,400	80	–

[a] Sw/ON/42729 and Sw/SK are the wholly avian viruses described in this report. A/Swine/Minnesota/593/99 is a triple-reassortant H3N2 reference virus, and A/Swine/Indiana/1726/88 is a classical swine H1N1 reference virus.
[b] –, HI titers <100 (MAb ascites) or <10 (serum).

reveal a North American waterfowl origin. We do not have data to address the extent of transmission of these viruses among the swine population. Nonetheless, it is important for diagnostic virologists to know that H1 and H3 viruses that are genetically and antigenically distinct from the H1 and H3 viruses currently circulating among pigs in North America may be present within the swine population. Finally, the isolation of these viruses reemphasizes the fact that bird-to-pig interspecies transmission of influenza viruses may occur not just in Asia but also in North America.

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to the gene sequences determined for this report are as follows: AY619970 to AY619977 for Sw/ON/42729, AY619962 to AY619969 for Sw/ON/K01477, AY619954 to AY619961 for Sw/SK, AY619979 for A/Swine/Wisconsin/238/97, and AY619978 for A/Swine/Wisconsin/458/98.

This work was supported in part by a grant to C.W.O. from the USDA National Research Initiative Competitive Grants Program.

We thank Gabriele Landolt for technical assistance and many helpful discussions.

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