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Identification of Human H1N2 and Human-Swine Reassortant H1N2 and H1N1 Influenza A Viruses among Pigs in Ontario, Canada (2003 to 2005)†

Alexander I. Karasin,[1] Suzanne Carman,[2] and Christopher W. Olsen[1]*

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin,[1] *and Animal Health Laboratory, Laboratory Services Division, University of Guelph, Box 3612, Guelph, Ontario, Canada*[2]

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Since 2003, three novel genotypes of H1 influenza viruses have been recovered from Canadian pigs, including a wholly human H1N2 virus and human-swine reassortants. These isolates demonstrate that human-lineage H1N2 viruses are infectious for pigs and that viruses with a human PB1/swine PA/swine PB2 polymerase complex can replicate in pigs.

The segmented nature of the RNA genomes of influenza A viruses allows genetic reassortment to occur when two or more viruses coinfect a single cell (24, 45). Reassortment in pigs has been postulated to be one mechanism for generating influenza viruses with pandemic potential for the human population (3, 37–39, 44, 45), and thus monitoring the swine population for novel viruses may be an important component of pandemic surveillance. Since 1998, triple reassortant H3N2 influenza viruses containing human, classical swine, and avian virus lineage genes have been isolated from pigs in the United States (20, 36, 43, 48) and Canada (C. W. Olsen and S. Carman, unpublished data). In the United States, these viruses underwent further reassortment with classical H1N1 swine influenza viruses to create reassortant H1N2 viruses (5, 6, 17, 19) and reassortant H1N1 viruses (30, 32; R. J. Webby, K. D. Rossow, S. M. Goyal, S. L. Krauss, and R. G. Webster, Sci. Prog. Abstr. Am. Soc. Virol. 21st Ann. Mtg., abstr. W1-3, 2002; C. W. Olsen, unpublished data). These reassortant H1N2 and H1N1 viruses also contained genes from human, classical swine, and avian influenza virus lineages. During this same time period, H1N2 viruses were also isolated from human beings in many countries around the world. These viruses, first isolated during the 2001 to 2002 influenza season, were reassortants between human H3N2 and H1N1 viruses and did not contain genes of animal influenza virus origin (9, 14, 16, 22, 26, 46). We now report the interspecies transmission of one of these wholly human H1N2 viruses to a pig in Ontario, Canada, in 2003, and the subsequent isolation of reassortant H1N2 viruses from pigs in Ontario with hemagglutinin (HA) genes closely related phylogenetically to the 2003 H1N2 virus isolate and other genes of mixed human and swine virus lineages. In addition, we describe reassortant H1N1 viruses of a unique genotype not previously recovered from pigs, containing a human virus lineage PB1 polymerase gene and all remaining genes of classical swine virus lineage.

The H1N2 and reassortant H1N1 swine isolates described here were recovered from either nasal swab or lung tissue samples inoculated onto Madin-Darby canine kidney cells as previously described (18), with the exception of A/Swine/Ontario/11112/04, which was isolated in embryonated chicken eggs. Full-length, viral RNA segments were amplified by reverse transcription-PCR, and their nucleotide sequences were determined by cycle sequencing (ABI BigDye; PE Applied Biosystems, Foster City, Calif.) by using previously described techniques (17, 18, 20) and primers (17, 21, 49). In addition, genes from two contemporary, classical H1N1 swine influenza viruses isolated (in MDCK cells) from pigs in Ontario and Alberta, Canada, in 2003 (A/Swine/Ontario/57561/03, A/Swine/Alberta/56626/03), as well as from previously described (31) classical H1N1 swine viruses isolated (in eggs) from pigs in the United States (A/Swine/Wisconsin/125/97, A/Swine/Wisconsin/238/97, A/Swine/Wisconsin/458/98, and A/Swine/Wisconsin/464/98), were also sequenced to provide comparative information for phylogenetic analyses. Viruses were passaged no more than once beyond initial isolation before sequencing.

BLAST analyses (1) were conducted on each sequence to identify related reference viruses. Sequence comparisons to reference viruses were conducted using DNASTAR software (version 5.0 for Win32), and phylogenetic relationships between viruses were estimated by the method of maximum-parsimony (PAUP software, v.4.0b10; David Swofford, Smithsonian Institution, and Sinauer Associates, Sunderland, Mass.) using a bootstrap resampling method (200 replications) with a fast heuristic search algorithm.

Three H1N2 viruses were identified (Table 1). A/Swine/Ontario/52156/03 (Sw/ONT/52156) was isolated in October, 2003, from a group of approximately 6-week-old pigs suffering unexpected, sudden deaths. A/Swine/Ontario/48235/04 (Sw/ONT/48235) and A/Swine/Ontario/55383/04 (Sw/ONT/55383) were isolated in October and November 2004 from approximately 4- and 8-week-old pigs, respectively, with signs of respiratory disease. These three H1N2 viruses were isolated from three independent farms distributed across an approximately 15-by-20-square-mile region of Ontario. Phylogenetic analyses

* Corresponding author. Mailing address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Dr., Madison, WI 53706. Phone: (608) 265-8681. Fax: (608) 263-0438. E-mail: olsenc@svm.vetmed.wisc.edu.

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TABLE 1. Subtypes and genotypes of viruses featured in this study

Virus name	Subtype	Phylogenetic lineage of RNA segments							
		PB2	PB1	PA	HA	NP	NA	M	NS
A/Swine/Ontario/52156/03	H1N2	Human	Human	Human	Human	Human	Human	Human	Human
A/Swine/Ontario/48235/04	H1N2	Classical swine	Human	Classical swine	Human	Classical swine	Human	Classical swine	Classical swine
A/Swine/Ontario/55383/04	H1N2	Classical swine	Human	Classical swine	Human	Classical swine	Human	Classical swine	Classical swine
A/Swine/Ontario/53518/03	H1N1	Classical swine	Human	Classical swine	Classical swine	Classical swine	Classical swine	Classical swine	Classical swine
A/Swine/Ontario/11112/04	H1N1	Classical swine	Human	Classical swine	Classical swine	Classical swine	Classical swine	Classical swine	Classical swine
A/Swine/Ontario/23866/04	H1N1	Classical swine	Human	Classical swine	Classical swine	Classical swine	Classical swine	Classical swine	Classical swine

demonstrated that all eight RNA segments of the 2003 isolate, Sw/ONT/52156, were most closely related to the H1N2 viruses that circulated among human beings throughout the world beginning during the 2001 to 2002 influenza season. In contrast, phylogenetic analyses of the H1N2 viruses isolated from pigs in Ontario in 2004 revealed that these viruses were reassortants. Although their HA genes were closely related phylogenetically to the HA gene of Sw/ONT/52156, their neuraminidase (NA) and PB1 polymerase genes were of human influenza virus lineages distinct from the 2001 to 2002 human H1N2 viruses, and all of their remaining genes were of classical swine virus lineage. (By way of example, the phylograms for the H1 HA, N2 NA, matrix [M], and PB1 genes of the viruses featured in the present study are available in the supplemental material.)

Beyond these phylogenetic evaluations, we also sought to determine whether there were deduced amino acid sequence differences between the HA and NA proteins of the reassortant Sw/ONT/48235 and Sw/ONT/55383 viruses isolated in 2004 compared to the HA and NA proteins of the Sw/ONT/52156 virus initially isolated in 2003 that might reflect adaptation to infection of swine. The issue of potential swine adaptation mutations is of particular interest in light of our recent studies demonstrating that wholly human influenza A viruses are restricted in their infectivity for primary swine respiratory epithelial cells in vitro (G. A Landolt, A. I. Karasin, R. A. Brockman-Schneider, J. E. Gern, K. Tewari, M. Suresh, A. S. Gambaryan, K. Shinya, Y. Kawaoka, and C. W. Olsen, unpublished data) and for pigs in vivo (25, 25a). Within the HA protein, Sw/ONT/48235 and Sw/ONT/55383 have mutations, compared to Sw/ONT/52156, at the following eight sites (numbering from the start of the open reading frame, with the original Sw/ONT/52156 amino acid given before each residue number): P19T, A42T, Y111H, V145I, S158R, N414S, S467G, and V541I (Table 2). None of these sequence differences occur in residues previously implicated in forming the receptor binding site for H1 HA molecules and, more specifically, none of the isolates contain I or V at residue 168 (155 using the H3 numbering system) that typify H1 swine viruses that can bind NGc rather than NAc-containing sialic acids (28). In addition, none of these differences affect potential N-linked glycosylation sites. The amino acids in Sw/ONT/48235 and Sw/ONT/55383 at residues 19 and 42 are identical in sequence to the earlier A/New Caledonia/22/99 human virus and are thus unlikely to be associated with swine adaptation. However, three of the sequence differences (Y111H, V145I, and S158R) fall within phylogenetically important regions (PIRs) in the H1 HA (PIRs D, F, and G, respectively) (11) and thus may reflect swine adaptation mutations. Finally, the S158R mutation is located within a known antigenic site (4, 27) and thus may also be due to antigenic drift.

Within the NA proteins, both the Sw/ONT/48235 and the Sw/ONT/55383 2004 isolates differ in sequence from the 2003 Sw/ONT/52156 isolate at the following sites (numbering from the start of the open-reading frame, with the original Sw/ONT/52156 amino acid given before each residue number): T24M,

TABLE 2. Sites of putative amino acid sequence differences in the HA and NA proteins of the H1N2 viruses described in this study

Amino acid residue[a]	A/Swine/Ontario/52156/03[b]	A/Swine/Ontario/48235/04[c]	A/Swine/Ontario/55383/04[c]	PIRs[d]
HA 19	P	T	T	
HA 42	A	T	T	
HA 111	Y	H	H	D
HA 145	V	I	I	F
HA 158	S	R	R	G
HA 414	N	S	S	
HA 467	S	G	G	
HA 541	V	I	I	
NA 24	T	M	M	
NA 40	Y	Y	Q	A′
NA 147	D	D	N	C′
NA 172	K	R	R	
NA 216	S	G	G	
NA 265	T	I	I	
NA 332	S	F	F	F′
NA 339	D	N	D	F′
NA 399	D	E	E	
NA 401	D	G	G	K′
NA 402	N	N	S	K′
NA 412	V	I	V	
NA 431	N	K	K	
NA 432	Q	E	E	
NA 435	E	K	K	
NA 437	L	W	W	

[a] Numbering is based on defining the first amino acid of the open reading frame of each gene as amino acid 1.

[b] A/Swine/Ontario/52156/03 is a wholly human H1N2 virus.

[c] A/Swine/Ontario/48235/04 and A/Swine/Ontario/55383/04 are reassortant viruses with HA, NA, and PBI genes of human influenza virus lineages and the remaining genes of classical swine virus lineage.

[d] PIRs were previously defined by Taubenberger and coworkers (10, 11).

K172R, S216G, T265I, S332F, D399E, D401G, N431K, Q432E, E435K, and L437W. In addition, Sw/ONT/55383 is uniquely different from the other two isolates at three sites (Y40Q, D147N, and N402S), and Sw/ONT/48235 is uniquely different from the other two isolates at two sites (D339N and V412I) (Table 2). None of these sites of amino acid differences fall within the previously defined active site of the NA (8), and only the N402S mutation affects (eliminates) a potential N-linked glycosylation site. The amino acids in Sw/ONT/48235 and Sw/ONT/55383 at residues 24, 216, 401, and 431 are identical in sequence to the earlier A/Moscow/10/99 human virus and are thus unlikely to be associated with swine adaptation. However, the differences in Sw/ONT/48235 and/or Sw/ONT/55383 compared to Sw/ONT/52156 at residues 40, 147, 332, 339, 401, and 402 fall within previously defined (10) N1 NA PIRs (A′, C′, F′, F′, K′, and K′, respectively) and thus may reflect swine adaptation mutations.

In the course of the surveillance work that identified the reassortant H1N2 viruses described above, we also discovered a previously unreported genotype of reassortant H1N1 viruses. These viruses were isolated from pigs that ranged in age from 3-week-old nursery piglets to adult sows and that exhibited various clinical signs typical of swine influenza illness (fever, coughing, dyspnea, inappetence, and inactivity). Complete sequencing of all eight RNA segments of three of the isolates (A/Swine/Ontario/53518/03 [Sw/ONT/53518], A/Swine/Ontario/11112/04 [Sw/ONT/11112], and A/Swine/Ontario/23866/04 [Sw/ONT/23866]) (Table 1; see also the supplemental material) and partial sequencing of six additional viruses (data not shown), revealed that these viruses contained PB1 polymerase genes of human influenza virus lineage, while all of their remaining genes were of classical H1N1 swine influenza virus lineage. These nine reassortant H1N1 viruses were isolated between October 2003 and February 2005 from pigs on nine independent swine farms located over an 80-by-85-square-mile region of Ontario, indicating that viruses of this reassortant H1N1 genotype spread widely within the swine population of Ontario.

The fact that the reassortant H1N2 and H1N1 viruses described here all contained human influenza virus-lineage PB1 genes is of interest, given that the reassortant H3N2, H1N2, and H1N1 viruses that recently emerged among pigs in the United States also consistently contained human PB1 genes (5, 17, 19, 20, 30, 32, 43, 48; Webby et al., Sci. Prog. Abstr. Am. Soc. Virol. 21st Ann. Mtg.; Olsen, unpublished). The triad of PA, PB1, and PB2 polymerase proteins of influenza A viruses function as complexes to synthesize cRNA, vRNA, and mRNA (2, 12, 13, 34, 35). Human, classical swine, equine, and avian phylogenetic lineages have been defined for each of the three polymerase genes (15, 23, 29, 44), and there is evidence for contributions to species specificity and host range determination by individual polymerase genes (7, 33, 41, 42, 47). However, it is also clear that constellations of polymerase proteins from different phylogenetic origins can function together to create infectious viruses, as evidenced by the combinations of human PA and PB2 and avian PB1 genes in the 1957 and 1968 human pandemic viruses (23) and the human PB1 and avian PA and PB2 genes in the reassortant H3N2, H1N2, and H1N1 viruses isolated from pigs in the United States since 1998 (5, 17, 19, 20, 30, 32, 43, 48; Webby et al., Sci. Prog. Abstr. Am. Soc. Virol. 21st Ann. Mtg.; Olsen, unpublished). For the reassortant swine isolates from the United States, it has been suggested that the combination of avian PA and PB2 genes confers a replication advantage for these viruses in pigs (43). However, given our findings herein of reassortant H1N2 and H1N1 swine viruses possessing human lineage PB1 genes and classical swine rather than avian lineage PA and PB2 genes, it is clear that a human influenza virus lineage PB1 is able to function in cooperation with PA and PB2 genes of variable lineages to produce reassortant influenza viruses that are infectious for and competent to replicate in pigs.

Taken in total, our results document that reassortment involving human influenza viruses continues to occur among pigs in North America. In light of the fact that avian influenza viruses have also been isolated from pigs in Canada since 1999 (18, 21), it is clear that the potential for pandemic influenza virus emergence through reassortment of avian and human influenza viruses in pigs exists not just in the traditionally defined "influenza epicenter" of Asia (40) but also in North America.

Nucleotide sequence accession numbers. The GenBank numbers assigned to the gene sequences of the viruses investigated in the present study are as follows: A/Swine/Ontario/52156/03 (human H1N2) (DQ280221 to DQ280228), A/Swine/Ontario/48235/04 (reassortant H1N2) (DQ280229 to DQ280236), A/Swine/Ontario/55383/04 (reassortant H1N2) (DQ280205 to DQ280212), A/Swine/Ontario/53518/03 (reassortant H1N1) (DQ280213 to DQ280220), A/Swine/Ontario/11112/04 (reassortant H1N1) (DQ280245 to DQ280252), A/Swine/Ontario/23866/04 (reassortant H1N1) (DQ280237 to DQ280244), A/Swine/Ontario/57561/03 (classical swine H1N1) (DQ280189 to DQ280196), A/Swine/Alberta/56626/03 (classical swine H1N1) (DQ280197 to DQ280204), A/Swine/Wisconsin/125/97 (classical swine H1N1) (DQ280260), A/Swine/Wisconsin/238/97 (classical swine H1N1) (DQ280257 to DQ280259), A/Swine/Wisconsin/458/98 (classical swine H1N1) (DQ280256), and A/Swine/Wisconsin/464/98 (classical swine H1N1) (DQ280253 to DQ280255).

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