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Characterization of H1N1 Swine Influenza Viruses Circulating in Canadian Pigs in 2009▽

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The 2009 pandemic H1N1 (pH1N1), of apparent swine origin, may have evolved in pigs unnoticed because of insufficient surveillance. Consequently, the need for surveillance of influenza viruses circulating in pigs has received added attention. In this study we characterized H1N1 viruses isolated from Canadian pigs in 2009. Isolates from May 2009 were comprised of hemagglutinin and neuraminidase (NA) genes of classical SIV origin in combination with the North American triple-reassortant internal gene (TRIG) cassette, here termed contemporary SIV (conSIV) H1N1. These conSIV H1N1 viruses were contiguous with the North American αH1 cluster, which was distinct from the pH1N1 isolates that were antigenically more related to the γH1 cluster. After the initial isolation of pH1N1 from an Alberta pig farm in early May 2009, pH1N1 was found several times in Canadian pigs. These pH1N1 isolates were genetically and antigenically homogeneous. In addition, H1N1 viruses bearing seasonal human H1 and N1 genes together with the TRIG cassette and an NA encoding an oseltamivir-resistance marker were isolated from pigs. The NS gene of one of these seasonal human-like SIV (shSIV) H1N1 isolates was homologous to pH1N1 NS, implicating reassortment between the two strains. Antigenic cross-reactivity was observed between pH1N1 and conSIV but not with shSIV H1N1. In summary, although there was cocirculation of pH1N1 with conSIV and shSIV H1N1 in Canadian pigs after May 2009, there was no evidence supporting the presence of pH1N1 in pigs prior to May 2009. The possibility for further reassortants being generated exists and should be closely monitored.

Influenza, a disease shared between humans, pigs, and other animals, is caused by influenza A viruses belonging to the family *Orthomyxoviridae*. Influenza A virus is a single-stranded RNA virus with a segmented genome composed of eight gene segments now recognized to encode for 12 protein products (28). These are the polymerase basic protein 2 (PB2) encoded by segment 1; PB1, PB1-F2, and N40 encoded by segment 2; polymerase acidic protein (PA) encoded by segment 3; hemagglutinin (HA) encoded by segment 4; nucleoprotein (NP) encoded by segment 5; neuraminidase (NA) encoded by segment 6; matrix and ion channel proteins (M1 and M2) encoded by segment 7 and 2 nonstructural proteins (NS) encoded by segment 8. Sixteen HA and 9 NA types have been identified and form the basis for classifying influenza A viruses into subtypes. All subtypes exist in aquatic birds, which serve as the natural reservoir for all influenza A viruses (24).

The first characterized subtype of swine influenza virus (SIV), the classical H1N1 (cSIV), was isolated from pigs in the United States in 1930 and is believed to have been acquired from humans during the 1918 influenza pandemic (18). Prior to the 1968 human H3N2 pandemic, cSIV remained the sole agent responsible for influenza in North American pigs. In the late 1990s, however, reassortment between human H3N2 and cSIV resulted in double-reassortant viruses capable of causing disease in pigs (29). Additional reassortment with an avian influenza virus resulted in the generation of a triple-reassortant (TR) H3N2 virus, which subsequently further reassorted with cSIV, giving rise to TR H3N1, H1N1, and H1N2 viruses (24). However, the subtypes found most frequently in pigs in North America currently are TR H3N2, H1N1, and H1N2. Almost all contemporary Canadian and U.S. swine viruses have an internal gene combination comprised of the PB1 of human influenza A origin, PB2 and PA of avian influenza A origin, and NP, M, and NS of swine influenza A origin (24). This triple-reassortant internal gene (TRIG) cassette appears to have a high potential for accepting HA and NA from different influenza A subtypes thereby increasing the evolutionary rate of SIV. Phylogenetic studies that have been carried out on H1 subtype viruses circulating in swine in the United States have led to grouping of the SIV H1 subtype into α, β, γ, and δ clusters (13, 23). Although α-, β-, and γ-cluster viruses are related to the classical H1 SIV lineage, all have evolved by drift mutations, as well as reassortment with triple-reassortant H3N2 viruses. The H1 gene segment of δ-cluster viruses is derived from seasonal human H1N1 and is thus the most divergent. SIV H1N1 bearing human seasonal influenza A-origin HA and NA with a TRIG cassette have repeatedly emerged in the United States since 2005 (23). In addition, completely human H1N1 and double-reassortant H1N1 and H1N2 viruses were isolated from pigs in Ontario, Canada,

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TABLE 1. GenBank accession numbers and related information for 2009 SIV H1N1 viruses from Canada

Isolate[a]	Strain[b]	Sampling date	Sequenced segments[c]	Accession nos.
A/SW/QC/1488-2/09	conSIV	5 May 2009	Full genome	JF713848-JF713855
A/SW/QC/1488-3/09	conSIV	5 May 2009	HA, NA, and M	JF713856-JF713858
A/SW/QC/1488-4/09	conSIV	5 May 2009	HA, NA, and M	JF713859-JF713861
A/SW/QC/1488-5/09	conSIV	5 May 2009	HA, NA, and M	JF713862-JF713864
A/SW/QC/3399-3/09	conSIV	6 May 2009	HA, NA, and M	JF713946-JF713948
A/SW/QC/3399-6/09	conSIV	6 May 2009	Full genome	JF713949-JF713956
A/SW/QC/1531-1/09	conSIV	7 May 2009	HA, NA, and M	JF713865-JF713867
A/SW/QC/1531-2/09	conSIV	7 May 2009	HA, NA, and M	JF713868-JF713870
A/SW/QC/1531-3/09	conSIV	7 May 2009	Full genome	JF713871-JF713878
A/SW/QC/1533-1/09	conSIV	7 May 2009	HA, NA, and M	JF713879-JF713881
A/SW/QC/1533-5/09	conSIV	7 May 2009	HA, NA, and M	JF713882-JF713884
A/SW/QC/3453/09	conSIV	7 May 2009	HA, NA, and M	JF713957-JF713959
A/SW/QC/368/09	conSIV	8 May 2009	HA, NA, and M	JF713820-JF713822
A/SW/QC/370/09	conSIV	8 May 2009	Full genome	JF713823-JF713830
A/SW/QC/1588/09	conSIV	8 May 2009	HA, NA, and M	JF713885-JF713887
A/SW/QC/3639-3/09	conSIV	12 May 2009	HA, NA, and M	JF713960-JF713962
A/SW/QC/3639-4/09	conSIV	12 May 2009	HA, NA, and M	JF713963-JF713965
A/SW/QC/398/09	conSIV	13 May 2009	Full genome	JF713831-JF713838
A/SW/QC/3777/09	conSIV	14 May 2009	HA, NA, and M	JF713966-JF713968
A/SW/QC/1695-3/09	conSIV	15 May 2009	Full genome	JF713888-JF713895
A/SW/QC/1697-1/09	conSIV	15 May 2009	Full genome	JF713896-JF713903
A/SW/QC/1697-2/09	conSIV	15 May 2009	HA, NA, and M	JF713904-JF713906
A/SW/QC/1697-3/09	conSIV	15 May 2009	HA, NA, and M	JF713907-JF713909
A/SW/QC/1697-4/09	conSIV	15 May 2009	HA, NA, and M	JF713910-JF713912
A/SW/QC/1697-5/09	conSIV	15 May 2009	HA, NA, and M	JF713913-JF713915
A/SW/QC/3845/09	conSIV	19 May 2009	HA, NA, and M	JF713969-JF713971
A/SW/QC/410-A/09	conSIV	20 May 2009	HA, NA, and M	JF713839-JF713841
A/SW/QC/410-B/09	conSIV	20 May 2009	HA, NA, and M	JF713842-JF713844
A/SW/QC/4015-1/09	conSIV	22 May 2009	HA, NA, and M	JF713972-JF713974
A/SW/QC/4015-2/09	conSIV	22 May 2009	HA, NA, and M	JF713975-JF713977
A/SW/QC/1792-1/09	conSIV	26 May 2009	HA, NA, and M	JF713916-JF713918
A/SW/QC/1792-1/09	conSIV	26 May 2009	HA, NA, and M	JF713919-JF713921
A/SW/QC/4110/09	conSIV	26 May 2009	HA, NA, and M	JF713978-JF713980
A/SW/QC/4112/09	conSIV	26 May 2009	HA, NA, and M	JF713981-JF713983
A/SW/QC/4113/09	conSIV	26 May 2009	HA, NA, and M	JF713984-JF713986
A/SW/QC/1805-1/09	conSIV	27 May 2009	HA, NA, and M	JF713922-JF713924
A/SW/QC/1805-2/09	conSIV	27 May 2009	HA, NA, and M	JF713925-JF713927
A/SW/QC/1805-3/09	conSIV	27 May 2009	HA, NA, and M	JF713928-JF713930
A/SW/QC/1805-4/09	conSIV	27 May 2009	HA, NA, and M	JF713931-JF713933
A/SW/QC/1805-5/09	conSIV	27 May 2009	HA, NA, and M	JF713934-JF713936
A/SW/QC/1814-1/09	conSIV	27 May 2009	HA, NA, and M	JF713937-JF713939
A/SW/QC/1814-3/09	conSIV	27 May 2009	HA, NA, and M	JF713940-JF713942
A/SW/QC/1814-5/09	conSIV	27 May 2009	HA, NA, and M	JF713943-JF713945
A/SW/QC/4150/09	conSIV	27 May 2009	HA, NA, and M	JF713987-JF713989
A/SW/QC/438-B/09	conSIV	29 May 2009	HA, NA, and M	JF713845-JF713847
A/SW/MB/5-5/09	conSIV	4 Jun 2009	Full genome	JF714017-JF714024
A/SW/MB/10422/09	pH1N1	6 Jul 2009	Full genome	JF714033-JF714040
A/SW/SK/11-16/09	shSIV[d]	20 Jul 2009	Full genome	JF714006-JF714013
A/SW/SK/11-35/09	shSIV	20 Jul 2009	HA, NP, and NA	JF714014-JF714016
A/SW/SK/12-71/09	pH1N1[e]	20 Jul 2009	Full genome	JF713998-JF714005
A/SW/MB/22/09	pH1N1	17 Aug 2009	HA, NA, and M	JF714077-JF714079
A/SW/QC/705-A/09	conSIV	26 Aug 2009	HA and NA	JF714133, JF714134
A/SW/QC/705-B/09	conSIV	26 Aug 2009	HA and NA	JF714135, JF714136
A/SW/MB/25-3/09	pH1N1	31 Aug 2009	Full genome	JF714041-JF714048
A/SW/QC/729-B/09	conSIV	4 Sep 2009	HA and NA	JF714129, JF714130
A/SW/QC/3193/09	conSIV	11 Sep 2009	HA and NA	JF714125, JF714126
A/SW/QC/1036/09	conSIV	18 Sep 2009	HA and NA	JF714131, JF714132
A/SW/MB/31/09	pH1N1	18 Sep 2009	Full genome	JF714049-JF714056
A/SW/MB/32-2/09	pH1N1	18 Sep 2009	Full genome	JF714057-JF714064
A/SW/MB/33/09	pH1N1	28 Sep 2009	HA and NA	JF714065, JF714066
A/SW/MB/35-1/09	pH1N1	29 Sep 2009	HA and NA	JF714067, JF714068
A/SW/MB/36/09	pH1N1	6 Oct 2009	Segments 2 to 8	JF714080-JF714086
A/SW/MB/46/09	pH1N1	2 Nov 2009	Full genome	JF714025-JF714032
A/SW/QC/3868/09	pH1N1	6 Nov 2009	HA and NA	JF714105, JF714106
A/SW/MB/54/09	pH1N1	15 Nov 2009	Full genome	JF714069-JF714076
A/SW/SK/59-6/09	pH1N1	16 Nov 2009	Full genome	JF713990-JF713997
A/SW/QC/3973-1/09	pH1N1	16 Nov 2009	HA and NA	JF714095, JF714096
A/SW/QC/3974-5/09	pH1N1	16 Nov 2009	Full genome	JF714097-JF714104

Continued on following page

TABLE 1—*Continued*

Isolate[a]	Strain[b]	Sampling date	Sequenced segments[c]	Accession nos.
A/SW/QC/7692/09	pH1N1	17 Nov 2009	HA and NA	JF714113, JF714114
A/SW/QC/4040-2/09	pH1N1	18 Nov 2009	HA and NA	JF714121, JF714122
A/SW/QC/7780/09	pH1N1	23 Nov 2009	HA and NA	JF714115, JF714116
A/SW/QC/7780-PO/09	pH1N1	23 Nov 2009	HA and NA	JF714117, JF714118
A/SW/QC/4036-5/09	pH1N1	26 Nov 2009	HA and NA	JF714087, JF714088
A/SW/QC/7918-E/09	conSIV	30 Nov 2009	HA and NA	JF714123, JF714124
A/SW/QC/F8037/09	pH1N1	4 Dec 2009	HA and NA	JF714111, JF714112
A/SW/QC/1014-1/09	pH1N1	10 Dec 2009	HA and NA	JF714107, JF714108
A/SW/QC/1014-2/09	pH1N1	10 Dec 2009	HA and NA	JF714109, JF714110
A/SW/QC/1500/09	pH1N1	10 Dec 2009	HA and NA	JF714119, JF714120
A/SW/QC/1196903-1/09	pH1N1	10 Dec 2009	HA and NA	JF714137, JF714138
A/SW/QC/F8154/09	conSIV	11 Dec 2009	HA and NA	JF714127, JF714128
A/SW/QC/4362-1/09	pH1N1	17 Dec 2009	HA and NA	JF714089, JF714090
A/SW/QC/4362-2/09	pH1N1	17 Dec 2009	HA and NA	JF714091, JF714092
A/SW/QC/4362-3/09	pH1N1	17 Dec 2009	HA and NA	JF714093, JF714094

[a] QC, Quebec; MB, Manitoba; SK, Saskatchewan; AB, Alberta.
[b] pH1N1, pandemic H1N1 isolates from swine, turkeys, and humans; conSIV, contemporary SIV H1N1 isolates from swine; shSIV, seasonal human-like SIV H1N1 from swine.
[c] "Full genome" means all influenza A gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS) were sequenced and deposited in GenBank with the accession numbers for each gene segment for each virus arranged in the corresponding order from segment 1 to segment 8. For the rest of the isolates, accession numbers are arranged in the order in which the indicated sequenced segments are presented.
[d] Novel reassortment with NS from pandemic H1N1.
[e] Novel reassortment with NP from TRIG cassette of contemporary SIV.

between 2003 and 2005 (10). These events demonstrate the potential of swine as a "mixing vessel" for influenza A viruses from which new subtypes with pandemic potential for humans could emerge. It also indicates the need for increased biosecurity in swine operations since human influenza A viruses can directly or indirectly contribute to disease in swine.

Occasional infection of humans with swine-origin influenza resulting in clinical disease has been known to occur, with infrequent hospitalization and mortality also described. However, persistent human-to-human transmission of SIV hardly occurred prior to 2009 (14). In March to April 2009, a previously unrecognized H1N1 subtype containing a gene combination of North American and Eurasian SIV was isolated from humans in the United States and Mexico (3). This apparent swine-origin virus spread rapidly among humans prompting the World Health Organization (WHO) to declare a phase 6 pandemic in June 2009 (2009 pH1N1). Shortly after the first isolation of the virus from humans in the United States and Mexico, virus isolates from pigs in Alberta, Canada, were found to be phylogenetically similar to 2009 pH1N1 (8, 26). Subsequently, more findings of natural and experimental disease in pigs due to 2009 pH1N1 were published (2, 12, 16, 17, 27). Experimental and epidemiological data indicate that the 2009 pH1N1 can transmit between pigs, as well as from pigs to humans and vice versa (2, 8, 17, 25). Therefore, this virus is capable of establishing a stable lineage in swine (2), with the possibility of further reassortment with existing and/or new influenza A viruses of swine. The importance of monitoring the evolution of the 2009 pH1N1 and other SIV can therefore not be overemphasized.

It has been suggested that the 2009 pH1N1 might have circulated in pigs unnoticed for some time (6). SIV isolated from swine in Canada in 2009 were thus characterized to better understand the types of viruses circulating, as well as whether pH1N1 may have preexisted in the Canadian swine population. Furthermore, an evaluation of current SIV isolates from pigs enables monitoring of the evolution of the 2009 pH1N1 virus in pigs, as well as its interaction with previously characterized and/or emerging SIV. Our data indicate that after May 2009, pH1N1 cocirculated in Canadian pigs with contemporary SIV (conSIV) H1N1, seasonal human-like SIV (shSIV) H1N1, and TR H3N2. However, virus isolates collected from other provinces in Canada at about the same time as the first isolation of pH1N1 in Alberta were exclusively conSIV and/or TR H3N2, suggesting that pH1N1 was not present in Canadian pigs prior to May 2009.

MATERIALS AND METHODS

Viruses, virus isolation, and quantification. Viruses in the present study were isolated from pigs in Quebec, Manitoba, Saskatchewan, and Alberta (see Table 1) in 2009. Nasal swabs and/or lung tissues specimens were collected in transport medium and sent to the National Centre for Foreign Animal Disease in Winnipeg for analysis. Initial screening and identification of pandemic versus nonpandemic H1N1 isolates was accomplished by real-time reverse transcription-PCR (RT-PCR) assays that are specific for the 2009 N1 and classic North American swine lineage N1 genes (David Suarez, unpublished assay).

Virus isolation was done by inoculation of Madin-Darby canine kidney cell (MDCK) cultures or the allantoic cavity of 9- to 10-day-old embryonated chicken eggs as previously described (26). Culture supernatants or allantoic fluids were harvested and tested for the presence of SIV by real-time matrix RT-PCR assay and by hemagglutination assay as described below.

For real-time RT-PCR, total RNA was extracted from 0.5 ml of culture supernatants or allantoic fluid by using an RNeasy minikit (Qiagen) and tested as previously described (26).

Cloning, sequencing, and phylogenetic analysis of SIV genes. Full-length HA, NA, and M gene segments for all of the SIV isolates and the remaining five gene segments for representative isolates from different farms were amplified and sequenced. Nucleic acid amplification by a one-step RT-PCR was performed using universal primer sets (7) and the Superscript III One-Step RT-PCR kit (Invitrogen). RT-PCR products were gel purified (in some cases), cloned into pCR4-TOPO vector (Invitrogen), and then used to transform One-Shot TOP10 *E. coli*. Bacterial colonies containing plasmids with the gene of interest were further amplified in liquid broth cultures and plasmid DNA purified with a QIAprep spin miniprep kit (Qiagen). Three clones of each gene were sequenced using the BigDye terminator chemistry and an ABI 3130xl genetic analyzer (Applied Biosystems). Contiguous sequences were generated, and the consensus

sequence of the three clones for each gene segment obtained by using the DNASTAR package by Lasergene.

Sequence alignments were carried out using CLUSTAL W, and the generation of phylogenetic trees was performed by using molecular evolutionary genetics analysis version 4 (MEGA4 [21]). Phylogenetic trees were generated with the close-neighbor joining and 500 bootstrap replicate options of the maximum-parsimony method.

Antigenic characterization. Reference sera from swine infected with representative isolates of the 2008 USA SIV clusters (α, β, γ, and δ) were produced at the National Animal Disease Center, USDA-ARS, Ames, IA, as previously described (13). Briefly, 4-week-old pigs that screened negative for influenza A virus antibodies were immunized with inactivated virus combined with commercial adjuvant by the intramuscular route. Two doses of vaccine were given 2 to 3 weeks apart. Pigs with hemagglutination inhibition (HI) titers of <1:80 after the second dose were given a third dose of vaccine prior to the final blood collection. Hyperimmune 2009 pH1N1 serum was obtained from pigs that were immunized with inactivated 2009 pH1N1 and then at 22 days postimmunization were challenged with live 2009 pH1N1. Serum was then collected at 42 days postimmunization (20 days postchallenge). Sera were treated with a 20% kaolin suspension (Sigma-Aldrich) and hemadsorbed with a 0.5% suspension of turkey red blood cells (TRBC) in order to remove nonspecific hemagglutinins (25). Ferret postinfection antiserum against A/Brisbane/59/07, the 2010 seasonal human influenza H1N1 vaccine strain (1) was kindly provided by Yan Li, National Microbiology Laboratory, Winnipeg, MB, Canada. A hemagglutination assay was performed to determine the HA titer for each virus by adding an equal volume of 0.5% TRBC suspension to serial 2-fold dilutions of virus. The HA titration endpoint was the highest dilution of virus that caused complete hemagglutination of TRBC, the reciprocal of which was considered the HA titer for that virus.

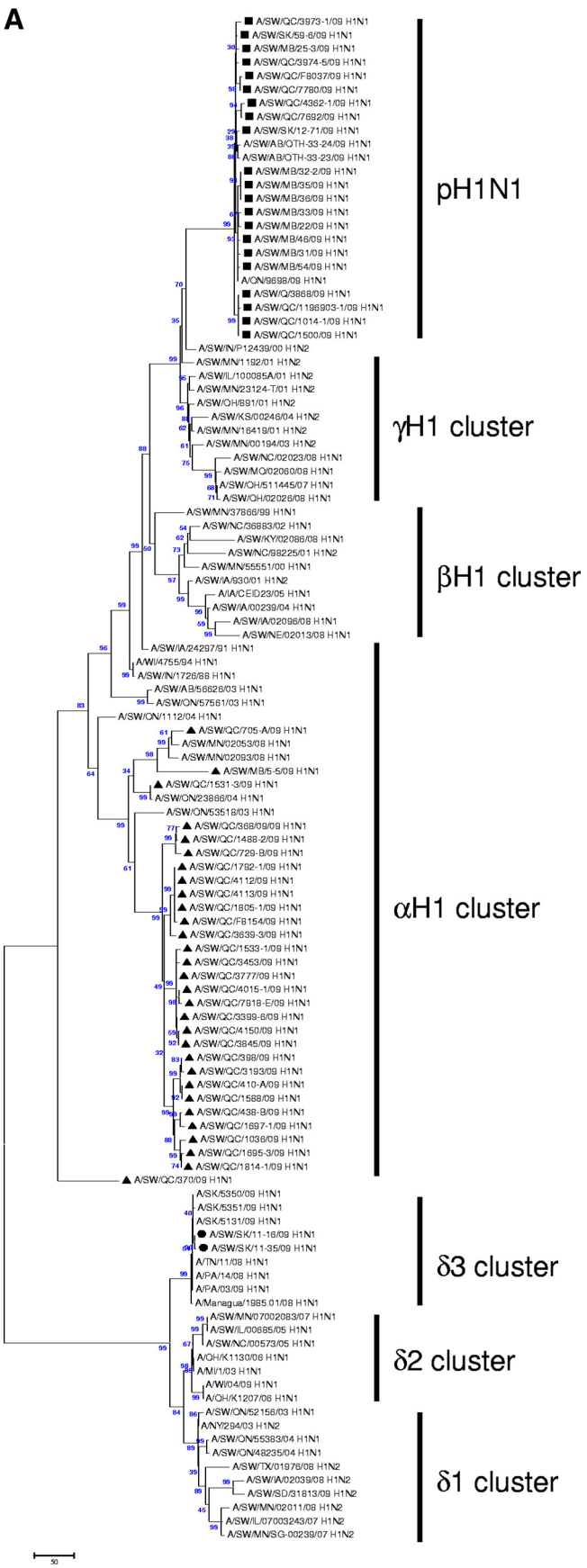
HI assays were then performed by mixing four HA units of virus with serial 2-fold dilutions of each reference serum, followed by the addition of 0.5% TRBC suspension (*WHO Influenza Manual*). The reciprocal of the highest dilution of serum to completely prevent agglutination of TRBC by virus was considered the HI titer for that virus. Antigenic drift and/or relatedness was inferred by comparisons between the HI titer of homologous and test viruses and by antigenic cartography as described below.

Antigenic cartography. Using the HI data generated from the serum-virus combinations above, the quantitative analyses of the antigenic properties of swine influenza A (H1) viruses were performed using antigenic cartography, previously used for human and swine influenza A (H3N2) viruses (4, 20), swine origin A (H1N1) influenza virus in humans (6), and U.S. SIV (13). The result is an antigenic map showing the antigenic relationships among the Canada 2009 SIV, USA 2008 SIV, and other SIVs, including 2009 pH1N1. The distance between points in an antigenic map best represents the antigenic distance among strains as measured by the HI assay. Antigenically similar antigens are closer to each other and vice versa for more distant antigens. Because antisera were tested against multiple antigens and antigens were tested against multiple antisera, many measurements are used to determine the position of the antigen and antiserum points in an antigenic map, thus potentially increasing the accuracy of point placement beyond that of individual HI measurements.

RESULTS

Phylogenetic characterization. The sequence data for the viruses in the current report have been deposited in GenBank under the accession numbers given in Table 1. The HA, NA, and M genes for the bulk of the virus isolates were initially sequenced and analyzed. Subsequently, some of these viruses were selected for full genome sequencing based on the clustering of the HA, NA and M genes on the phylogram.

The HA genes of the nonpandemic H1N1, except A/SW/SK/11-16/09 and A/SW/SK/11-35/09, were all of classical swine H1 origin. With the exception of A/SW/QC/370/09, these HA gene sequences of cSIV origin clustered with the previously described αH1 clade (Fig. 1A). Nevertheless, the HA genes of these viruses showed some genetic drift, differing from their closest relatives in the αH1 cluster by 5 to 6% (data not shown). The HA of A/SW/QC/370/09 was an outlier, showing only 92% nucleotide identity with A/SW/St-Hyacinthe/106/1991 and failing to fall within any of the known H1 clusters.



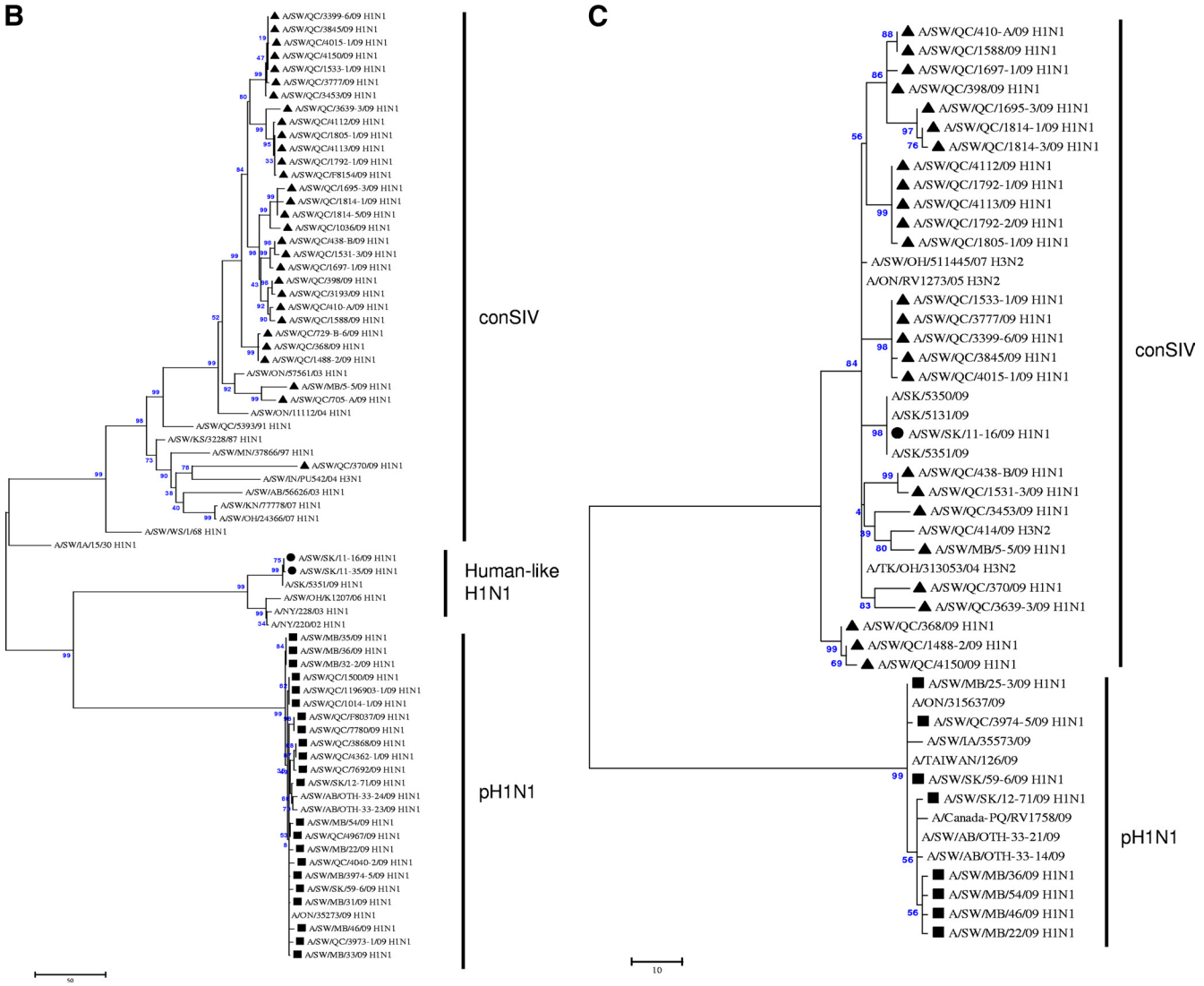


FIG. 1. Evolutionary relationships of the HA (A), NA (B), and M (C) gene segments of H1N1 viruses isolated from Canadian pigs in 2009. Phylogenetic analysis of contemporary (▲), human-like (●), and pandemic (■) 2009 Canadian H1N1 virus HA (A), NA (B), and M (C) gene segments was conducted using MEGA4. The evolutionary history was inferred using the maximum-parsimony method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Representative of viruses isolated from the same location on the same dates (Table 1) are shown. The M gene segment was sequenced for a subset of the pH1N1 isolates only.

Similar to HA, the NA gene sequences of all of these viruses indicated a cSIV N1 origin. The classical N1 clustered with previously characterized cSIV H1N1 and TR H1N1 (Fig. 1B).

Viruses (A/SW/SK/11-16/09 and A/SW/SK/11-35/09) isolated from a Saskatchewan pig farm in which there was a history of human respiratory illness were shown to be reassortants between triple-reassortant SIV and human seasonal influenza virus and formed a subcluster δ3, probably representing the third documented introduction of human-lineage HA into the North American swine influenza gene pool (Fig. 1A). Other subclusters (δ1 and δ2) of δH1 cluster have previously been described in Canada (10) and the United States (13). The seasonal human-like SIV (shSIV) H1N1 HA genes closely matched (99%) that of A/SK/5350/2009 and A/SK/5351/2009 isolated from Saskatchewan swine workers in June 2009 (1) a month prior to the isolation from pigs in July 2009. Likewise, the NA gene sequences of these isolates had as top matches A/SK/5351/2009 and A/Managua/1409.01/2008 (seasonal human H1N1) (Fig. 1B) and encoded the H275Y amino acid substitution implicated in oseltamivir resistance.

The HA, NA, and M genes of SIV isolates from the same farms were 98 to 100% identical and, for the most part, clustered together on phylogenetic trees (Fig. 1). Therefore, PB2, PB1, PA, NP, and NS genes of representative isolates from each farm and/or cluster were sequenced to complete the full genome (Table 2). Based on the top matches for each gene sequence, these H1N1 isolates, including shSIV H1N1, contained the North American SIV TRIG cassette. Therefore,

TABLE 2. Top matches and genetic lineage for gene sequences of 2009 contemporary H1N1 swine influenza viruses in Canada[a]

Isolate	Top matching sequence (genetic ancestry)			
	PB2	PB1	PA	HA
A/SW/QC/370/09 H1N1	A/TK/ON/31232/05 H3N2 (avian)	A/TK/MN/366767/05 H3N2 (human)	A/SW/ON/33853/05 H3N2 (avian)	A/SW/St-Hyacinthe/106/91 H1N1(cSIV)
A/SW/QC/1488-2/09 H1N1	A/TK/ON/31232/05 H3N2 (avian)	A/TK/OH/313053/04 H3N2 (human)	A/Mink/NS/1055488/07 H3N2 (avian)	A/SW/ON/23866/04 H1N1 (cSIV)
A/SW/QC/1531-3/09 H1N1	A/TK/ON/31232/05 H3N2 (avian)	A/TK/OH/313053/04 H3N2 (human)	A/Mink/NS/1055488/07 H3N2 (avian)	A/SW/ON/23866/04 H1N1 (cSIV)
A/SW/QC/1697-1/09 H1N1	A/SW/ON/33853/05 H3N2 (avian)	A/TK/OH/313053/04 H3N2 (human)	A/Mink/NS/1055488/07 H3N2 (avian)	A/SW/ON/23866/04 H1N1 (cSIV)
A/SW/QC/398/09 H1N1	A/TK/ON/31232/05 H3N2 (avian)	A/SW/MN/66853/06 H3N2 (human)	A/Mink/NS/1055488/07 H3N2 (avian)	A/SW/ON/23866/04 H1N1 (cSIV)
A/SW/QC/3399-6/09 H1N1	A/TK/ON/31232/05 H3N2 (avian)	A/TK/OH/313053/04 H3N2 (human)	A/SW/QC/4001/05 H3N2 (avian)	A/SW/ON/23866/04 H1N1 (cSIV)
A/SW/QC/1695-3/09 H1N1	A/TK/ON/31232/05 H3N2 (avian)	A/SW/ON/33853/05 H3N2 (human)	A/SW/QC/4001/05 H3N2 (avian)	A/SW/ON/23866/04 H1N1 (cSIV)
A/SW/MB/05-5/09 H1N1	A/TK/OH/313053/04 H3N2 (Avian)	A/SW/MN/66853/06 H3N2 (human)	A/TK/OH/313053/04 H3N2 (avian)	A/SW/MN/02093/08 H1N1 (cSIV)
A/SW/SK/11-16/09 H1N1	A/SK/5350/09 H1N1 (avian)	A/SK/5351/09 H1N1 (human)	A/SK/5351/09 H1N1 (avian)	A/SK/5351/09 H1N1 (human)

[a] Each SIV gene was sequenced from cloned RT-PCR products and top matching sequences obtained from the NCBI nucleotide database using the basic local alignment search tool (BLAST).

these viruses probably derived from reassortments between TRIG-bearing SIV and either classical swine or seasonal human influenza A viruses. In addition, shSIV H1N1 A/SW/SK/11-16/09 possessed an NS gene that closely matched that of the 2009 pH1N1 (Table 2) and clustered together with 2009 pH1N1 NS1 on the phylogram (Fig. 2A). Since the nonpandemic SIV possessing cSIV HA and NA were possibly all TRIG cassette-bearing reassortant viruses, we hereby refer to them as contemporary SIV (conSIV) H1N1 to distinguish these from the cSIV.

Based on the HA, NA, and M genes, the 2009 pH1N1 viruses isolated from Canadian pigs in 2009 were clearly distinct from the conSIV H1N1 isolates (Fig. 1). These genes were 99 to 100% identical for all swine pH1N1 isolates. The HA, NA, and M gene sequences of pH1N1 clustered with previously described pH1N1 isolates from swine and humans (Fig. 1). Phylogenetically, the γH1 SIV cluster appeared to be the closest ancestral link to the 2009 pH1N1 HA gene (Fig. 1A). Full-genome sequencing of selected isolates confirmed a 2009 pH1N1gene constellation composed of NA and M of Eurasian SIV lineage, HA of North American cSIV lineage, and the rest of the internal genes derived from the North American SIV TRIG cassette (data not shown). However, the NP gene of A/SW/SK/12-71/09 (pH1N1) clustered phylogenetically with the NP gene of conSIV H1N1 (Fig. 2B). This suggests that A/SW/SK/12-71/09 might have acquired the NP

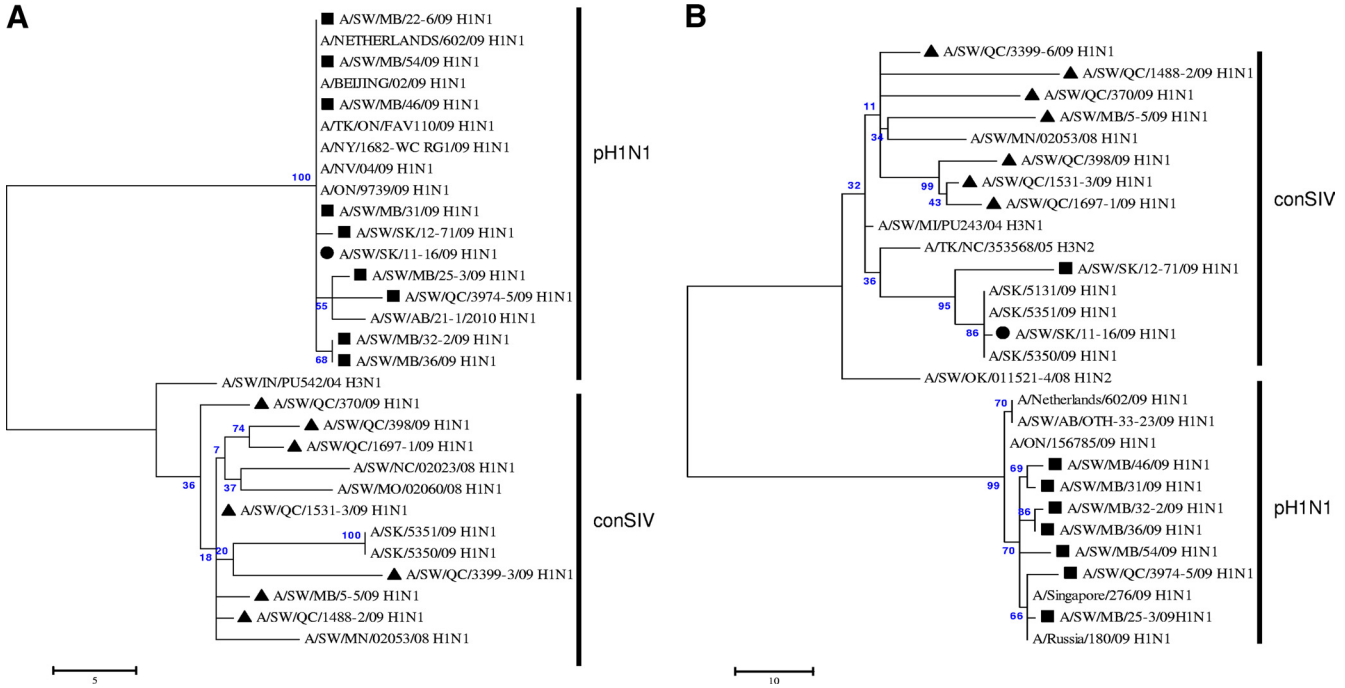


FIG. 2. Evolutionary relationships of the NS (A) and NP (B) gene segments of selected H1N1 viruses isolated from Canadian pigs in 2009. Phylogenetic analysis of contemporary (▲), human-like (●), and pandemic (■) 2009 Canadian H1N1 virus-NS1 (A) and NP (B) gene segments was conducted in MEGA4. The evolutionary history was inferred using the maximum-parsimony method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. A/SW/SK/11-16/09 (seasonal human-like H1N1) NS1 gene segment clusters with pandemic H1N1 NS1 (A) and A/SW/SK/12-71/09 (pH1N1) NP gene segment clusters with contemporary SIV H1N1 NP (B), suggesting the occurrence of gene reassortment events. Only selected viruses were sequenced for these gene segments.

TABLE 2—*Continued*

Top matching sequence (genetic ancestry)			
NP	NA	M	NS
A/SW/MI/PU243/04 H3N1 (cSIV)	A/SW/IN/1726/88 H1N1(cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/OK/008722/07 H3N2 (cSIV)	A/SW/ON/57561/03 H1N1(cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/OK/008722/07 H3N2 (cSIV)	A/SW/ON/57561/03 H1N1(cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/OK/008722/07 H3N2 (cSIV)	A/SW/ON/57561/03 H1N1(cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/MI/PU243/04 H3N1 (cSIV)	A/SW/ON/57561/03 H1N1(cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/MI/PU243/04 H3N1 (cSIV	A/SW/Korea/CAS08/05 H1N1 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/AB/14722/05 H3N2 (cSIV)	A/SW/ON/57561/03 H1N1(cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SW/MI/PU243/04 H3N1 (cSIV)	A/SW/MN/02053/08 H1N1 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)	A/TK/OH/313053/04 H3N2 (cSIV)
A/SK/5351/09 H1N1 (cSIV)	A/SK/5351/09 H1N1 (human)	A/TK/OH/313053/04 (cSIV)	A/Netherland/602/09 pH1N1 (cSIV)

gene of a TRIG cassette-bearing nonpandemic reassortant SIV, possibly due to coinfection and reassortment in pigs.

Using a human 2009 pH1N1 isolate from Mexico (A/Mexico/InDRE/4487/2009) as a reference, a number of amino acid substitutions were previously identified in the HA, NA, and M genes of isolates from the first 2009 pH1N1-infected pig farm in Canada (26). The amino acid sequences for the viruses described in the current study were also compared to those of the Mexican pH1N1 and previous Canadian swine isolates. A small number of apparently random amino acid substitutions was observed in the HA and NA proteins (Tables 3 and 4). HA mutations identified in many of the viruses included S203T and Q293H in HA1 and K402T in HA2 (Table 3). Similarly, V106I and N248D NA mutations were present in most isolates (Table 4). None of the HA and NA AA substitutions seen in the previous 2009 pH1N1 isolates from swine in Canada (26) were present in the viruses in the present study. However, as with other 2009 pH1N1 viruses, the M2 gene of the pH1N1 from swine in Canada encoded the adamantine resistance marker (data not shown).

Antigenic characterization. Classical or conventional SIV in the United States has been well characterized over the past decade (23–25). On the other hand, there is a paucity of data on these viruses in Canada. Therefore, to evaluate the antigenic evolution of 2009 conSIV Canada, we used reference antisera specific for the α, β, γ, and δ clusters of USA swine H1 subtype viruses from 2008 (23). Most of the 2009 conSIV Canada isolates tested by HI assay cross-reacted with all but one of the αH1 and γH1 reference antisera (Table 5). There was minimal or no cross-reactivity with the βH1 and δH1 clusters of SIV 2008 USA. The human-like H1N1 Canada isolates (A/SW/SK/11-16/09 and A/SW/SK/11-35/09) cross-

TABLE 3. Amino acid sequence alignment of the HA1 and HA2 protein of 2009 pandemic H1N1 SIV from pigs in Canada

Isolate	Amino acid alignment[a]																						
	HA1															HA2							
	32	44	97	127	170	194	203	216	222	223	234	270	283	293	298	374	385	402	460	477	520	529	547
A/Mexico/InDRE4487/09	L	L	D	D	G	N	S	I	D	Q	V	T	K	Q	I	E	K	K	I	V	V	S	I
A/SW/AB/OTH-33-1/09																							
A/SW/AB/OTH-33-2/09																							
A/SW/AB/OTH-33-3/09					E				V														
A/SW/AB/OTH-33-23/09				E													E						
A/SW/AB/OTH-33-24/09																							
A/SW/QC/4362-1/09	I									R									V		A		
A/SW/QC/3973-1/09					R		T																
A/SW/QC/3868/09		I	N			S	T									K							
A/SW/QC/1014-1/09		I	N			S	T									K							
A/SW/QCF8037/09							T			R	I		N										
A/SW/QC/7692/09	I																		V		A		
A/SW/QC/7780/09							T				I		N									P	
A/SW/QC/3974-5/09							T	V	E														V
A/SW/QC/1500/09		I	N			S	T									K							
A/SW/QC/1196903-1/09		I	N	E		S	T									K							
A/SW/SK/59-6/09							T																
A/SW/SK/12-71/09																							
A/SW/MB/46/09														H				T					
A/SW/MB/25-3/09							T								T								
A/SW/MB/31/09														H				T					
A/SW/MB/32-2/09												A		H				T					
A/SW/MB/33/09										R				H				T		I			
A/SW/MB/35/09												A		H				T					
A/SW/MB/36/09												A		H			R	T					
A/SW/MB/22/09														H				T					
A/SW/MB/54/09														H				T					

[a] Amino acid sequences of the 2009 pandemic H1N1 isolates from pigs in Canada were compared to the human isolate A/Mexico/InDREA/4487/09. Substitutions relative to the human isolate are shown. Empty cells mean there were no substitutions.

TABLE 4. Amino acid sequence alignment of the NA protein of 2009 pandemic H1N1 SIV from pigs in Canada

Isolate	Amino acid alignment[a]											
	13	16	71	95	106	166	168	248	257	309	394	415
A/Mexico/InDRE4487/09	V	T	N	S	V	V	S	N	R	L	V	L
A/SW/AB/OTH-33-1/09												
A/SW/AB/OTH-33-2/09				G								
A/SW/AB/OTH-33-3/09				G								
A/SW/AB/OTH-33-23/09				G								
A/SW/AB/OTH-33-24/09				G								
A/SW/QC/3973-1/09					I			D				
A/SW/QC/3868/09								D			I	
A/SW/QC/1014-1/09					I			D	K			
A/SW/QC/F8037/09					I	I		D				
A/SW/QC/7692/09								D				
A/SW/QC/7780/09					I	I		D				
A/SW/QC/1500/09					I			D	K			
A/SW/QC/3974-5/09					I			D				
A/SW/QC/4040-2/09	A		S		I			D				
A/SW/QC/4362-1/09								D			I	
A/SW/QC/4967/09					I			D			I	
A/SW/MB/46/09					I		T	D				
A/SW/MB/31/09					I			D				
A/SW/MB/32-2/09					I			D				
A/SW/MB/33/09		A			I			D				
A/SW/MB/35/09					I			D				
A/SW/MB/36/09					I			D				
A/SW/MB/22/09	I				I			D				
A/SW/MB/54/09					I			D			I	M
A/SW/SK/59-6/09					I			D		D		
A/SW/SK/12-71/09												

[a] Amino acid sequences of the 2009 pandemic H1N1 isolates from pigs in Canada were compared with the human isolate A/Mexico/InDREA/4487/09. Substitutions relative to the human isolate are shown. Empty cells mean there were no substitutions. Note that all of the viruses listed in the table code for a histidine at position 275, which is associated with oseltamivir susceptibility.

reacted with none of the SIV 2008 USA reference antisera (Table 5), including the δH1 cluster to which they are phylogenetically related (Fig. 1A). However, both viruses cross-reacted with ferret antiserum against the 2010 seasonal human H1N1 vaccine strain A/Brisbane/59/07 with HI titers of 640, further supporting that A/SW/SK/11-16/09 and A/SW/SK/11-35/09 resulted from a recent reassortment between influenza A viruses of human and swine origin.

We also evaluated the serologic cross-reactivity between Canadian swine 2009 pH1N1 isolates and the USA 2008 reference antiserum panel. Cross-reactivity of 2009 pH1N1 was mainly observed within the γH1 cluster of SIV 2008 USA, involving all seven 2009 pH1N1 Canada swine isolates and four of six antisera used (Table 5). The HI cross-reactivity was confirmed by virus neutralization using a representative 2009 pH1N1 strain from swine. Complete neutralization of virus infectivity by antisera to the γH1 cluster was observed up to a dilution of 1/80, in contrast to an absence of any noticeable effect by antibodies to the other clusters of SIV 2008 USA (data not shown).

On the other hand, when we assayed for cross-reactivity between Canadian 2009 conSIV H1N1 isolates and swine 2009 pH1N1, pH1N1 antiserum reacted consistently and for the most part, homogenously with all 2009 pH1N1 viruses and also cross-reacted significantly with Canadian conSIV H1N1 viruses (Table 6). The human-like H1N1 (A/SW/SK/11-16/09 and A/SW/SK/11-35/09) viruses did not react with pH1N1 antiserum.

Antigenic cartography. In an antigenic map, HI measurements are computed so that the distances between the influenza strains (colored dots) represent antigenic relatedness (20). Therefore, strains with less distance between them are more antigenically similar. In the antigenic map (Fig. 3), most of the 2009 conSIV Canada isolates are represented by the large cyan-colored dots clustered together, suggesting close antigenic relatedness between these viruses. Furthermore, these 2009 conSIV Canada isolates were antigenically closely related to representative strains of the αH1 (small cyan dots) and γH1 (pink dots) but widely distant from the δH1 (gold dots) clusters of 2008 USA SIV.

Similarly, most of the 2009 pH1N1 Canadian SIV (large red dots) isolates clustered together and were also antigenically closely related to pH1N1 isolates from U.S. swine and humans (small red dots). The 2009 pH1N1 Canada isolates were also antigenically most closely related to 2009 conSIV Canada (αH1 cluster, cyan dots) and γH1 clusters of SIV 2008 USA.

DISCUSSION

Earlier reports have dealt with the characterization of classical SIV H1N1 isolates from Quebec (19) and Ontario (10), Canada, although fewer than 10 viruses were examined in each of these. In the present study more than 70 SIV H1N1 viruses isolated from five Canadian provinces in 2009 were characterized and demonstrated the cocirculation of conSIV H1N1, pH1N1, and shSIV H1N1. In addition to the H1N1 viruses described here, triple-reassortant H3N2 viruses were also isolated from Canadian pigs in 2009 (15).

The fact that the conSIV H1N1 viruses isolated in Canada in 2009 all cluster within the αH1 clade infers that the H1 gene

TABLE 5. Antigenic characterization of 2009 H1N1 swine influenza viruses in Canada by hemagglutination inhibition assay using reference antiserum for 2008 SIV USA influenza A isolates representing different H1 clusters

Isolate[a]	HI titer[b]																							
	αH1 cluster				βH1 cluster								γH1 cluster						δH1 cluster					
	MN/02053		MN/02093		IA/02096		KY/02086		NE/02013		NC/02084		NC/02023		OH/02026		MO/02060		TX/01976		IA/02039		MN/02011	
SW/AB/OTH33-2 (p)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	40	80	40	<20	<20	<20	<20	<20	<20
SW/AB/OTH33-3 (p)	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	40	40	0	80	160	160	<20	<20	<20	<20	20	20
TK/ON/FAV110/04 (p)	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	<20	<20	<20	20	0	20	20	20	<20	<20	<20	<20	<20	<20
TK/ON/FAV114/04 (p)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	40	40	40	<20	<20	<20	<20	<20	<20
SW/MB/35-1 (p)	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	40	<20	80	160	80	<20	<20	<20	<20	<20	<20
SW/QC/4362-2 (p)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	20	40	<20	40	80	80	<20	<20	<20	<20	<20	<20
SW/QC/3974-5 (p)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	40	80	80	<20	<20	<20	<20	<20	<20
SW/QC/3868 (p)	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	20	20	40	<20	80	80	80	<20	<20	<20	20	<20	20
SW/QC/1500-1 (p)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	20	40	40	<20	<20	<20	<20	<20	<20
SW/QC/1196903 (p)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	40	<20	80	80	80	<20	<20	<20	<20	<20	<20
Homologous HI titer	320	320	160	320	80	320	80	640	640	160	160	80	320	320	80	1,280	1,280	2,560	40	40	320	40	2,560	2,560
SW/QC/1531-3 (con)	160	160	80	160	20	20	<20	20	<20	<20	<20	20	40	160	<20	640	1,280	640	<20	<20	<20	<20	<20	<20
SW/QC/368 (con)	160	320	80	160	20	20	20	<20	40	<20	40	20	40	160	20	320	640	640	<20	<20	<20	<20	<20	<20
SW/QC/1805-5 (con)	80	160	80	160	<20	20	<20	<20	<20	<20	20	20	40	40	<20	320	640	640	<20	<20	<20	<20	<20	<20
SW/QC/1792-2 (con)	40	80	40	80	<20	20	<20	<20	<20	<20	<20	<20	20	40	<20	320	320	320	<20	<20	<20	<20	<20	<20
SW/QC/705-A (con)	<20	20	20	20	<20	20	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	80	40	<20	<20	<20	<20	<20	<20
SW/QC/705-B (con)	<20	<20	20	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	80	20	<20	<20	<20	<20	<20	<20
SW/QC/370 (con)	20	40	40	40	<20	20	20	20	40	<20	<20	20	20	20	<20	80	160	80	<20	<20	<20	<20	<20	<20
SW/QC/1805-3 (con)	40	160	40	80	20	20	<20	20	20	<20	40	40	40	40	<20	320	640	320	<20	<20	<20	<20	<20	<20
SW/QC/1805-4 (con)	40	80	40	80	<20	20	<20	<20	<20	<20	20	20	20	40	<20	160	320	160	<20	<20	<20	<20	<20	<20
SW/QC/1805-2 (con)	40	160	40	80	20	40	20	20	<20	<20	40	40	40	80	<20	320	1280	640	<20	<20	<20	<20	<20	<20
SW/QC/4015-1 (con)	20	80	40	80	<20	<20	<20	<20	<20	<20	<20	<20	40	80	<20	160	640	640	<20	<20	<20	<20	<20	<20
SW/QC/3399-6 (con)	20	80	40	80	<20	<20	<20	<20	<20	<20	<20	<20	20	40	<20	160	320	320	<20	<20	<20	<20	<20	<20
SW/QC/1697-3 (con)	40	80	160	80	20	20	<20	<20	<20	<20	<20	<20	20	40	<20	640	640	640	<20	<20	<20	<20	<20	<20
SW/QC/1792-1 (con)	20	80	20	40	<20	20	<20	<20	<20	<20	20	20	20	40	<20	160	320	320	<20	<20	<20	<20	<20	<20
SW/MB/5-5 (con)	20	80	80	80	20	40	40	40	20	<20	40	40	40	40	<20	80	160	80	<20	<20	<20	<20	<20	20
SW/SK/11-16 (sh)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
SW/SK/11-35 (sh)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	20	<20	<20	<20

[a] p, 2009 pandemic H1N1 isolates from swine, turkeys, and humans; con, 2009 contemporary SIV H1N1 isolates from swine; sh, 2009 seasonal human-like SIV H1N1 from swine.

[b] HI titers for each virus were obtained as the reciprocal of the highest dilution of anti-reference virus antibody that completely inhibited agglutination of 0.5% turkey red blood cells by that virus. Titers under each reference antiserum are the results from two separate determinations.

TABLE 6. Antigenic characterization of 2009 H1N1 swine influenza viruses in Canada by hemagglutination inhibition assay using 2009 pandemic H1N1 hyperimmune antiserum

Isolate	Strain[a]	HI titer[b]			
		Serum 1	Serum 2	Serum 3	Serum 4
A/SW/AB/OTH-33-2/09	pH1N1	1,280	2,560	2,560	2,560
A/Mexico/InDREA4487/09	pH1N1	1,280	2,560	2,560	1,280
A/SW/AB/OTH-33-3/09	pH1N1	1,280	2,560	2,560	2,560
A/TK/ON/FAV-110/04/09	pH1N1	640	2,560	2,560	2,560
A/TK/ON/FAV-114/04/09	pH1N1	2,560	2,560	2,560	2,560
A/SW/MB/35-1/09	pH1N1	320	2,560	1,280	1,280
A/SW/QC/4362-2/09	pH1N1	640	2,560	2,560	1,280
A/SW/QC/3974-5/09	pH1N1	1,280	2,560	2,560	2,560
A/SW/QC/3868/09	pH1N1	640	2,560	2,560	2,560
A/SW/QC/1500-1/09	pH1N1	320	2,560	1,280	1,280
A/SW/QC/1196903-1/09	pH1N1	1,280	2,560	2,560	2,560
Homologous HI titer	pH1N1	1,280	1,280	1,280	2,560
A/SW/QC/1531-3/09	conSIV H1N1	160	160	160	320
A/SW/QC/368/09	conSIV H1N1	160	320	160	320
A/SW/QC/1805-5/09	conSIV H1N1	80	160	160	320
A/SW/QC/1792-2/09	conSIV H1N1	160	160	160	320
A/SW/QC/705-A/09	conSIV H1N1	320	640	640	640
A/SW/QC/705-B/09	conSIV H1N1	160	640	320	320
A/SW/QC/370/09	conSIV H1N1	80	320	320	640
A/SW/QC/1805-3/09	conSIV H1N1	160	320	320	320
A/SW/QC/1805-4/09	conSIV H1N1	320	640	320	640
A/SW/QC/1805-2/09	conSIV H1N1	160	320	320	320
A/SW/QC/4015-1/09	conSIV H1N1	160	320	160	160
A/SW/QC/3399-6/09	conSIV H1N1	80	160	80	160
A/SW/QC/1697-3/09	conSIV H1N1	160	320	320	640
A/SW/QC/1792-1/09	conSIV H1N1	160	320	320	320
A/SW/MB/5-5/09	conSIV H1N1	80	640	320	640
A/SW/SK/11-16/09	shSIV H1N1	<20	<20	<20	<20
A/SW/SK/11-35/09	shSIV H1N1	<20	<20	<20	<20

[a] pH1N1, pandemic H1N1 isolates from swine, turkeys, and humans; conSIV H1N1, contemporary SIV H1N1 isolates from swine; shSIV H1N1, seasonal human-like SIV H1N1 from swine.

[b] HI titers for each virus were obtained as the reciprocal of the highest dilution of anti-reference virus antibody that completely inhibited agglutination of 0.5% turkey red blood cells by that virus.

originated from the North American cSIV lineage and that this lineage must of had a relatively high prevalence compared to other North American swine H1 clusters. Similarly, the NA gene of the conSIV H1N1 viruses was also found to be of cSIV origin. However, the TRIG cassette constitutes the remaining gene segments, demonstrating that these viruses are all triple-reassortant SIV. In reality, most swine viruses characterized in the 21st century in the United States are known to possess the TRIG cassette (13, 23–25). This, taken together with our current data, suggests that the North American cSIV, which remained genetically stable for over 7 decades since 1930, has been replaced by triple-reassortant SIV H1N1. The foregoing implies that TRIG cassette-bearing SIV strains have a selective advantage over their counterparts that lack this cassette. As recently as 2003 and 2004, cSIV isolated from Ontario pigs lacked the TRIG cassette but had a PB1 of human influenza A origin (10). Although these viruses were thought to have spread widely in Ontario pigs, it is not known whether they persisted within the Canadian swine population.

Following the emergence of the triple-reassortant H3N2 in the late 1990s, influenza A viruses bearing human-like HA and NA with a TRIG backbone have been isolated from humans and swine in the United States and Canada (1, 24). These viruses resulted from reassortments between seasonal human H1N1 and swine TRIG-containing viruses, which was the case with the human-like H1N1 viruses described in this report. However, the HAs of the viruses in the present study form a cluster distinct from those of 2004 and other similar viruses in the United States, suggesting independent and recent transmission and reassortant events. In contrast, the 2004 human-like swine isolates from Ontario contained cSIV internal genes instead of the TRIG cassette, with the exception of PB1, which was of human influenza A origin. The shSIV H1N1 viruses described here were highly similar to A/SK/5351/2009 and, based on the fact that isolation from humans preceded the isolation from pigs by a month, it is likely that this virus was transmitted from humans to pigs (1). Continued surveillance will be required to know whether this most recent shSIV, representing the δ3 HA phylogenetic cluster, will cocirculate with conSIV. This example highlights the possibility of shSIV and human seasonal H1 viruses recycling between pigs and humans. Because the HA and NA are of seasonal human influenza A origin, the human population is likely to have immunity to these viruses as indicated by the mild disease in the case reported by Bastien et al. (1). However, human seasonal H1N1 appears to have been replaced by pH1N1, and immunity to the former in the human population may wane over the coming years, whereas shSIV could continue to evolve in the swine population. The fact that the NS gene segment of the shSIV A/SW/SK/11-16/09 closely matched 2009 pH1N1 NS suggests that reassortment had oc-

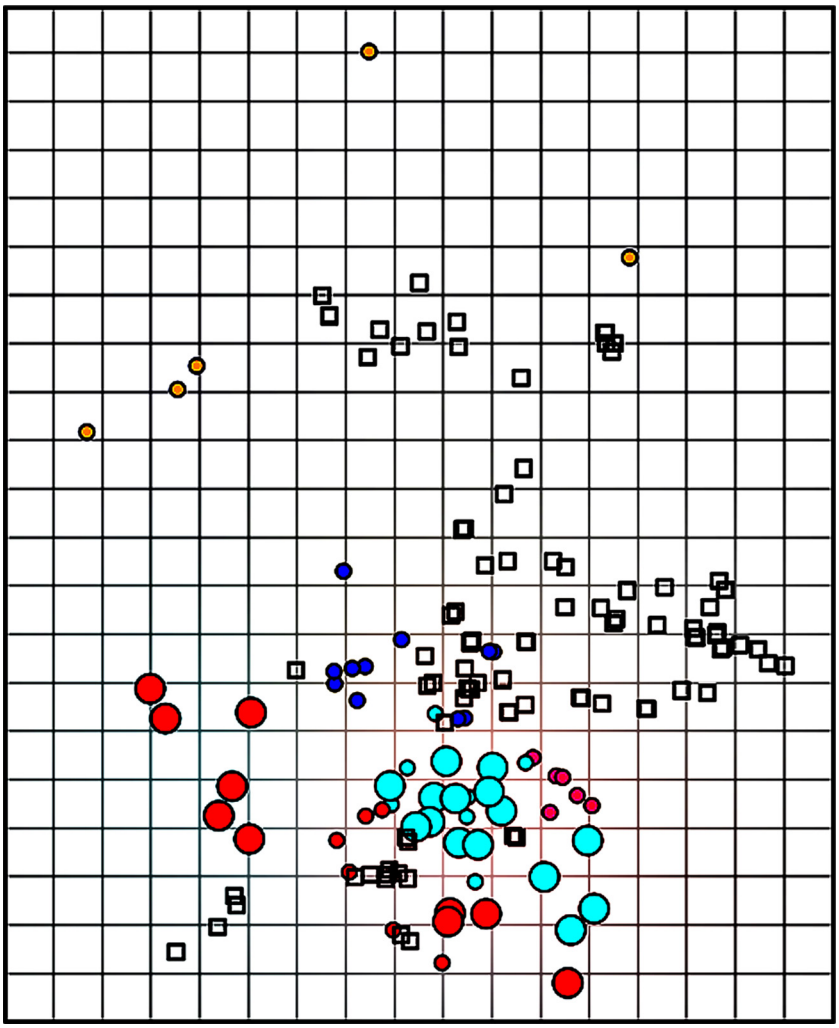


FIG. 3. Antigenic map of swine influenza A and 2009 pandemic H1 viruses: the relative positions of strains (colored dots) and swine hyperimmune antisera (squares) were computed such that the distances between strains and antisera in the map with the least possible error represent the corresponding HI measurements (20). Small dots represent strains αH1 (cyan), γH1 (pink), βH1 (blue), and δH1 (gold) clusters USA and pandemic H1N1 (red). The large dots represent contemporary H1N1 (cyan) and pandemic H1N1 (red) 2009 SIV Canada, including two pandemic H1N1 isolates from turkeys (A/TK/ON/FAV-110/04/09 and A/TK/ON/FAV-114/04/09). Each grid square represents one unit of antigenic distance, corresponding to a 2-fold difference in titer in the HI assay.

curred between these two viral lineages and highlights the probability for further reassortment between different influenza A viruses of swine and pH1N1.

Influenza A is disease shared by pigs and humans with documented interspecies transmission events in both directions. Previous human cases with swine lineage viruses have resulted in occasional hospitalizations and/or deaths and limited human-to-human transmission. In contrast, infection with 2009 pH1N1 became the first outbreak of likely swine-origin influenza to infect and spread efficiently from humans to humans. Not surprisingly, being of likely swine origin, this virus was isolated from pigs in Canada and Argentina shortly after the virus emerged in Mexico and the United States (8, 17). Subsequent experimental challenge studies (2, 12, 26) supported the field data that pigs could be readily infected by pig-to-pig transmission and that possibly pig-to-human transmission (8) was also occurring. Our data show that the 2009 pH1N1 spread in the Canadian swine population in 2009 and probably continued to do so in 2010 (5). The relatively wide distribution of this virus in swine suggests that 2009 pH1N1 might establish itself in the North American swine population, cocirculating with conSIV H1N1, shSIV H1N1, and TR H3N2, increasing the chances of further reassortants being generated, as we observed with the pH1N1 A/SW/SK/12-71/09, which possessed an NP gene segment of conSIV origin.

Nevertheless, it was only after the first isolation of pH1N1 from pigs in Alberta that this virus was subsequently isolated from pigs elsewhere in Canada (5, 16). Indeed, SIV isolated from other Canadian provinces prior to and at about the same time as the first pH1N1 isolates in Alberta were found to be conSIV H1N1 and/or TR H3N2 (15), suggesting that there was no pH1N1 in Canadian pigs prior to May 2009.

Antigenic drift of HA and NA genes can lead to the emergence of viruses capable of escaping existing herd immunity,

leading to seasonal influenza epidemics. Determination of antibody cross-reactivity between viruses can thus reveal the degree of antigenic evolution and/or relatedness over time. The Canadian isolates were more antigenically related to αH1 and γH1 SIV 2008 USA clusters based on HI cross-reactivity and their convergence by antigenic cartography with previously characterized αH1 and γH1 SIV in the United States (13).

The complete lack of HI cross-reactivity between the Canadian 2009 shSIV H1N1 and the α, β, and γ H1 clusters and the pH1N1 observed here is expected given that the HA of shSIV H1N1 differs antigenically and genetically from conSIV and pH1N1 HA. However, there was also a complete lack of cross-reactivity of these human-like H1N1 viruses with δH1 cluster of SIV 2008 USA, which is comprised of other viruses with shSIV HA. This supports the idea that these Canadian isolates represent a recent independent reassortment event involving seasonal human H1N1 influenza and SIV. This was substantiated by the demonstration of significant cross-reactivity between this shSIV H1N1, as well as the related isolates from humans and reference antiserum to the 2010 seasonal influenza vaccine strain (1), and further supports its designation as a distinct subcluster (δ3).

Our observation of serologic cross-reactivity between Canada 2009 pH1N1 and γH1 cluster of conSIV 2008 USA is consistent with our phylogenetic data and previous reports (22). This suggests the possibility of limited protection against 2009 pH1N1 in pigs previously exposed to some strains of conSIV H1N1. Indeed, vaccination studies confirmed partial protection against 2009 pH1N1 by multivalent commercial vaccines containing γH1 (22). However, full protection against 2009 pH1N1was only achieved using an autogenous vaccine, thus representing a better option for protection of naive pigs against this virus (22).

Our data also indicate that at the time the 2009 pH1N1 viruses were antigenically uniform, and thus previously infected pigs are likely to be immune to re-exposure to pH1N1 strains. This antigenic uniformity is consistent with genetic stability of the HA gene, as noted here and in other reports (6). This is significant in that immunized pigs might be protected from acquiring this virus from humans and vice versa. The role of the developing population immunity in humans and swine on pressure for the virus to change remains to be seen.

On the other hand, pH1N1 is likely cocirculating with other SIV subtypes in the Canadian swine population. It is likely that some naive pigs might first encounter pH1N1 before any other SIV. A relevant question concerns the chances that such pigs would be protected against subsequent exposure to the conSIV H1N1. The consistent cross-reactivity between the Canadian conSIV H1N1 viruses and pH1N1 hyperimmune serum raises the possibility that pigs previously exposed to pH1N1 could have partial protection against some of the currently circulating conSIV H1N1 in Canada. Unexpectedly, cross-reactive HI titers were previously observed between αH1 SIV and sera from pigs vaccinated with 2009 pH1N1 (22). However, this is in agreement with our data, given that the Canada 2009 conSIV H1N1 in this report belonged to αH1 cluster.

The 2009 pandemic has emphasized the importance of swine as a potential source for influenza A viruses with human pandemic potential. The apparent cocirculation of conSIV H1N1 and pH1N1 and the sporadic emergence of shSIV H1N1 in the Canadian pig population further highlight the potential for reassortant viruses emerging from the human-pig interface. Furthermore, pigs have also been shown to be at least transiently infected with influenza A viruses of nonmammalian subtypes from avian reservoirs (9, 11). Since the currently characterized SIV isolates bear the TRIG cassette, which appears to accept HA and NA from other influenza A viruses, it is highly likely that new reassortant viruses will emerge from swine. Therefore, a comprehensive surveillance of influenza A viruses circulating in swine should be given serious consideration in order that any new strains with potential public and animal health importance be identified quickly.

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