



## ABSTRACT

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3 In 2007, a H3N2 influenza A virus was isolated from Canadian mink. It was found to be  
4 phylogenetically related to a triple reassortant influenza virus which emerged in Canadian  
5 swine in 2005, but it is antigenically distinct. The transmission of the virus from swine to  
6 mink seems to have occurred following the feeding of animals with a ration composed of  
7 uncooked meat bi-products of swine obtained from slaughterhouse facilities. Serological  
8 analyses suggest that the mink influenza virus does not circulate in the swine population.  
9 Presently, the prevalence of influenza virus in Canadian farmed and wild mink  
10 populations is unknown. The natural occurrence of influenza virus infection in mink with  
11 the presence of clinical signs is a rare event that deserves to be reported.

12

1

2           In 1998, a H3N2 triple reassortant influenza virus emerged in US swine  
3 population (10, 19, 21). The origins of its 8 RNA viral genes were identified to be 1) of  
4 human virus lineage for the hemagglutinin (HA), neuraminidase (NA), and RNA  
5 polymerase (PB1) genes; 2) of classical swine virus lineage for the nucleoprotein (NP),  
6 matrix (M), and nonstructural (NS) genes; and 3) of North American avian virus lineage  
7 for RNA polymerase (PA and PB2) genes. Unfortunately, the H3N2 triple reassortant  
8 subtype of influenza virus emerged in Canadian swine and turkey population in 2005 and  
9 is now widespread across the country (17). The H3N2 subtype of swine influenza virus  
10 (SIV) had not crossed the US-Canadian border prior to 2005, which explains why it had  
11 not been reported in Canadian swine population since the middle of the 90's (17). Today,  
12 the SIV subtypes that are mostly found in Canada are usually H1N1 and H3N2 (8, 17).  
13 Other subtypes such as H1N2, H3N3 and H4N6 were recently reported to be present in  
14 the Canadian swine population and are sporadically found (8, 9, 11).

15           Mink have been previously known to be susceptible to influenza virus infection  
16 (14-16, 20). However, an influenza virus outbreak in mink population associated with  
17 the development of clinical signs in infected animals is a rare event, and it has been  
18 reported only on a few occasions, such as the 1984 Sweden outbreak caused by an avian  
19 H10N4 influenza virus (12). Therefore, it is believed that most of the natural influenza  
20 virus infections in mink are asymptomatic with the consequence that no specific  
21 influenza virus subtype has been known to circulate in mink population. Noteworthy, in  
22 experimental conditions it was demonstrated that several subtypes of avian, human and  
23 equine influenza viruses were able to infect mink (5, 6, 14, 15, 20). Avian and swine are

1 currently considered to be the most important species epidemiologically involved in  
2 mechanisms that possess highly pandemic potential for the human population, such as  
3 reassortment. It is very important to do epidemiological surveillance of influenza virus  
4 not only within the common susceptible animals like avian and swine, but for all other  
5 species where intensive production and high geographic density of animals may favor the  
6 appearance of new influenza virus isolates. Herein, the genomic and antigenic  
7 characterization of a H3N2 triple reassortant influenza virus isolated from Canadian mink  
8 is reported.

9 In 2006, mink ranches in the province of Nova Scotia, Canada, were experiencing  
10 an increase in respiratory problems such as pneumonia, with varying rates of mortality.  
11 The mortality rate was higher in ranches where other pathogens were also present, like  
12 Aleutian disease virus of mink. Two animals of 6 months of age experiencing clinical  
13 signs such as dry cough were necropsied. No macroscopic lung lesion was observed.  
14 Histopathological examination revealed the presence of mild interstitial pneumonia and  
15 mild to moderate bronchiolitis. Bacteriological cultures of the lungs were negative. A  
16 PCR diagnostic test using a specific primer set previously described by others (18) was  
17 performed on lung tissues. The result was positive for the presence of influenza A virus.  
18 Afterwards, another PCR diagnostic test (3) able to differentiate between H1, H3, N1 and  
19 N2 subtypes, was realized. The result was positive for the H3N2 subtype. Furthermore,  
20 immunohistochemistry (IHC) using a polyclonal anti-NP antibody (National Institute of  
21 Allergy and Infectious Disease, Bethesda, MD) was positive in lungs. Unfortunately,  
22 attempts to isolate the virus from lung homogenates following inoculation of Madin-  
23 Darby canine kidney (MDCK) cells or embryonated chicken eggs were unsuccessful. In

1 2007, lung samples of mink from the same geographical region were taken for an  
2 influenza A virus surveillance program. Some samples were positive for influenza A  
3 virus by PCR. Then, attempts to isolate the virus were realized. In one case, an influenza  
4 A virus (A/Mink/Nova Scotia/1055488/07; abbreviated as Mk/NS/1055488/07) was  
5 isolated only in MDCK cells. The presence of the virus was confirmed by PCR (18) and  
6 electron microscopy (13). Surprisingly, no viruses could be isolated in embryonated  
7 chicken eggs. The lung sample from which the virus was isolated originated from an  
8 animal raised in a Nova Scotia mink ranch which never experienced any respiratory  
9 problems.

10 The viral RNA was isolated from the MkNS/1055488/07 virus using a  
11 commercial kit (QIAamp Viral RNA Mini Kit, Qiagen, Mississauga, Ontario). The full-  
12 length of the viral RNA segments were amplified by reverse transcription-PCR (RT-  
13 PCR), the PCR products were purified (QIAquick PCR purification kit; Qiagen) and both  
14 strands of the purified DNA PCR products were sequenced by using the same primer sets  
15 (7, 10) used in the RT-PCR reaction with standard automated sequencing methods  
16 (Faculté de médecine vétérinaire Sequencing Laboratory, Bigdye terminator version 3.1,  
17 sequencer: ABI 310, Applied Biosystems; Foster City, California, USA). Resulting  
18 sequences were compared with other sequences of SIV references strains available in the  
19 Diagnostic Veterinary Virology Laboratory (DVVL) of the Université de Montréal (such  
20 as a virus isolated in the province of Quebec during the 2005 H3N2 Canadian swine  
21 influenza virus outbreak: A/Swine/Quebec/4001/05 (Sw/Qc/4001/05)) and in GenBank  
22 database. Software (BioEdit Sequence Alignment Editor version 7.0.9, Ibis Therapeutics;  
23 Carlsbad, California, USA) using the CLUSTAL W alignment method was utilized and

1 an unrooted phylogenetic tree of the HA gene was constructed by using the distance-based  
2 neighbor-joining method. Bootstrap values were calculated on 1000 repeats of the  
3 alignment. The nucleotide (nt) identities between Mk/NS/1055488/07 and the most  
4 recent 2005 Canadian swine (A/Swine/Manitoba/12707/05, A/Swine/Alberta/14722/05,  
5 A/Swine/British Columbia/28103/05, A/Swine/Ontario/33853/05, abbreviated as  
6 Sw/Mn/12707/05, Sw/Ab/14722/05, Sw/BC/28103/05 and Sw/On/33853/05,  
7 respectively, and Sw/Qc/4001/05), turkey (A/Turkey/Ontario/31232/05 abbreviated as  
8 Tk/On/31232/05), and human (A/Ontario/RV1273/05 abbreviated as On/RV1273/05)  
9 triple reassortant influenza virus isolates (17) were determined. They were established to  
10 be ranging from 97.5% to 98.4% for HA, 98.0% to 98.9% for NA, 98.3% to 99.1% for  
11 PB1, 98.1% to 99.0% for PB2, 98.7% to 99.3% for PA, 99.2% to 99.4% for NP, 99.2% to  
12 99.3% for M and 99.0% to 99.1% for NS genes (data not shown). Following the use of  
13 the classification adopted by Dr Olsen and his collaborators (2006) (17), an HA nt  
14 unrooted phylogenetic tree was constructed using reference strains of each clusters and  
15 all 2005 H3N2 previously reported Canadian reference strains (Figure 1). As illustrated  
16 in figure 1, the Mk/NS/1055488/07 isolate was classified within the same cluster (IV) as  
17 all 2005 Canadian H3N2 SIV isolates previously classified (17). Also, the genetic  
18 relatedness of Mk/NS/1055488/07 to older Canadian H3N2 such as the  
19 A/Swine/Quebec/150/90 (Sw/Qc/150/90) (1, 2) was much lower compared to recent  
20 H3N2 Canadian strains (Figure 1). When a BLAST analysis (from  
21 <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was realized in June 2008 with all the viral RNA  
22 genome segments of the Mk/NS/1055488/07, the recent triple reassortant Canadian

1 influenza viruses reported by Olsen and collaborators (2006) (17) were the most  
2 genetically closely related to the mink H3N2 influenza virus (data not shown).

3         The antigenic comparison of the Mk/NS/1055488/07 isolate with 1) the  
4 Sw/Qc/4001/05 Quebec reference strain that has been previously classified in the same  
5 cluster (IV) (Figure 1); 2) a reference strain of cluster II (A/Swine/Iowa/8548-1/98,  
6 abbreviated as Sw/Ia/8548-1/98); and 3) a Quebec H3N2 older strain (Sw/Qc/150/90)  
7 was realized by using the hemagglutination inhibition assay (HI). The sera used in the HI  
8 were obtained following immunization of chicks with formalin inactivated viruses  
9 (ck/150/90 and ck/Ia/98) or from naturally infected pigs (sw/4001/05). The HI was  
10 performed as previously described with minor modifications (4). The H3N2 influenza  
11 specific sera possessed various HI titers against all H3N2 influenza strains, but their HI  
12 titer was higher against their homologous strain (Table 1). The most striking result was  
13 that all H3N2 influenza specific reference sera reacted poorly against the mink influenza  
14 virus isolate (Table 1). As an example, the HI titer of the pig serum specific for the most  
15 recent influenza virus isolated in Quebec (sw/4001/05) had a HI titer of 1280 against its  
16 homologous strain (Sw/Qc/4001/05) compared to a HI titer of 80 against  
17 Mk/NS/1055488/07 (Table 1). Similarly, the HI titer of ck/150/90 and ck/Ia/98 sera were  
18 1280 and 160, respectively, for their homologous strains and were only 80 and 20,  
19 respectively, against Mk/NS/1055488/07. These latest results clearly indicate that the  
20 Mk/NS/1055488/07 isolate is antigenically distinct from other strains and also distinct  
21 from a strain previously classified in the same genomic cluster IV, like the  
22 Sw/Qc/4001/05 strain (Figure 1, Table 1).

1           Unfortunately, the spread of influenza virus in the mink population could not be  
2 evaluated because there were not enough samples submitted to the diagnostic laboratory.  
3 On the other hand, thousands of swine samples are submitted each year to the DVVL  
4 diagnostic laboratory. Consequently, the spread of the mink influenza virus in the swine  
5 population was evaluated. Thus, the HI titers against the Sw/Qc/4001/05 and  
6 Mk/NS/1055488/07 strains of 100 pig sera obtained between July 2007 and March 2008  
7 were evaluated. All sera were obtained from pigs housed in farms in Quebec, Canada,  
8 and each serum was obtained from a different farm. To establish if the HI sera titers  
9 obtained were significantly different, the nonparametric Wilcoxon matched pairs t test  
10 was applied using the GraphPad Prism version 4 software. Twenty-three percent of the  
11 tested sera were HI negative against both viruses (data not shown). The HI mean values  
12 of HI positive sera were 292.3 and 79.9 for Sw/Qc/4001/05 and Mk/NS/1055488/07,  
13 respectively (Figure 2a). The difference between Sw/Qc/4001/05 and  
14 Mk/NS/1055488/07 HI titers was significant with a  $P < 0.0001$  (Figure 2a). Furthermore,  
15 no pig serum had a HI titer higher against Mk/NS/1055488/07 compared to  
16 Sw/Qc/4001/05, with the exception of two sera that had the same HI titers against both  
17 strains (Figure 2b), suggesting that this particular mink influenza virus isolate does not  
18 seem to circulate in the swine population.

19           Herein, the present manuscript reports a rare event which is the natural occurrence  
20 of influenza virus infection in mink with the presence of clinical signs (5). The mink  
21 influenza virus isolate was identified to be genetically related to the H3N2 triple  
22 reassortant swine influenza virus that emerged in Canadian swine and turkey populations  
23 in 2005 (Figure 1) (17). It is difficult to establish if the virulence of the disease found in



1 mink is different from the one found in H3N2 influenza virus infected swine and turkey.  
2 Occasionally, the disease in mink seemed to be more severe, but it was exacerbated by  
3 the presence of other pathogens such as mink parvovirus. If we assume that the origin of  
4 the virus in mink population is swine, then what is the link between both species? The  
5 only hypothesis that could answer this question is the type and origin of food that was  
6 given during the 2006 outbreak in mink. All of the mink ranches that experienced the  
7 influenza like syndrome were fed a ration prepared in large batches at the same location,  
8 referred to as a kitchen. The ration was composed of uncooked meat bi-products of  
9 swine, beef and poultry obtained from slaughterhouse facilities. The swine bi-product  
10 included ground lung purchased in large frozen blocks from areas of the country where  
11 swine influenza H3N2 is known to be prevalent. Swine influenza had not been detected  
12 in Nova Scotia's swine in surveillance testing previous to the mink influenza outbreak.  
13 To our knowledge, no other animal species except mink were fed with this ration of  
14 uncooked meat bi-products of swine. Even if the mink influenza virus was isolated from  
15 asymptomatic animals, the animals of that ranch were fed in 2006 with the same batch of  
16 feed that was related to the appearance of the disease in several other Nova Scotia  
17 ranches. Depending on the level of biosecurity of each ranch, it is possible that the mink  
18 influenza virus could be transmitted to wild mink or other wild species, but until now, it  
19 is only a speculation. In the future, the mink industry should be careful of their  
20 management procedures, like the use of slaughterhouse uncooked meat bi-products, to  
21 avoid emerging diseases in mink which might favor the appearance of pathogens, such as  
22 influenza virus, that could have a harmful impact on public health safety. Unfortunately,  
23 the prevalence of influenza virus in Nova Scotia's farmed and wild mink populations is

1 unknown and it would be interesting to conduct new epidemiological studies to follow  
2 the genetic evolution and distribution of the influenza virus in their populations.

3

4 **Nucleotide sequence accession numbers.** The GenBank accession numbers assigned to  
5 the gene sequences determined for this report are as follows: EU826543 to EU826550 for  
6 Sw/Qc/4001/05 and EU826551 to EU826558 for Mk/NS/1055488/07.

7

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15



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- 24

1 **FIGURE LEGENDS**

2

3 **Figure 1.** Phylogenetic tree of the HA gene nucleotide sequence of recent H3N2 triple  
4 reassortant Canadian influenza virus isolates. The GenBank accession numbers of each  
5 strains are indicated on the right, next to the name of the strains. The strains have been  
6 classified in 4 distinct clusters (I to IV) as previously described (17). Bootstrap values  
7 are indicated, excepted in cluster IV where some are non-indicated for clarity purpose.  
8 Horizontal scale bar indicates the distance between strains; a 0.1 distance means that the  
9 strains possess 90% nt identity.

10

11 **Figure 2.** Hemagglutination inhibition titers of swine sera against a 2005 H3N2  
12 Canadian SIV isolate and mink influenza virus. The HI titers of swine sera against the  
13 Quebec SIV reference strain (Sw/Qc/4001/05) were compared to the HI titers against the  
14 mink influenza virus isolate (Mk/NS/1055488/07). A) The mean HI titers of the tested  
15 positive sera are represented with the standard error of the mean (SEM). B) The absolute  
16 HI titers of each serum against both viruses are illustrated.

**Table 1. Antigenic reactivity of mink influenza virus isolate with reference specific antisera by hemagglutination inhibition assay**

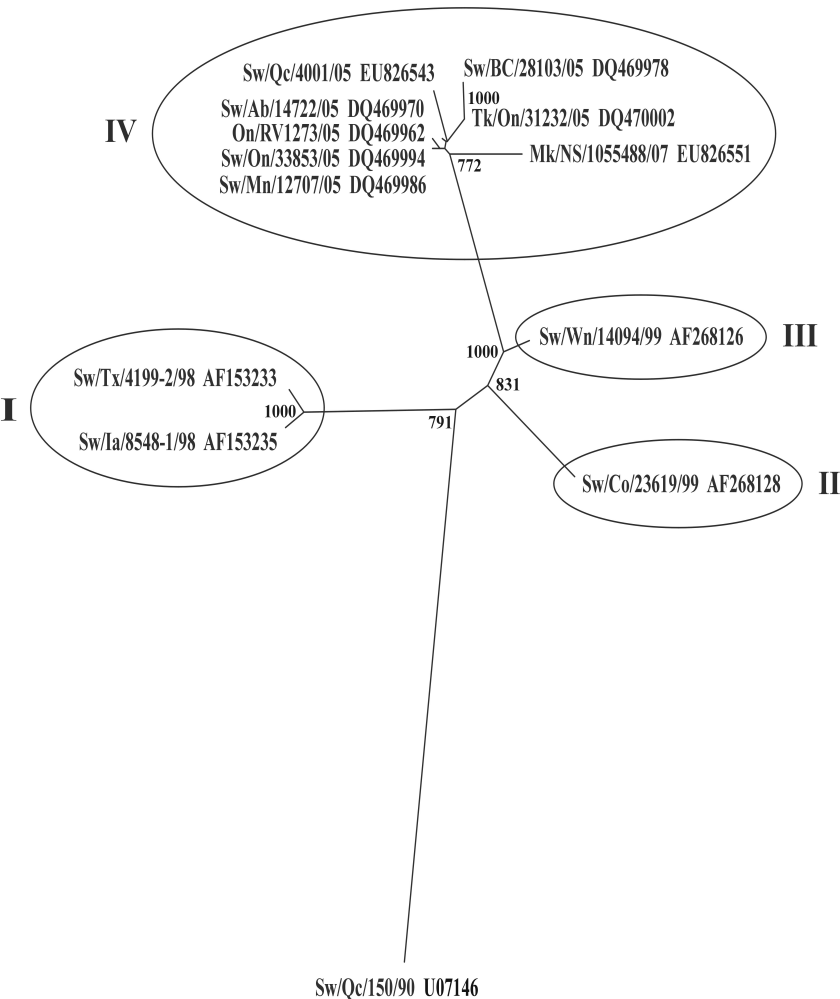
| Sera <sup>a</sup> | Strains           |                 |               |                  |
|-------------------|-------------------|-----------------|---------------|------------------|
|                   | Sw/Qc/150/90      | Sw/Ia/8548-1/98 | Sw/Qc/4001/05 | Mk/NS/1055488/07 |
| ck/150/90         | 1280 <sup>b</sup> | 40              | 640           | 80               |
| ck/Ia/98          | <10               | 160             | 160           | 20               |
| sw/4001/05        | 10                | 40              | 1280          | 80               |

<sup>a</sup>ck/150/90 and ck/Ia/98 are sera that were obtained following immunization of chicks with formalin inactivated viruses (Sw/Qc/150/90 and Sw/Ia/8548-1/98, respectively); sw/4001/05 is a serum that was obtained from naturally Sw/Qc/4001/05 infected pigs.

<sup>b</sup>HI titer

1

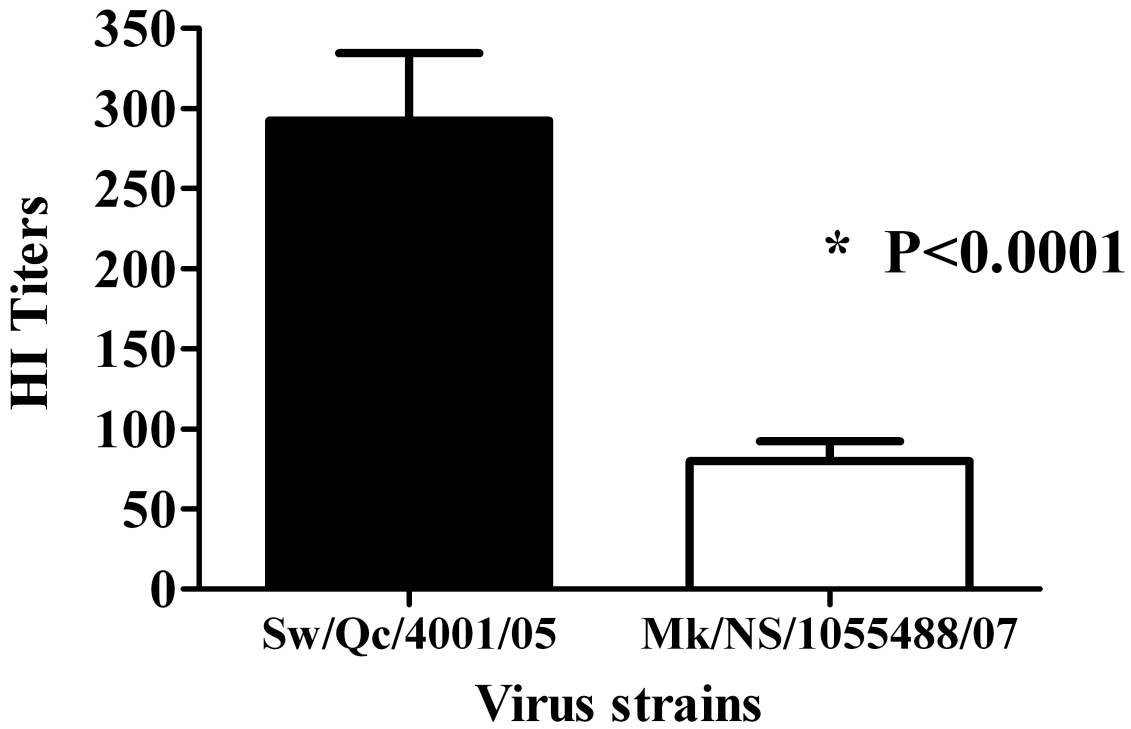
# Figure 1





# Figure 2

A)



B)

