Title:

H5N1 hybrid of avian and human influenza viruses in farmed minks with pandemic potential

Running Title:

H5N1 avian-human hybrid influenza virus in minks

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Abstract—50 words

We isolated avian, swine, human influenza viruses, and one hybrid influenza virus from minks in China. The H5N1 hybrid virus had pandemic potential because its seven genomic segments were from H1N1 human influenza virus and its HA gene was from H5N6 highly pathogenic avian influenza virus carrying multiple mammalian-adaptive mutations.

Keywords: influenza virus, mink, mutation, pandemic, reassortment, receptor binding

Text—1098 words

The ongoing devastating COVID-19 pandemic highlights the importance of early identification and elimination of viruses with pandemic potential. Minks (*Neovison vison*) could be an intermediate host for generating pandemic viruses. They are populous in multiple countries for fur farming and have numerous opportunities to catch viruses from multiple hosts, because they are frequently fed with raw materials including poultry, pork, and horse by-products and slaughter offal (*1-6*). Minks also support replication of various viruses, particularly influenza viruses, and could be a mixing vessel for generating novel viruses through genomic recombination or reassortment (*1-6*). Avian influenza viruses (AIVs), human influenza viruses (HuIVs), and swine influenza viruses (SIVs) have been isolated from minks in multiple countries (*2-6*). These viruses could hybridize in minks and generate novel subtypes of influenza virus with pandemic potential, like the example given in this report.

The study

During the years 2017–2019, we identified 14 influenza viruses from symptomatic minks in China (Table 1). Most of the symptomatic minks were young with dyspnea and declined appetite, and the fatality was approximately 10–20%. Pneumonia was found in all these symptomatic minks through autopsy examination. Swab samples collected from these minks were positive for influenza virus using the relevant RT-PCR assays (*7*). The influenza viruses were isolated by inoculating specific-pathogen-free embryonated eggs (Spafas, Jinan, China). The entire viral genomes of these 14 influenza viruses were sequenced using the methods described previously (*8*). The sequences were deposited in GenBank with the accession numbers of MK801253–MK801260, MK812851–MK812858, MK812971–MK812978, MK849807–MK849838, MK849855–MK849862, MK849867–MK849874, and MT510055–MT510094.

These genomic sequences were analyzed using the software package of MEGA along with some reference sequences for universal nomenclatures of influenza viruses (*9,10*). The phylogenetic analysis revealed that the 14 viruses from minks included 11 subtype H5N6 AIVs, one subtype H1N1 HuIV, one subtype H1N1 SIV, and one subtype H5N1 hybrid virus. The H5N1 hybrid virus A/Mink/Eastern China/0712/2018(H5N1), abbreviated as 0712/H5N1, was from genomic reassortment of a subtype H5N6 AIV (the donor of the HA gene) and a subtype H1N1 HuIV (the donor of other genes) (Figure 1). BLAST of the sequences of 0712/H5N1 at the NCBI website also suggested that 0712/H5N1 was the most similar to some H5N6 AIVs circulating in Asia after the year 2015 in their HA gene sequences, and 0712/H5N1 was the most similar to some H1N1 HuIVs circulating worldwide in 2017 or 2018 in their other gene sequences (sequences identities all >99.20%).

Detection of the viral RNA using an RT-PCR assay specific for subtype N6 NA gene was

58 negative for 0712/H5N1 and positive for the 11 H5N6 viruses listed in Table 1. Detection of the
59 viral RNA using an RT-PCR assay specific for subtype H1 HA gene was negative for
60 0712/H5N1 and positive for the two H1N1 viruses listed in Table 1. These data confirmed that
61 0712/H5N1 was a hybrid rather than a mixture of an H5N6 AIV and an H1N1 HuIV. As far as
62 we know, this is the first avian-human hybrid influenza virus identified in minks, although avian
63 and human influenza viruses have been isolated in minks in multiple countries (*2-6*).

64 The HA protein of 0712/H5N1 has multiple basic amino acid residues
65 (PLRERRRKR*GLF) at the cleavage site, categorizing it as a highly pathogenic AIV (HPAIV).
66 Phylogenetic analysis of the HA gene suggested that 0712/H5N1 was a variant of clade 2.3.4.4d
67 (Figure 1), which was the dominant clade of H5 HPAIVs in China in recent years (*11*).

68 The HA gene protein of 0712/H5N1 carried multiple mutations, including D94N, S107R,
69 T108I, L129del, S133A, I151T, S154N, T156A, and V182N (H5 numbering throughout). All
70 these mutations could aid the virus to bind to human-like receptors (alpha2,6-linked sialic acids),
71 and thus facilitate adaption of the virus to humans (*12,13*).

72 The receptor preference of 0712/H5N1 was evaluated using goose red blood cells (GRBCs)
73 which present both avian-like receptors (alpha2,3-linked sialic acids) and human-like receptors
74 (alpha2,6-linked sialic acids). The hemagglutination titers of 0712/H5N1 using GRBCs remained
75 unchanged using the GRBCs treated with 2,3-sialidase (Takara, Japan) to remove alpha2,3-
76 linked sialic acids (*14*), suggesting that 0712/H5N1 preferred to bind to human-like receptors.
77 Notably, 11 of the 14 viruses isolated from minks preferred to bind to human-like receptors, as
78 evaluated using the same assay (Table 1).

79 H5 HPAIVs have been endemic in Asia and Africa. They have caused sporadic severe
80 respiratory disease in humans for decades. H5 HPAIVs could spark a dangerous pandemic in

81 humans if they acquire enough adaptation in humans through genomic reassortment and site
82 mutations (*12,13,15*). Experimental studies showed that H5N1 hybrid viruses bearing H1N1
83 HuIV genes transmitted in guinea pigs by respiratory droplet (*15*). Therefore, 0712/H5N1 had
84 considerable pandemic potential because it not only harbored seven genomic segments of HuIVs,
85 but also carried multiple mammalian-adaptive mutations in the avian-origin HA gene. Similar
86 avian-human hybrids sparked human pandemics in 1957 and 1968 (*13*).

87 The hybrid 0712/H5N1 was isolated from a dying young mink, which showed dyspnea and
88 declined appetite four days after being vaccinated for mink viral enteritis and canine distemper
89 by outside veterinarians. The mink developed severe pneumonia as observed through autopsy
90 and histopathological examination (Figure 2). One month after the isolation, we randomly
91 collected 20 swab samples from this mink farm, and the samples were all negative for influenza
92 virus, suggesting that the hybrid virus had disappeared naturally. To minimize laboratory risk,
93 we disinfected all the viral materials using autoclave sterilization after we obtained the genomic
94 sequences and the receptor-binding data.

95 Minks could play a critical role in generating human-animal hybrid influenza viruses, due to
96 their special ecological features. Like in this study, minks could catch AIVs and SIVs from their
97 feed, and catch HuIVs from humans through close contact, and thus have the possibility to
98 generate hybrid viruses with considerable pandemic potential. To minimize this public health
99 risk, multiple sections of governments should collaborate and stipulate scientific regulations. The
100 regulations should cover requirements on mink farm sites, disease prevention measures, mink
101 feeds, reduction of human-animal contacts, management of mink waste, virus surveillance, and
102 management of infected minks. Moreover, we recommend that farmed minks in China should be
103 vaccinated against H5 HPAIVs to protect minks and minimize the public health risk.

Conclusions

We isolated 14 influenza viruses from minks in China in recent years, including eleven H5N6 AIVs, one H1N1 HuIV, one H1N1 SIV, and one H5N1 hybrid virus with HA gene from subtype H5N6 avian influenza virus and other genes from H1N1 human influenza virus. The H5N1 hybrid virus preferred to bind to human-like receptors and was of considerable pandemic potential. These findings suggested that minks for fur farming in China should be managed more strictly and vaccinated against H5 HPAIVs for public health.

Data Availability Statement

The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

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Ethics Statement

This study was conducted according to the animal welfare guidelines of the World Organization for Animal Health (Terrestrial Animal Health Code) and approved by the Committee on ethics from the College of Veterinary Medicine, Qingdao Agricultural University.

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Influenza viruses we isolated in China in recent years.

Virus isolates	Isolation province	Virus origin	Receptor-binding*
A/mink/Shandong/1121/2017(H1N1)	Shandong	SIV	1:1
A/Mink/Eastern China/0712/2018(H5N1)	Shandong	Hybrid	1:1
A/Mink/Eastern China/006/2018(H5N6)	Shandong	AIV	1:1
A/Mink/Eastern China/032/2018(H5N6)	Shandong	AIV	2:1
A/Mink/Northern China/110/2018(H5N6)	Hebei	AIV	1:1
A/Mink/Eastern China/149/2018(H5N6)	Shandong	AIV	1:1
A/Mink/Eastern China/528/2018(H5N6)	Shandong	AIV	1:1
A/Mink/Eastern China/571/2018(H5N6)	Shandong	AIV	1:1
A/Mink/Eastern China/0824/2018(H5N6)	Shandong	AIV	4:1
A/Mink/China/456/2018(H5N6)	Shandong	AIV	2:1
A/Mink/China/0509/2019(H1N1)	Shandong	HuIV	1:1
A/Mink/China/181/2019(H5N6)	Shandong	AIV	1:1
A/Mink/China/183/2019(H5N6)	Shandong	AIV	1:1
A/Mink/China/191/2019(H5N6)	Shandong	AIV	1:1

*The ratios were hemagglutination titers using untreated GRBCs versus those using GRBCs treated with 2,3-sialidase.

Figure 1. Phylogenetic relationships of a mink influenza virus (marked with the triangle) with some reference viruses for a universal nomenclature of influenza virus (*10*). A: based on the viral HA gene sequences (clades of H5 HPAIVs are given after strain names); B: based on the viral NA gene sequences.

Figure 2. Congestion, hemorrhage, edema, vasculitis, and compensatory emphysema in the lung of a symptomatic mink. Scale bar = 200 μm.