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# Potential Human Adaptation Mutation of Influenza A(H5N1) Virus, Canada

**To the Editor:** In December 2013, influenza associated with pandemic influenza A H5N1 was reported in Canada in a patient who had traveled to China; the patient died in January 2014. This case leaves unanswered questions.

In the absence of direct poultry contact by the patient, the possible route of transmission and infection, often influenced by receptor-binding properties of the virus, requires special attention. The full genome and phylogenetic analysis by Pabbaraju et al. (*1*) provides a summary of what can typically be deduced from the sequence. The authors also mention 2 novel mutations, R189K and G221R, in the hemagglutinin (HA) protein (R193K and G225R in H3 numbering, used hereafter). When mapped to the H5 HA protein structure by using FluSurver in GISAID (http://www.gisaid.org, http://flusurver.bii.a-star.edu.sg), both mutations are found in the immediate receptor-binding pocket, and G225R has been known to change specificity of an H3N2 virus toward human erythrocytes (*2*). The same position is also known for receptor recognition changes in the 2009 pandemic H1N1 virus (mutations D222G, D225G, or D239G in different numberings). Besides A/Alberta/01/2014 (clade 2.3.2.1c), the mutation G225R has been found in 3 other H5N1 sequences: A/duck/Hunan/15/2004 (clade 2.3.3), A/chicken/Xinjiang/53/2005, and A/chicken/Xinjiang/27/2006 (both clade 7, all lineage assignments made with LABEL, http://label.phiresearchlab.org/). Although few G225R mutations were found, they were all found in avian hosts, indicating that the mutation can occur sporadically and avian-like receptor-binding properties may not be fully abolished by G225R.

In the absence of glycan-binding data or crystal structures, which take longer to deduce, computational structural modeling is an efficient and safe alternative for fast preliminary assessment of these mutations in their natural structural context of H5N1 binding pockets. We have shown (*3*) that a method using the classical AMBER03 molecular mechanics force field (*4*) with an implicit solvation model in combination with short molecular dynamics simulations in YASARA (*5*) can reproduce relative preference for human-like α2,6-linked versus avian-like α2,3-linked sialic acid receptors. The interaction energies of all atoms in a system are described and combined with distance-dependent functions for different interaction types, including bonds, various angles, van der Waals, electrostatics, and solvent, which leads to consideration of the concerted effects of all residues in the binding pocket. By using this energy function, short molecular dynamics simulations enable all atoms to move for specified intervals within the constraints of their interactions. These simulations are used to minimize and finally predict the energies of the wild-type and mutant HA proteins for their ligand-bound and unbound states considering their respective ligands (see Methods section of [*3*] for details).

In this study, we further tested the computational structural model on mutations with known effect on receptor-binding properties (*2*,*3*,*6*–*9*) in H5N1 context based on recently resolved crystal structures and the respective ligand complexes (*9*). We limited this selection to mutations in the immediate vicinity of the crystallized receptor analog because the method should be most accurate for this scenario. The results showed that the binding preference of known mutations could be predicted at least qualitatively. Next, we tested the additional mutations found in A/Alberta/01/2014(H5N1). Our results (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/209/12-1200-Techapp1.pdf) suggest that G225R could incur a relative predicted increase in binding to the human-like receptors. Although the quantitative accuracy of computational methods in this regard is limited, the predicted numerical value suggests a possible similar extent of the effect to that of the well-known Q226L utation. It should also be noted that the predicted increase in binding to human-like receptors does not necessarily imply a concomitant loss of avian receptor binding. The role of R193K is less clear with a slight predicted tendency of favoring avian-like receptors. These preliminary findings highlight the necessity of verifying not only the receptor-binding properties of this virus through experimentation, but given the predicted increased preference for human receptors, also verifying potential roles in altered mammalian transmissibility.

These receptor-binding pocket mutations of the virus were not seen in the most closely related Asian H5N1 sequences of clade 2.3.2.1c (*1*), and no human contacts were known to be affected. From the epidemiologic perspective of this isolated human case, it is possible that this variant arose in the patient after initial infection and contributed to prolonged and severe infection and to the more unusual spread to brain tissue. If more avian strains with G225R mutations are found, the example of Q226L in H7N9 indicates that relative receptor-binding changes alone do not necessarily imply immediate mammalian transmissibility (*10*). It should also be noted that G225R was not among the mutations identified by recent controversial mammalian adaptation studies, (*7*,*8*) indicating that there may be more H5N1 host specificity markers than have been identified. Consequently, the functional roles of G225R in avian influenza should be further analyzed by conducting secure experiments and, pending verification, checking closely for its potential as an avian influenza host specificity marker.

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# Genetic Changes of Reemerged Influenza A(H7N9) Viruses, China

**To the Editor:** From March 30, 2013, through April 8, 2014, a total of 401 human infections with novel avian influenza A (H7N9) virus were reported in China (*1*). In the initial wave from February through May 2013, cases were laboratory confirmed for 133 patients (45 died), mainly in eastern China. From June through early October 2013, only 2 laboratory-confirmed cases were reported in China. One of these, identified on August 10, 2013, was the first case of influenza A(H7N9) virus infection in Guangdong Province (strain A/Guangdong/HZ-01/2013). However, a second wave of influenza A(H7N9) virus infection began on October 14, 2013 (*2*). As of April 8, 2014, a total of 266 laboratory-confirmed cases had been reported, mainly in Zhejiang Province in eastern China (92 cases, 37 deaths) and Guangdong Province in southern China (99 cases, 30 deaths).

Previous sequencing studies suggested that 6 of the 8 influenza A(H7N9) virus RNA segments were acquired from influenza A(H9N2) virus. This acquisition process involved at least 2 steps of sequential reassortment; the most recent event most likely occurred in the Yangtze River Delta area of eastern China (*3*–*5*). To date, nearly all analyses have been performed by using sequences obtained from viruses isolated during the first wave of infection; changes associated with viruses isolated during the second wave are largely unknown (*6*). We therefore conducted phylogenetic analyses of whole-genome sequence data for 15 influenza A(H7N9) viruses isolated from human patients in Guangdong from November 4, 2013, through January 15, 2014.

We estimated maximum-likelihood trees for all 8 RNA segments by using MEGA version 5.2 and the general time-reversible model (*7*). RNA segments encoding the hemagglutinin, neuraminidase, and matrix genes of A/Guangdong/H7N9 viruses isolated after November 2013 were genetically similar to those of A/Guangdong/HZ-01/2013 and H7N9 strains from the first wave of influenza (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/14-0250-Techapp1.pdf). An additional 4 RNA segments (nonstructural protein [NS], nucleocapsid protein [NP], polymerase basic proteins [PB] 1 and 2) of A/Guangdong/H7N9 influenza viruses isolated after November 2013 were clustered with A/Guangdong/HZ-01/2013 virus and were divergent from all currently sequenced subtype H7N9 viruses from the first wave in eastern China. The only exception was the NP segment of A/Guangdong/SZ-026/2014, which was found segregated into a separate cluster with subtype H9N2 viruses from Shandong Province. Moreover, analyses showed that RNA segments encoding NS, NP, PB1, and PB2 of A/Guangdong/H7N9 isolated after November 2013 were most similar to the same segments from influenza A(H9N2) viruses that had recently circulated in Guangdong (online Technical Appendix Figure, panels D–G). That is, NS, NP, PB1, and PB2 showed greater similarity to local subtype H9N2 viruses from Guangdong than to subtype H7N9 viruses from the first wave of influenza.

Notably, 2 separate clusters were observed for the phylogenetic tree of the RNA segment encoding the polymerase acidic gene (online Technical Appendix Figure, panel H). A/Guangdong/HZ-01/2013-like viruses clustered with subtype H7N9 viruses from the first wave of influenza. However, A/Guangdong/DG-02/2013-like viruses were clustered with subtype H9N2 influenza viruses circulating in Guangdong, suggesting that recent reassortment with circulating subtype H9N2 viruses occurred after the first case of infection with influenza A(H7N9) virus reported in Guangdong (online Technical Appendix Figure, panel H).

This study provides evidence that influenza A(H7N9) viruses isolated during the second wave of influenza in Guangdong differ genetically (in 5 of the 8 RNA segments) from that of influenza A(H7N9) viruses isolated during the first wave. High similarity of these 5 segments with those of locally circulating subtype H9N2 viruses suggests that rapid and continued reassortment with circulating subtype H9N2 viruses occurred during the second wave of the influenza A(H7N9) virus epidemic. Because reassortment and genetic changes can contribute to host fitness and infection capacity of reemerged influenza A(H7N9) viruses, studies of pathogenicity and transmission, to reveal the exact role of each genetic alteration, are needed.

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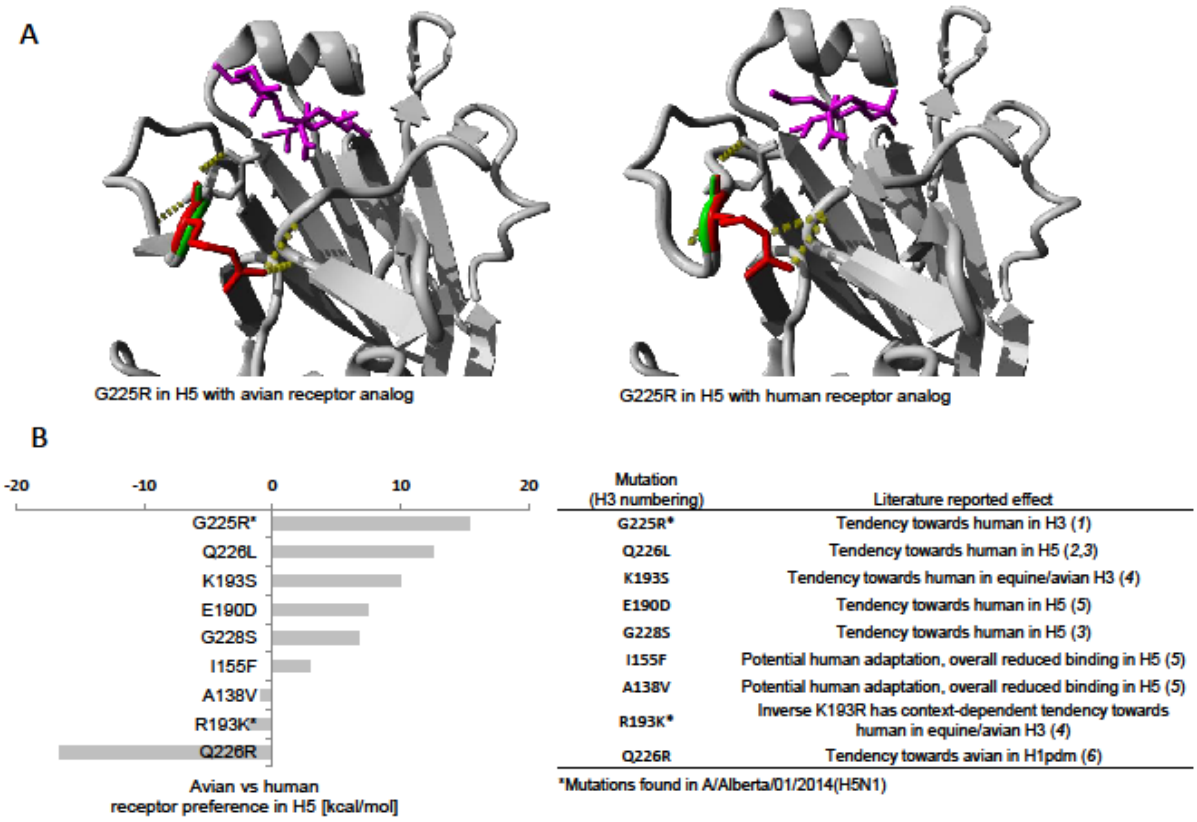
# Potential Human Adaptation Mutation of Influenza A(H5N1) Virus, Canada




| Mutation (H3 numbering) | Literature reported effect |
|---|---|
| G225R* | Tendency towards human in H3 (*1*) |
| Q226L | Tendency towards human in H5 (*2,3*) |
| K193S | Tendency towards human in equine/avian H3 (*4*) |
| E190D | Tendency towards human in H5 (*5*) |
| G228S | Tendency towards human in H5 (*3*) |
| I155F | Potential human adaptation, overall reduced binding in H5 (*5*) |
| A138V | Potential human adaptation, overall reduced binding in H5 (*5*) |
| R193K* | Inverse K193R has context-dependent tendency towards human in equine/avian H3 (*4*) |
| Q226R | Tendency towards avian in H1pdm (*6*) |

*Mutations found in A/Alberta/01/2014(H5N1)

Technical Appendix Figure. G225R, a new HA receptor binding pocket mutation in A/Alberta/01/2014(H5N1) is predicted by A) computational structural modeling that will B) alter specificity toward a relative increase in human receptor binding. A) Modeled hemagglutinin complex with the avian-like receptor that is based on PDB:3zp0 on the left and the human-like receptor based on PDB:3zp1 on the right side. G225R facilitates creation of new hydrogen bonds to the opposite disulfide-bridge stabilized scaffold (≈S137), thereby slightly moving the 225 loop holding critical residue Q226, which is in direct contact with the ligand and appears slightly altered in the model of the complex with the human-like ligand. This provides a hypothetical mechanism for the binding changes. Colors indicate properties as follows: green: Wildtype G225; red: Mutant 225R; magenta: Receptor analog; yellow: H-bonds. B) Predicted tendencies of receptor preference changes on the left; positive values indicate increased

preference for human and negative values for avian receptors, respectively. Exact values may not be accurate but qualitative tendencies have been shown to be reproducible compared to experiments. The method was also applied to several binding pocket mutations with known effect reported in the literature and the predictions match the experimental observations summarized on the right side.

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