Acta virologica **57**: 333 – 338, 2013 doi:10.4149/av_2013_03_333

Effects of the Q223R mutation in the hemagglutinin (HA) of egg-adapted pandemic 2009 (H1N1) influenza A virus on virus growth and binding of HA to human- and avian-type cell receptors

O. SUPTAWIWAT[1], W. JEAMTUA[1], CH. BOONARKART[1], A. KONGCHANAGUL[1,2], P. PUTHAWATHANA[1], P. AUEWARAKUL[1*]

[1]Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok, Thailand; [2]Institute of Molecular Biosciences, Mahidol University, 25/25 Phuttamonthon 4 Road, Nakhon Pathom, Thailand

Received October 22, 2012; accepted May 31, 2013

Summary. – The 2009 swine-origin influenza A virus (H1N1) and its initial reassortant vaccine strains did not grow well in embryonated eggs. The glutamine to arginine mutation at the amino acid position 223 (Q223R) of the hemagglutinin (HA) gene is the major mutation previously found in egg-adapted 2009 H1N1 strains and shown to enhance viral growth in embryonated eggs. However, the effect of this mutation on the receptor-binding preference had not been directly demonstrated. In this study, the Q223R mutation was shown to change the viral HA binding preference from the human-type receptor, α2,6-linked sialic acid, to the avian-type receptor, α2,3-linked sialic acid; and to enhance the viral growth in embryonated eggs but not in cell culture.

Keywords: influenza A virus; hemagglutinin; mutation Q223R; cell receptors; sialic acid

Introduction

Although the pandemic waves of the swine-origin H1N1 influenza have passed and the herd immunity may have been saturated in a part of the population, pockets of non-immune population exist and vaccination is still required. A significant antigenic drift, to the point that the immunity to the original 2009 strain does not provide protection, is also likely to occur in the near future. Generation of new vaccine strains to update the seasonal influenza vaccine will soon be necessary. In contrast to the previous set of seasonal influenza viruses, the 2009 swine-origin H1N1 influenza virus did not readily yield reassortant vaccine strains with a high growth property in eggs. At the same time, classical reassortant vaccine strains showed adaptive mutations (Robertson and Engelhardt, 2010). This became a problem for the generation of reassortants by reverse genetics. Some mutations in the HA and inclusion of indigenous PB1 gene were shown to improve the growth of reverse genetics-derived vaccine strains (Chen *et al.*, 2010; Suphaphiphat *et al.*, 2010; Wanitchang *et al.*, 2010). Aspartic acid to glycine (D222G) and the Q223R are the most common mutations identified in HA of egg-adapted strains and used in vaccine strains to enhance the viral replication in embryonated eggs (Robertson and Engelhardt, 2010). The D222G mutation was shown to be associated with an increased virulence and its effect on the receptor-binding property has been well characterized (Chutinimitkul *et al.*, 2010; Liu *et al.*, 2010; Abed *et al.*, 2011; Belser *et al.*, 2011; Pan *et al.*, 2012). On the other hand, the effect of Q223R on the receptor binding has not been directly demonstrated.

*Corresponding author. E-mail: prasert.aue@mahidol.ac.th; phone: +662-4198291.

Abbreviations: E0 = the initial virus for passage in egg; E9 = the 9th egg passage; HA = hemagglutination/hemagglutinin; NA = neuraminidase; NP = nucleoprotein; RG = reverse genetic; $TCID_{50}$ = tissue culture infectious dose; wt = wild type

Materials and Methods

Viruses. A/Thailand/104/2009 (H1N1) (wt pandemic (H1N1) virus) was isolated during the first wave of pandemic in Bangkok,

Thailand. The virus was isolated in Madin-Darby Canine Kidney (MDCK) cells maintained in Minimum Essential Medium (MEM) containing 1 μg/ml of TPCK-treated Trypsin. The virus at the passage number 4 in MDCK cells was inoculated into allantoic sacs of 9-day-old embryonated eggs, which were further incubated at 33°C for 72 hrs. The allantoic fluid was then harvested and inoculated into allantoic sacs of embryonated eggs for 9 continuous passages (the 9th egg passage = E9 virus).

Reverse genetics-derived viruses. The HA and neuraminidase (NA) genes of the virus before and after passages in embryonated eggs were amplified by RT-PCR using consensus primers for the full length gene amplification as previously described (Hoffmann *et al.*, 2001). The GenBank Acc. No. of the HA and NA gene of the isolate before passaging in eggs is ACR23302 and ACR23301, respectively. The amplified HA and NA were cloned into the reverse genetics plasmid pHw2000 (Hoffmann *et al.*, 2002). The plasmids were then used to generate viruses by co-transfection into HEK-293 and MDCK mixed cells together with the plasmids carrying the internal genes of PR8 as previously described (Hoffmann *et al.*, 2000). At 48 hrs post-transfection, culture supernatant was inoculated into allantoic sacs of 9-day-old embryonated eggs.

Virus growth experiments. Each virus was cultured in MDCK cells and embryonated eggs for viral growth assay. In cell culture, $6x10^5$ of MDCK cells were infected with $6x10^3$ $TCID_{50}$ of each virus. The viral supernatant was collected after 48 hrs. In eggs, 10^3 $TCID_{50}$ of each virus were inoculated into four eggs. Finally, the viral supernatants at 48hrs (cell) or end point 72 hrs (egg) were titrated for 50% tissue culture infectious dose ($TCID_{50}$) in MDCK cells using an influenza A nucleoprotein (NP)-specific ELISA as previously described (WHO, 2002).

Receptor binding assay. The receptor binding preference was analyzed by a solid-phase direct binding assay as previously described (Yamada *et al.*, 2006; Auewarakul *et al.*, 2007), using the sialylglycopolymer, which contains the *N*-acetylneuraminic acid linked to the galactose through either an α2,3 or an α2,6 bond (Neu5Acα2, 3LacNAcb-pAP, and Neu5Acα2,6LacNAcb-pAP) (Totani, *et al.*, 2003). Finally, the plate was read at $A_{450/630}$ in ELx 800 UV (BioTek®) microplate reader.

Results

Growth of parental and egg-adapted viruses in cell culture and embryonated eggs

In order to confirm the role of the Q223R mutation in the egg adaptation, we initially produced an egg-adapted strain from a 2009 H1N1 isolate. The parental isolate of A/Thailand/104/2009 (H1N1) grew well in MDCK cells and gave a HA titer of 640–2560 HAU/ml or 10^4–10^5 $TCID_{50}$/ml. When inoculated into allantoic sacs of embryonated eggs, it grew only moderately and yielded a titer of $10^{2.8}$ $TCID_{50}$/ml (HAU/ml = 320). This titer is relatively low as compared to an egg-adapted strain, such as PR8, which usually gives an HAU/ml of 10240–20480. In order to adapt the virus to eggs, we continuously propagated the virus in eggs for 9 passages. After 9 passages in eggs (E9), the virus gave a HA titer of 2,560 HAU/ml or $10^{5.75}$ $TCID_{50}$/ml. When directly compared with the initial virus (E0), the E9 virus gave a much higher titer in embryonated eggs, indicating that the virus has been adapted for replication in eggs (data not shown).

Growth of reverse genetics-derived virus

The HA and NA of the E9 virus were cloned and sequenced. The GenBank Acc. No. for HA and NA of the A/Thailand/104/2009 is GQ169382.1 and GQ179931.1, respectively. When compared to the original virus, only single mutation in each gene, Q223R and E249G, was identified in the HA and NA, respectively. The presence of the Q223R mutation in the E9 virus confirmed the role of this mutation in the egg adaptation. The HA and NA clones of E9 virus and wt pandemic (H1N1) virus were used to generate reverse genetics viruses in the PR8 genetic background. The reverse genetics-derived virus with HA and NA of the wt pandemic (H1N1) virus or the E9 virus separately or in combination (RG-wtHA-wtNA, RG-wtHA-E9NA, RG-E9HA-wtNA, RG-E9HA-E9NA) were rescued and used to study viral kinetics in MDCK cells and eggs. The reverse genetics-derived viruses carrying E9HA cultivated in eggs gave viral titers higher than those reverse genetics-derived viruses carrying the wt pandemic (H1N1) HA, while all viruses showed similar kinetics in MDCK cells (Fig. 1).

Binding of reverse genetics-derived virus to cell receptors

The receptor-binding property of the reverse genetic-derived viruses, A/Thailand/104/2009 (wt pandemic (H1N1) virus) as well as A/Thailand/12/06 (a seasonal H1N1) was tested. While the seasonal H1N1 and the wild type pandemic (H1N1) virus showed preferential binding to α2,6-linked sialic acid as previously shown for the swine-origin H1N1 virus, the virus carrying E9 HA showed a significant increase in the binding to the α2,3-linked sialic acid and a complete loss of the binding to the α2,6-linked sialic acid (Fig. 2). Because egg-adapted viruses have been shown to have an increased affinity to the α2,3-linked sialic acid, these data indicated that the Q223R mutation enhanced the viral replication in embryonated eggs by altering the receptor-binding preference.

Discussion

Because the availability of the α2,3-linked sialic acid in embryonated eggs, it is assumed that egg-adapted mutations

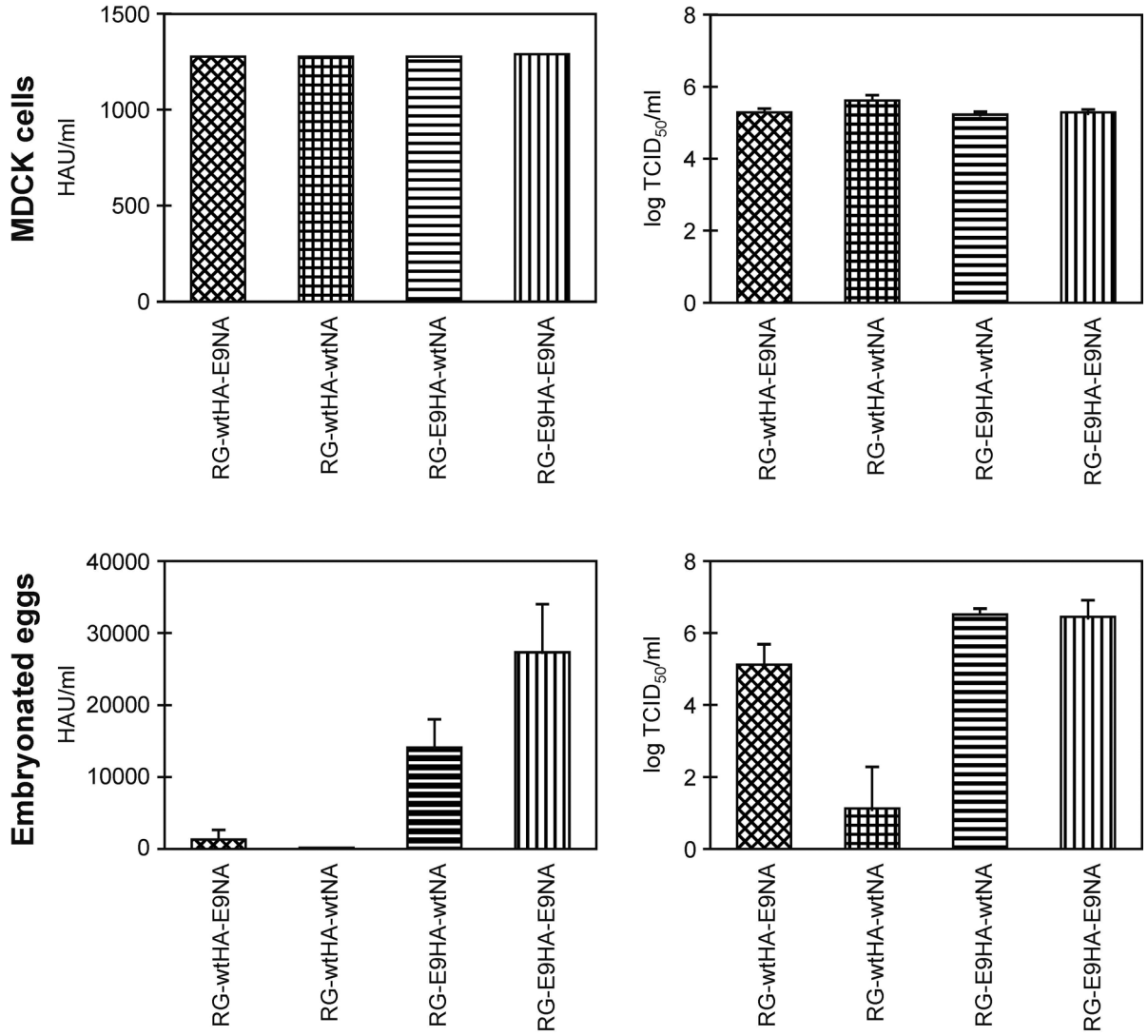

Fig. 1

The viral titer of all viruses in embryonated eggs and MDCK cells

All viruses (RG-104HA-NAE9, RG-104HA-NA, RG-E9HA-104NA, RG-E9HA-NA: 6+2 reverse genetic viruses in the PR8 genetic background carrying HA of A/Thailand/104/2009, A/Thailand/104/2009, E9, E9; and NA of E9, A/Thailand/104/2009, A/Thailand/104/2009, and E9, respectively) were infected into embryonated egg and MDCK cells and were titered by both hemagglutination assay (HA assay) and $TCID_{50}$ assay. The data were derived from 2 independent experiments.

usually lead to an increased affinity to this avian-type receptor (Ito, *et al.*, 1997). The D222G mutation, which enhances the viral growth in eggs and facilitates the production of vaccine strains, was shown to increase the viral affinity to the α2,3-linked sialic acid and decrease the affinity to the α2,6-linked sialic acid (Chen *et al.*, 2010; Liu *et al.*, 2010; Pan *et al.*, 2012). This mutation was also associated with an increased virulence in animals and lower respiratory tract infection in humans (Chutinimitkul *et al.*, 2010; Kilander *et al.*, 2010; Zheng *et al.*, 2010; Abed *et al.*, 2011; Chan *et al.*, 2011). Because of the availability of the α2,3-linked sialic acid in human lower airway, it is likely that the increased affinity to the α2,3-linked sialic acid is directly linked to the increased lower airway-tropism and virulence. The Q223R mutation was previously shown to enhance the viral infectivity in HEK-293 cells, and molecular modeling suggested that this might be due to an increase in the affinity to α2,6-linked sialic acid (Wang *et al.*, 2010). Our data clearly demonstrated that the Q223R mutation switched the receptor-binding preference from α2,6- to α2,3-linked sialic acid, and suggested that

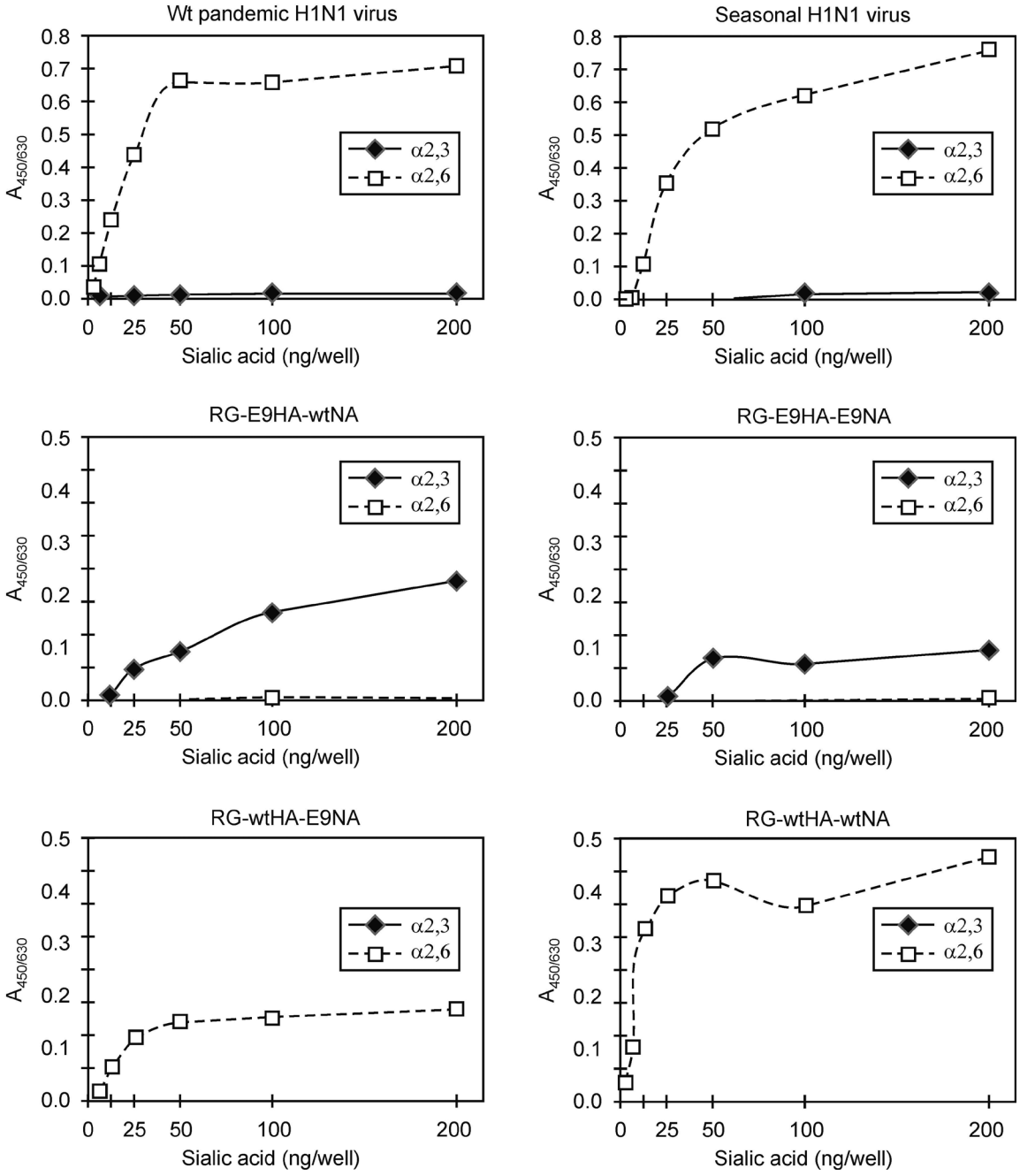

Fig. 2

The receptor binding preference of wt and reverse genetics-derived viruses

The wt viruses A/Thailand/104/2009 (H1N1), A/Thailand/12/06 (seasonal H1N1) and all reverse genetics-derived viruses (6+2): RG wtHA-NA, RG wtHA-E9NA, RG E9HA-wtNA, RG E9HA-wtNA were tested by a solid-phase direct binding assay as previously described (Yamada *et al.*, 2006) by using the sialylglycopolymer using specific 2–3 and 2–6 linked sialic acid.

the previous finding of the enhanced infectivity in cell culture may have been due to the expression of the α2,3-linked sialic acid in the cell line used in the study. A recent report found both D222G and Q223R as a minor population in the early phase of the pandemic and both mutations disappeared in the subsequent season, suggesting an incomplete adaptation to the human host in the early phase of the pandemic (Yasugi *et al.*, 2012). While the change in the receptor-binding of the

D222G resulted in an increased lower airway tropism and virulence, the effect of the Q223R on the viral virulence is unknown. The D222G and the Q223R together with some other mutations in the HA, such as K119N/E, K153/154E, and A186D, were found to increase the yield of the 2009 H1N1 influenza virus in eggs (Chen *et al.*, 2010). These mutations are likely to enhance the affinity to the α2,3-linked sialic acid. Because of the potential link between the affinity to the α2,3-linked sialic acid and the lower airway tropism, these mutations, if present in natural isolates, may serve as markers for increased virulence. Except for the D222G mutation, the role of these mutations in the viral virulence is not understood and requires further studies.

Acknowledgements. This work was supported by research grant No. M-00-10800 from the National Science and Technology Development Agency. O.S. and C.B. are supported by the research assistant program of the Faculty of Medicine Siriraj Hospital.

References

Abed Y, Pizzorno A, Hamelin ME, Leung A, Joubert P, Couture C, Kobasa D, Boivin G (2011): The 2009 pandemic H1N1 D222G hemagglutinin mutation alters receptor specificity and increases virulence in mice but not in ferrets. J. Infect. Dis. 204, 1008–1016. http://dx.doi.org/10.1093/infdis/jir483

Auewarakul P, Suptawiwat O, Kongchanagul A, Sangma C, Suzuki Y, Ungchusak K, Louisirirotchanakul S, Lerdsamran H, Pooruk P, Thitithanyanont A, Pittayawonganon C, Guo CT, Hiramatsu H, Jampangern W, Chunsutthiwat S, Puthavathana P (2007): An avian influenza H5N1 virus that binds to a human-type receptor. J. Virol. 81, 9950-9955. http://dx.doi.org/10.1128/JVI.00468-07

Belser JA, Jayaraman A, Raman R, Pappas C, Zeng H, Cox NJ, Katz JM, Sasisekharan R, Tumpey TM (2011): Effect of D222G mutation in the hemagglutinin protein on receptor binding, pathogenesis and transmissibility of the 2009 pandemic H1N1 influenza virus. PLoS One 6, e25091. http://dx.doi.org/10.1371/journal.pone.0025091

Chan PK, Lee N, Joynt GM, Choi KW, Cheung JL, Yeung AC, Lam P, Wong R, Leung BW, So HY, Lam WY, Hui DC (2011): Clinical and virological course of infection with haemagglutinin D222G mutant strain of 2009 pandemic influenza A (H1N1) virus. J. Clin. Virol. 50, 320–324. http://dx.doi.org/10.1016/j.jcv.2011.01.013

Chen Z, Wang W, Zhou H, Suguitan AL, Jr., Shambaugh C, Kim L, Zhao J, Kemble G, Jin H (2010): Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. J. Virol. 84, 44–51. http://dx.doi.org/10.1128/JVI.02106-09

Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, van Riel D, Schrauwen EJ, Bestebroer TM, Koel B, Burke DF, Sutherland-Cash KH, Whittleston CS, Russell CA, Wales DJ, Smith DJ, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Garcia-Sastre A, Perez DR, Fouchier RA (2010): Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. J. Virol. 84, 11802–11813. http://dx.doi.org/10.1128/JVI.01136-10

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000): A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. USA 97, 6108–6113. http://dx.doi.org/10.1073/pnas.100133697

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001): Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289. http://dx.doi.org/10.1007/s007050170002

Hoffmann E, Krauss S, Perez D, Webby R, Webster RG (2002): Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 20, 3165–3170. http://dx.doi.org/10.1016/S0264-410X(02)00268-2

Ito T, Suzuki Y, Takada A, Kawamoto A, Otsuki K, Masuda H, Yamada M, Suzuki T, Kida H, Kawaoka Y (1997): Differences in sialic acid-galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. J. Virol. 71, 3357–3362.

Kilander A, Rykkvin R, Dudman SG, Hungnes O (2010): Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A (H1N1) virus and severe clinical outcome, Norway 2009–2010. Euro Surveill. 15, 19498.

Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, Daniels R, Gregory V, Uhlendorff J, Kiso M, Klenk HD, Hay A, Feizi T, Matrosovich M (2010): Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. J. Virol. 84, 12069–12074. http://dx.doi.org/10.1128/JVI.01639-10

Pan D, Xue W, Wang X, Guo J, Liu H, Yao X (2012): Molecular mechanism of the enhanced virulence of 2009 pandemic Influenza A (H1N1) virus from D222G mutation in the hemagglutinin: a molecular modeling study. J. Mol. Model. 18, 4355–4366. http://dx.doi.org/10.1007/s00894-012-1423-2

Robertson JS, Engelhardt OG (2010): Developing vaccines to combat pandemic influenza. Viruses 2, 532–546. http://dx.doi.org/10.3390/v2020532

Suphaphiphat P, Franti M, Hekele A, Lilja A, Spencer T, Settembre E, Palmer G, Crotta S, Tuccino AB, Keiner B, Trusheim H, Balabanis K, Sackal M, Rothfeder M, Mandl CW, Dormitzer PR, Mason PW (2010): Mutations at positions 186 and 194 in the HA gene of the 2009 H1N1 pandemic influenza virus improve replication in cell culture and eggs. Virol. J. 7, 157. http://dx.doi.org/10.1186/1743-422X-7-157

Totani K, Kubota T, Kuroda T, Murata T, Hidari KI, Suzuki T, Suzuki Y, Kobayashi K, Ashida H, Yamamoto K, Usui T (2003): Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides

with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses. Glycobiology 13, 315–326. http://dx.doi.org/10.1093/glycob/cwg032

Wang W, Castelan-Vega JA, Jimenez-Alberto A, Vassell R, Ye Z, Weiss CD (2010): A mutation in the receptor binding site enhances infectivity of 2009 H1N1 influenza hemagglutinin pseudotypes without changing antigenicity. Virology 407, 374–380. http://dx.doi.org/10.1016/j.virol.2010.08.027

Wanitchang A, Kramyu J, Jongkaewwattana A (2010): Enhancement of reverse genetics-derived swine-origin H1N1 influenza virus seed vaccine growth by inclusion of indigenous polymerase PB1 protein. Virus Res. 147, 145–148. http://dx.doi.org/10.1016/j.virusres.2009.10.010

WHO (2002): WHO Manual on Animal Influenza Diagnosis and Surveillance.

Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y (2006): Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 444, 378–382. http://dx.doi.org/10.1038/nature05264

Yasugi M, Nakamura S, Daidoji T, Kawashita N, Ramadhany R, Yang CS, Yasunaga T, Iida T, Horii T, Ikuta K, Takahashi K, Nakaya T (2012): Frequency of D222G and Q223R hemagglutinin mutants of pandemic (H1N1) 2009 influenza virus in Japan between 2009 and 2010. PLoS One 7, e30946. http://dx.doi.org/10.1371/journal.pone.0030946

Zheng B, Chan KH, Zhang AJ, Zhou J, Chan CC, Poon VK, Zhang K, Leung VH, Jin DY, Woo PC, Chan JF, To KK, Chen H, Yuen KY (2010): D225G mutation in hemagglutinin of pandemic influenza H1N1 (2009) virus enhances virulence in mice. Exp. Biol. Med. 235, 981–988. http://dx.doi.org/10.1258/ebm.2010.010071