Extensive Mammalian Ancestry of Pandemic (H1N1) 2009 Virus

Natalia A. Ilyushina,1 Jeong-Ki Kim,1 Nicholas J. Negovetich,1 Young-Ki Choi,1 Victoria Lang, Nicolai V. Bovin,2 Heather L. Forrest, Min-Suk Song, Philippe Noriel Q. Pascua, Chul-Joong Kim,3 Robert G. Webster, and Richard J. Webby

We demonstrate that the novel pandemic influenza (H1N1) viruses have human virus-like receptor specificity and can no longer replicate in aquatic waterfowl, their historic natural reservoir. The biological properties of these viruses are consistent with those of their phylogenetic progenitors, indicating longstanding adaptation to mammals.

In 2009, a new H1N1 influenza virus (pandemic [H1N1] 2009) emerged in Mexico, spread to the United States (1), and subsequently caused the first influenza pandemic of the 21st century (2). The emergence of pandemic (H1N1) 2009 virus is imperfectly understood, but an early switch in hemagglutinin (HA) receptor specificity is essential to allow interspecies transmission (3–5).

Pandemic (H1N1) 2009 virus strains were recently reported to be reassortants of the North American and European swine lineages (6). Phylogenetic evidence suggests that this reassortment event occurred 10–17 years ago (7). These data suggest that the current pandemic (H1N1) 2009 virus strains should have receptor specificity typically found in the HA of mammalian viruses ( Neu.5Acα2,6Gal). In addition, they may have lost the ability to replicate in avian hosts, the natural reservoir species. To test these hypotheses, we examined the biological properties of pandemic (H1N1) 2009 virus, including receptor specificity, erythrocyte binding, and ability to replicate in avian species.

The Study

We first tested species-specific erythrocyte agglutination by the pandemic (H1N1) 2009 isolates A/California/04/2009 and A/Tennessee/1-560/2009 and by other isolates from humans, swine, and birds (Table 1). The pandemic (H1N1) 2009 isolates showed reduced or absent agglutination of goose and chicken erythrocytes. Human and swine H1N1 viruses were agglutinated by turkey, guinea pig, chicken, and goose erythrocytes, and all erythrocytes we tested except those of swine were agglutinated by avian isolates (Table 1).

We next measured the receptor binding of the 2 pandemic (H1N1) 2009 isolates to sialic substrates, both natural (fetuin) and synthetic (3′-sialyllactose [3′SL] and 6′-sialyllactosamine [6′SLN] attached to a polyacrylic carrier) (Figure). The binding pattern to fetuin was identical among all isolates tested (association constant $K_a = 5.8 \pm 0.5, 1/\mu M$ sialic acid). The currently circulating human and pandemic influenza (H1N1) viruses showed a preference for 6′SLN and negligible binding to the avian-type 3′SL.

A similar pattern was observed for 2 recent swine viruses, which bound only to 6′SLN receptors with nearly equal affinity as pandemic (H1N1) 2009 isolates. As expected, the 2 avian H1 viruses bound strongly only to 3′SL (Figure).

To assess the infectivity and pathogenicity of pandemic (H1N1) 2009 virus strain A/California/04/2009 in terrestrial (chickens, quails) and aquatic (domestic and wild ducks) avian species, we inoculated 10 birds of each species by intranasal, intraocular, and intratracheal instillation with 10^6.0 of the 50% egg infectious dose (EID$_{50}$) of the virus. We then observed the birds for the next 2 weeks for death and for viral shedding and signs of illness. No birds showed obvious clinical signs of disease. Virus was detected only on postinoculation day 1 in infected chickens and ducks and only in tracheal samples at low titers (≤ 1.7 log$_{10}$ of the EID$_{50}$/mL [8]) (Table 2). However, no later shedding of virus was observed, indicating that the virus detected on postinoculation day 1 could have been caused by residual virus particles after inoculation. In contrast, our results revealed that the A/California/04/2009 strain efficiently infected quails with significantly higher titers (≥ 3.4 log$_{10}$ EID$_{50}$/mL until postinoculation day 5; p<0.05) in both oropharyngeal and cloacal swab specimens (Table 2). The virus was detected in the trachea (1.7 log$_{10}$ EID$_{50}$/g), lungs (2.3 log$_{10}$ EID$_{50}$/g), and cecal tonsil (0.8 log$_{10}$ EID$_{50}$/g) of quails on postinoculation day 5.

1These authors contributed equally to this article.
The potential bird-to-bird intraspecies transmission of the A/California/04/2009 pandemic (H1N1) 2009 virus strain in avian species was also examined by introducing 3 contact birds to the inoculated birds’ cages on postinoculation day 1. There was no subsequent evidence of viral shedding through the upper respiratory tract or fecal-oral route in any group of birds except 1 of 3 contact quails (Table 2). Oropharyngeal virus titers in this quail were 1.7 and 1.5 log_{10} EID_{50}/mL on postinoculation days 3 and 5, indicating that productive viral replication was occurring.

Conclusions

The A/California/04/2009 pandemic (H1N1) 2009 virus strain showed minimal replication and no transmission in chickens and ducks (domestic and wild), but the virus replicated and had limited transmissibility in quails. Our finding is consistent with those of Swayne et al. (9). The inability of the virus to replicate efficiently in chickens and ducks could very well be linked to its human virus–like receptor recognition.

The ability of influenza A viruses to agglutinate erythrocytes from a variety of hosts may reflect the viruses’ receptor specificity (10,11). We observed similar binding patterns for the mammalian influenza (H1N1) viruses, with the exception that the pandemic strains had reduced binding to chicken erythrocytes. This binding pattern was also observed with 1 of the swine isolates, suggesting it might be a trait of swine-adapted viruses. Taken together, a difference in the hemadsorption phenotype observed with erythrocytes from species with either less Neu5Acα2,6Gal or less Neu5Gc linkage overall could be explained by the mammalian origin of the novel pandemic (H1N1) 2009 influenza viruses.

To test this possibility, we measured the HA affinity of H1 influenza viruses from various species of origin for synthetic receptor analogues. All mammalian H1 viruses showed a typical human virus–like preference for the Neu5Asc02,6Gal-containing receptor 6′SLN. Compared with the currently circulating H1N1 human viruses, both pandemic (H1N1) 2009 strains and contemporary swine influenza virus (H1N1) strains were able to bind substantially more strongly (5–12×) to an α2,6-containing glycopolymer; the currently circulating subtype H1N1 human viruses are strictly adapted to this receptor (12). This feature demonstrated that pandemic H1N1 strains, which have a HA gene

### Table 1. Erythrocyte agglutination by representative human, pandemic, swine, and avian H1 influenza virus isolates

| Virus isolate                  | Subtype | Hemagglutination titer of erythrocytes from indicated species, HAU*† |
|-------------------------------|---------|---------------------------------------------------------------------|
|                               |         | Turkey‡ | Guinea pig‡ | Chicken§ | Goose§ | Horse¶ | Swine# |
| Human isolates                |         |         |           |          |        |        |        |
| A/Brisbane/59/2007            | H1N1    | 64      | 64        | 64       | 64     | <2     | <2     |
| A/New Jersey/15/2007          | H1N1    | 32      | 32        | 16       | 16     | <2     | <2     |
| Pandemic isolates             |         |         |           |          |        |        |        |
| A/California/04/2009          | H1N1    | 64      | 64        | 4        | 16     | <2     | <2     |
| A/Tennessee/1-560/2009        | H1N1    | 32      | 32        | <2       | 8      | <2     | <2     |
| Swine isolates                |         |         |           |          |        |        |        |
| A/swine/North Carolina/007270/2008 | H1N1 | 32    | 64        | 8        | 16     | <2     | <2     |
| A/swine/Iowa/003479/2009      | H1N1    | 64      | 64        | 32       | 32     | 2      | <2     |
| Avian isolates                |         |         |           |          |        |        |        |
| A/mallard/Alberta/66/2007     | H1N4    | 64      | 64        | 32       | 32     | 16     | <2     |
| A/mallard/Alberta/496/2008    | H1N4    | 64      | 64        | 32       | 32     | 16     | <2     |

*HAU, hemagglutination units. †Titers are expressed as the reciprocal of the highest virus dilution that yields complete HA agglutination. ‡Neu5Acα2,6Gal > Neu5Asc02,3Gal. §Neu5Acα2,6Gal < Neu5Asc02,3Gal. ¶Neu5Asc02,3Gal. #Neu5Asc02,6Gal > Neu5Gc02,3Gal.
of swine lineage, have retained their current swine virus–
like binding characteristics despite their efficient spread in
humans.

To identify substitutions in the HA molecule that
could be responsible for the human-like receptor bind-
ning phenotype of the pandemic and contemporary swine
influenza (H1N1) isolates, we compared the H1 HA se-
quences deposited in the Influenza Research Database
(www.fludb.org). We observed that 99.99% of all swine
viruses isolated after 1980 have Asp190 or Asn190. HA
sequences of swine viruses isolated before 2000 harbor
virus–like binding characteristics despite their ef
cient spread in mammals. The human-like amino acids encoded at HA po-
positions 190 and 225 in the novel pandemic and swine in-
fluenza (H1N1) viruses may at least partially explain their
innate affinity for the human-type receptor.

Recent phylogenetic analysis showed that each seg-
ment of the pandemic (H1N1) 2009 virus is nested within
a well-established swine influenza lineage for >10 years
before the recent outbreak (7). Hence, the ancestors of this
virus circulated undetected for about a decade before the
virus emerged in humans. Our finding that contemporary
swine viruses acquired the ability to recognize 6′SLN with
at least 5-fold higher affinity than did human strains and
completely lost the ability to bind to Neu5Ac2,3Gal pro-
duces clear evidence to support this hypothesis. It is pos-
sible that the progenitors of pandemic (H1N1) 2009 virus
were accumulating enough mammal-associated changes to
allow a refinement of their receptor-binding properties. Our
findings substantiate that strong mammalian-like receptor
specificity is a critical barrier to infection of various hosts
with pandemic (H1N1) 2009 virus. Other biological fac-
tors associated with their adaptation and tissue tropism in
humans will likely be identified in the future.

Acknowledgments

We thank Marie R. Gramer for provision of A/swine/North
Carolina/007270/2008 (H1N1) and A/swine/Iowa/003479/2009
(H1N1) influenza A virus strains; Elena A. Govorkova for helpful
discussion; Jon P. Seiler for technical assistance; Betsy Williford
for illustrations; and David Galloway and Sharon Naron for edito-
rial assistance.

This research was funded through contract no. HH-
SN266200700005C from the National Institute of Allergy and
Infectious Diseases, National Institutes of Health, Department
of Health and Human Services; a TBP grant from the Korea
Research Council of Fundamental Science & Technology; grant no.
KGM3110912 from the Korea Research Institute of Bioscience
and Biotechnology Research Initiative Program of Korea; a Presi-
dium Grant “Molecular and Cell Biology” from the Russian
Academy of Sciences; and the American Lebanese Syrian Asso-
ciated Charities.

Dr Ilyushina is a postdoctoral research fellow in the Division
of Virology, Department of Infectious Diseases, St. Jude Chil-
dren’s Research Hospital, Memphis, Tennessee, USA. She has
research interests in the molecular basis of viral pathogenesis and
antiviral drug usage for the control of influenza infection, includ-
ing emerging influenza (H1N1) and (H5N1) viruses.

References

1. Centers for Disease Control and Prevention. Swine influenza A
(H1N1) infection in two children—southern California, March–April
2009. MMWR Morb Mortal Wkly Rep. 2009;58:400–2.
2. World Health Organization. Global Alert and Response. Pandemic
(H1N1) 2009—update 58. 2009 Jul 6 [cited 2009 Jul 9]. http://www.
who.int/csr/don/2009_07_06/en/index.html
3. Nicholls JM, Chan RWY, Russell RJ, Air GM, Peiris JSM. Evolving
complexities of influenza virus and its receptors. Trends Microbiol.
2008;16:149–57. DOI: 10.1016/j.timi.2008.01.008
4. Stevens J, Blixt O, Glaser L, Taubenberger JK, Palese P, Paulson
JC, et al. Glycan microarray analysis of the hemagglutinins from
modern and pandemic influenza viruses reveals different recep-
tor specificities. J Mol Biol 2006;355:1143–55. DOI: 10.1016/j.
jbmb.2005.11.002
5. Matrosovich M, Tuzikov A, Bovin N, Gambaryan AS, Klimov A,
Castrucci MR, et al. Early alterations of the receptor-binding prop-
erties of H1, H2, and H3 avian influenza virus hemagglutinins after
their introduction into mammals. J Virol. 2000;74:8502–12. DOI:
10.1128/JVI.74.18.8502-8512.2000
6. Garten RJ, Davis CD, Russell CA, Shu B, Lindstrom S, Bal-
ish A, et al. Antigenic and genetic characteristics of swine-origin
2009 A (H1N1) influenza viruses circulating in humans. Science.
2009;325:197–201. DOI: 10.1126/science.1176225
7. Smith GJD, Vijaykrishna D, Bahl J, Lye C, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 2009;459:1122–5. DOI: 10.1038/nature08182
8. Reed LJ, Muench H. A simple method for estimating fifty percent endpoints. Am J Hyg. 1938;27:493–7.
9. Swayne DE, Pantin-Jackwood M, Kapczynski D, Spackman E, Suarez DL. Susceptibility of poultry to pandemic (H1N1) 2009 virus [letter]. Emerg Infect Dis [serial on the Internet]. 2009 Dec [cited 2009 Nov 18]. http://www.cdc.gov/EID/content/15/12/2061.htm
10. Ito T, Suzuki Y, Mithauli L, Vines A, Kida H, Kawaoka Y. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology. 1997;227:493–9. DOI: 10.1006/viro.1996.8323
11. Medeiros R, Escrivó N, Naffakh N, Manuguerra JC, van der Werf S. Hemagglutinin residues of recent human A (H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology. 2001;289:74–85. DOI: 10.1006/viro.2001.1121
12. Mochalova L, Gambaryan A, Romanova J, Tuzikov A, Chinnaraj D, et al. Receptor-binding properties of modern human influenza viruses primarily isolated in Vero and MDCK cells and chicken embryonated eggs. Virology. 2003;313:473–80. DOI: 10.1016/S0042-6822(03)00377-5
13. Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, et al. The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science. 2004;303:1838–42. DOI: 10.1126/science.1093155

Address for correspondence: Robert G. Webster, Department of Infectious Diseases, St. Jude Children’s Research Hospital, 262 Danny Thomas Pl, Memphis, TN 38105-3678, USA; email: robert.webster@stjude.org