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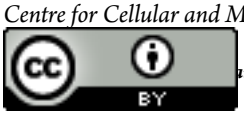
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SAXENA, S., MISHRA, N., SAXENA, R., SWAMY, M., SAHGAL, P., SAXENA, S., TIWARI, S., MATHUR, A., NAIR, M.. Structural and antigenic variance between novel influenza A/H1N1/2009 and influenza A/H1N1/2008 viruses. The Journal of Infection in Developing Countries, North America, 4, nov. 2009. Available at:

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Emerging Problems in Infectious Diseases

Structural and antigenic variance between novel influenza A/H1N1/2009 and influenza A/H1N1/2008 viruses

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Abstract

Background: The emergence of influenza A/H1N1/2009 is alarming. The severity of previous epidemics suggests that the susceptibility of the human population to H1N1 is directly proportional to the degree of changes in hemagglutinin/HA and neuraminidase/NA; therefore, H1N1/2009 and H1N1/2008 were analyzed for their sequence as well as structural divergence.

Methodology: The structural and sequence divergence of H1N1/2009 and H1N1/2008 strains were analyzed by aligning HA and NA amino acid sequences by using ClustalW and ESyPred3D software. To determine the variations in sites of viral attachment to host cells, a comparison between amino acid sequences of HA and NA glycosylation sites was performed with NetNGlyc software. The antigenic divergence was executed by CTL epitope prediction method.

Results: The amino acid homology levels of H1N1/2009 were 20.32% and 18.73% compared to H1N1/2008 for HA and NA genes, respectively. In spite of the high variation in HA and NA amino acid composition, there was no significant difference in their structures. Antigenic analysis proposes that great antigenic differences exist between both the viral strains, but no addition of a new site of glycosylation was observed.

Conclusions: To our knowledge, this is the first report suggesting that the circulating novel influenza virus A/H1N1/2009 attaches to the same glycosylation receptor sites as its predecessor influenza A/H1N1/2008 virus, but is antigenically different and may have the potential for initiating a significant pandemic. Our study may facilitate the development of better therapeutics and preventive strategies, as well as impart clues for novel H1N1 diagnostic and vaccine development.

Key words: swine flu, Influenza A H1N1 2009, H1N1

J Infect Dev Ctries 2010; 4(1):001-006.

(Received 1 October 2009 – Accepted 22 November 2009)

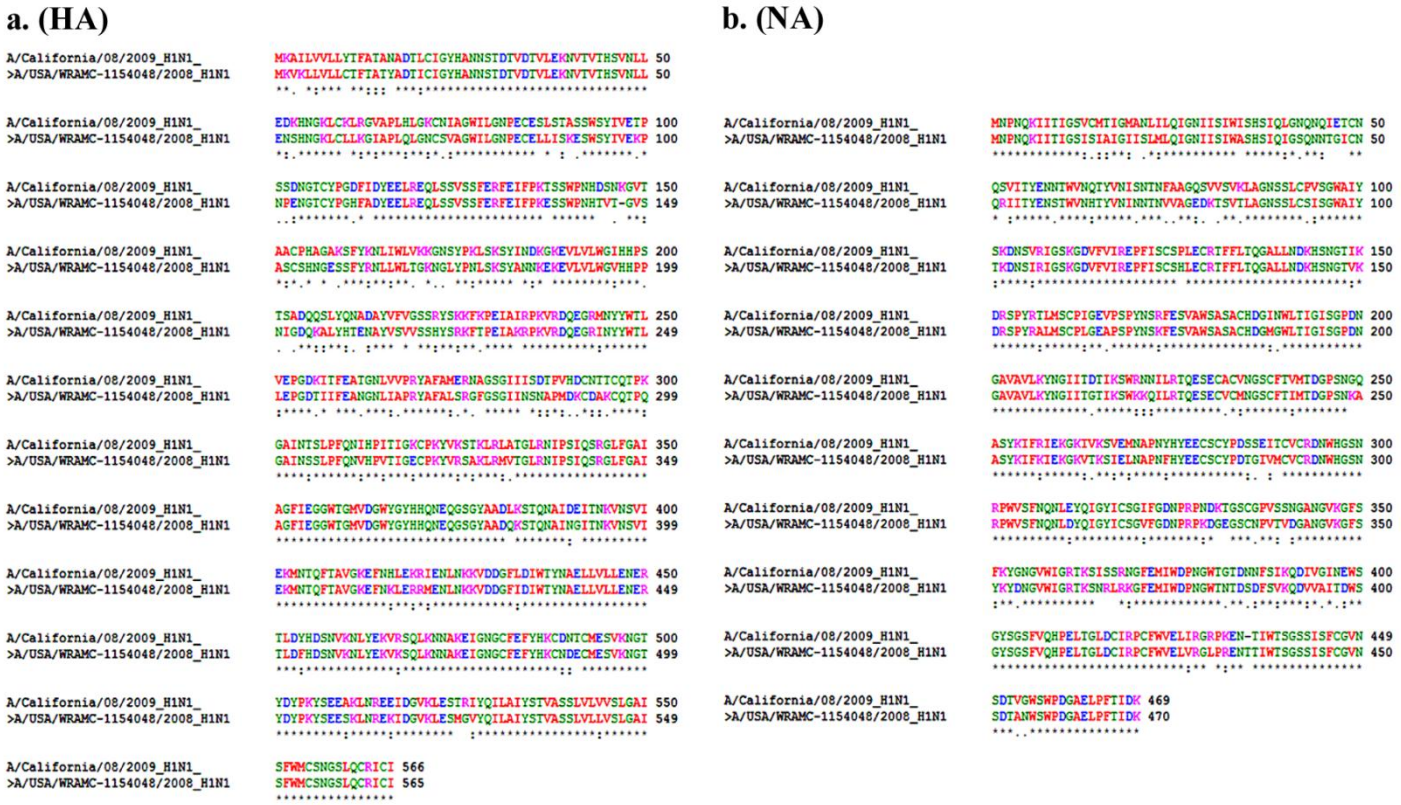
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Introduction

A global pandemic of swine flu, a new strain of influenza A virus subtype H1N1, is currently underway. According to the World Health Organization (WHO), the epidemic started in early April and by September 20, 2009, over 318,925 cases with at least 3,917 deaths (CFR ~1.2%) were reported globally [1], including 10,233 cases and 315 deaths in India [2]. H1N1/2009 originated due to triple reassortment among North American swine (30.6%) and avian influenza (34.4%), human influenza (17.5%), and classical swine influenza virus (17.5%) [3]. Influenza A/H1N1 causes acute febrile respiratory tract infection by infecting epithelial respiratory cells. H1N1 continues to circulate and causes annual epidemics that kill approximately 0.25 to 0.5 million people worldwide [4,5,6,7]. H1N1 has a unique capacity for genetic variation. Hemagglutinin (HA) and Neuraminidase (NA) are two surface glycoproteins of the virus and are the most important antigens for inducing protective immunity in the host [8]. The severity of previous epidemics suggests that susceptibility of the human population to H1N1 is directly proportional to the degree of change in HA and NA. Greater change results in lower herd immunity and higher susceptibility [9].

We therefore analyzed HA and NA of the novel circulating influenza A/H1N1/2009 strain and compared it with its predecessor influenza A/H1N1/2008 virus for sequence variation as well as

Figure 1. ClustalW amino acid sequences alignment of HA (a) and NA (b) proteins of influenza H1N1/2009 and its predecessor H1N1/ 2008.



The amino acid sequences of segment 4 of HA protein (a) and segment 6 of NA protein (b) are shown for the Influenza A/California/08/2009(H1N1) and A/District of Columbia/WRAMC-1154048/2008(H1N1) viruses as described earlier. Standard single-letter abbreviations for the amino acids are used. The collinear sequences were aligned by online use of ClustalW. Amino acid alignment exhibits non-conservative substitutions (" "), conservative substitutions (":") and semi-conservative substitutions ("."). Conserved regions are represented as ("*"). There are 20.32% differences in 566 positions.

structural and antigenic divergence for a better understanding of viral pathogenesis and antigenicity.

Materials and Methods

Sequences used in study

For comparison between circulating 2009 and 2008 strains, we used Influenza A/California/08/2009 [submitted to NCBI by Shu *et al.* (29 April 2009) with accession numbers FJ971076 for HA and FJ966973 for NA] and Influenza A/USA/WRAMC-1154048/2008 [submitted to NCBI by Houng *et al.* (1 Feb 2008) with accession numbers CY038770 for HA and CY038772 for NA] H1N1 strains. These sequences were used because A/California/08/2009 was the primary strain that led to the swine flu 2009 pandemic and A/USA/WRAMC-1154048/2008 was its predecessor H1N1 isolated in 2008.

Sequence divergence

We analyzed the sequence divergence of H1N1/2009 and H1N1/2008 strains by aligning HA (Figure 1a) and NA (Figure 1b) amino acid sequences by using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

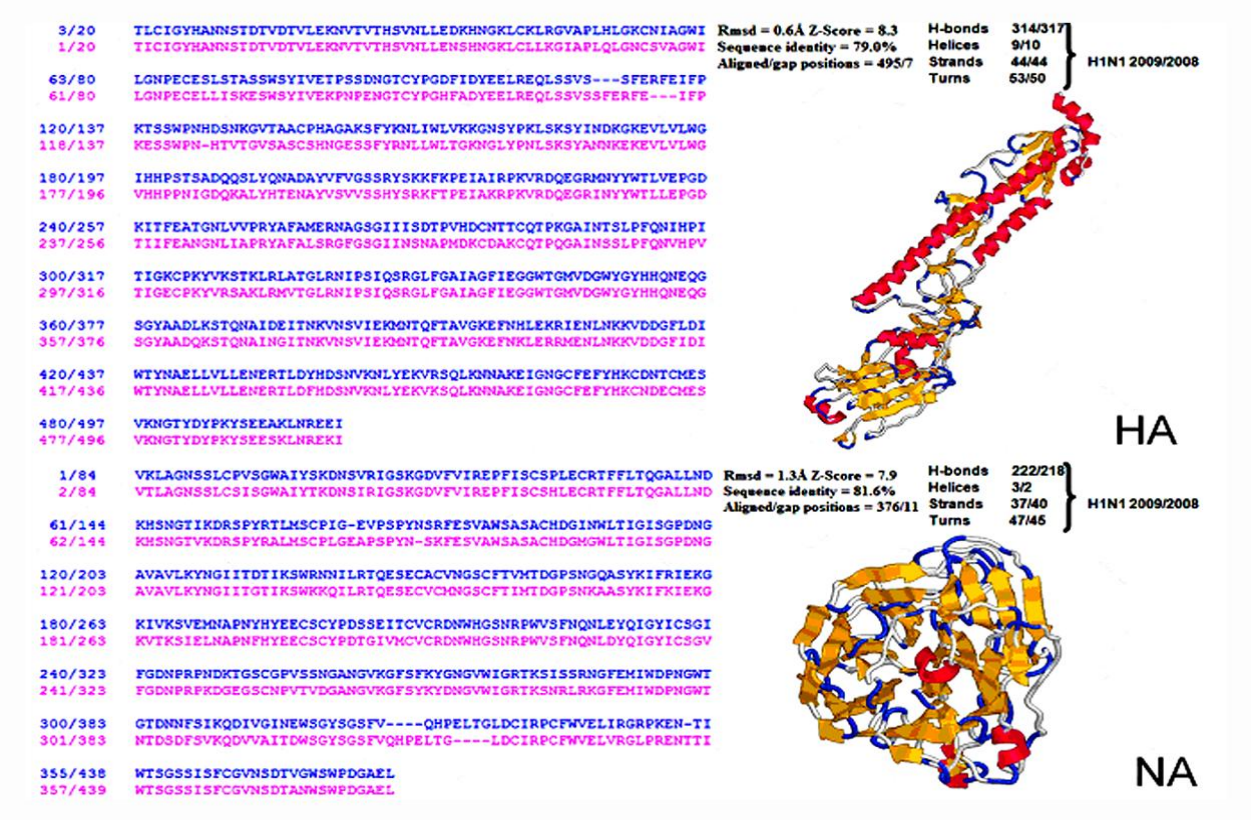
Differentiation in glycosylation pattern

To determine the variation in the sites of viral attachment to host cells, a comparison between amino acid sequences of HA and NA glycosylation sites was performed with NetNGlyc 1.0 software (http://www.cbs.dtu.dk/services/NetNGlyc/) (Table 1).

Antigenic vitiations

The antigenic divergence between 2009 and 2008 influenza A strains was executed by the CTL epitope prediction method. The amino acid sequences of HA and NA were evaluated separately with CTLPred software (http://www.imtech.res.in/raghava/ctlpred/) using consensus approach. The predicted antigenic sites were compared for HA and NA, respectively (Table 2).

Figure 2. Structural comparison of HA and NA of 2009 and 2008 strains of influenza A/H1N1.



Amino acid sequences were submitted to ESyPred3D software to derive structures for the HA and NA protein for both isolates A/California/08/2009(H1N1) and A/District of Columbia/WRAMC-1154048/2008(H1N1). The obtained PDB files were visualized by RasMol 2.7.5 and secondary structures are compared as shown in the figure. The obtained structure of both the proteins has been also shown in the figure. Both the PDB files of 2009 and 2008 strains for HA and NA proteins were submitted to CE server for structural deviation. The structural alignment and the alignment characteristics are presented in the figure.

Structural divergence

To determine the structural divergence in HA and NA proteins, the amino acid sequences of HA and NA were independently analyzed with ESyPred3D software (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/). Deviations between obtained structures were calculated (http://cl.sdsc.edu/ce/ce_align.html) considering high sequence similarity (Figure 2). Structures were visualized by RasMol 2.7.5. Secondary structures of both proteins from both isolates were compared.

Results

The most abrupt changes in antigenic specificity occurred through the HA and NA genes. Analysis exhibited an overall sequence homology of 79% in HA and 81% in NA among 2009 and 2008 viral strains. Our results revealed that the amino acid homology levels of H1N1/2009 were 20.32% (conservative 9.89%, semi-conservative 4.95% and non-conservative substitutions 5.48%) and 18.73% (conservative 9.15%, semi-conservative 5.32% and non-conservative 4.26%) compared to H1N1/2008 for HA (Figure 1a) and NA (Figure 1b), respectively. Secondary structure comparison suggests that the HA protein of H1N1/2009 (H-bonds: 314; Helices: 9; turns: 53; and strands: 44) and H1N1/2008 isolates (H-bonds: 317; Helices: 10; turns: 50; and strands: 44) as well as the NA protein of H1N1/2009 (H-bonds: 222; Helices: 3; turns: 47; and strands: 37) and H1N1/2008 isolates (H-bonds: 218; Helices: 2; turns: 45; and strands: 40) have variations. However, our structural comparison based on CE server analysis between H1N1/2009 and H1N1/2008 suggests that in spite of high variations in HA and NA amino acid composition and differences in secondary structure, there was no significant difference in their structure (HA; Rmsd = 0.6 angstrom and NA; Rmsd = 1.3 angstrom) (Figure 2).

Table 1. Comparison of N-glycosylation sites between HA and NA of 2009 and 2008 influenza A/H1N1 strains.

Protein	N-glycosylation			
	A/H1N1/2009		A/H1N1/2008	
	Position	Sequence	Position	Sequence
HA	28	NSTD	28	NSTD
	40	NVTV	40	NVTV
	104	NGTC	104	NGTC
	304	NTSL	303	NSSL
	498	NGTY	497	NGTY
	557	NGSL	556	NGSL
	–	–	71	NCSV
	–	–	176	NLSK
	–	–	142	NHTV
NA	50	NQSV	44	NNTG
	58	NNTW	58	NSTW
	63	NQTY	63	NHTY
	68	NISN	70	NNTN
	88	NSSL	88	NSSL
	146	NGTI	146	NGTV
	235	NGSC	235	NGSC
	–	–	434	NTTI

The table shows a comparison of predicted N-glycosylation sites in amino acid sequences for segment 4 (HA) and segment 6 (NA) sequences of the isolate A/California/08/2009(H1N1) and A/District of Columbia/WRAMC-1154048/2008(H1N1). N-glycosylation potential (0.5) is taken as cutoff.

* Red colour indicates the differences between N-glycosylation sites of isolates A/California/08/2009(H1N1) and A/District of Columbia/WRAMC-1154048/2008(H1N1)

glycosylation in H1N1/2009 (Table 1); however, antigenic analysis proposes considerable antigenic differences between both the viral strains (Table 2). To our knowledge, this is the first report suggesting that the circulating virus H1N1 uses the same glycosylation sites for its attachment to receptors, but is antigenically different.

Discussion

Considering the penetrance and global spread of swine flu, the World Health Organization has declared a world pandemic for the viral illness [10]. Sequence BLAST analysis of HA and NA genes of 2009 viral strains reveals that the closest relatives of H1N1/2009 are A/Swine/Indiana/P12439/00 and A/Swine/England/195852/92 [9]. This observation suggests that somehow these viruses were transported from the United States of America and the United Kingdom to Mexico and transmitted to swine. The present report of the current outbreak suggests that

H1N1/2009 is neither similar to the 1918 pandemic influenza virus (18% different) nor to the 1976 swine flu (12% different) [9,11]. Our amino acid sequence divergence analysis suggests a large variation in HA and NA proteins, but most of the substitutions are conservative and semi-conservative. We therefore have not observed any significant difference in the structure of HA and NA proteins, a finding which highlights the large structural flexibilities of HA and NA proteins. Contrary to H1N1/2009, the most successful pandemic influenza viruses have retained the core proteins of the virus and changed only HA and NA.

There is no difference in glycosylation sites between the presently circulating virus and H1N1/2008. Glycosylation of HA and NA represent the characteristic of the pathogen to escape the host defense through co-evolution with the host and identification of the host receptor [12]. Our antigenic analysis shows that H1N1 strains of 2009 and 2008 have large differences in antigenicity. This finding might be correlated with the large penetrance of H1N1/2009 because this strain has novel antigenicity; therefore, the human population lacks herd immunity [8]. High variation in amino acid sequences and the unique antigenicity of H1N1/2009 suggest that although the virus infection currently is not severe, it has further pandemic potential [9,13]. Developing countries have higher risk of infection, circulation for a longer time period, and further pandemic evolution [13]. Co-infections during bouts of influenza might play a crucial role in the evolution of H1N1 and may cause the development of resistance to known antivirals. Although the current strain of H1N1 has low virulence, mortality during infection has been observed [5].

Table 2. Comparison of antigenicity between HA and NA of 2009 and 2008 influenza A/H1N1 strains.

Antigenicity				
Protein	A/H1N1/2009		A/H1N1/2008	
	Position	Sequence	Position	Sequence
HA	53	KHNGKLCKL	53	SHNGKLCLL
	157	GAKSFYKNL	156	GESSFYRNL
	160	SFYKNLIWL	159	SFYRNLLWL
	395	KVNSVIEKM	394	KVNSVIEKM
	437	TYNAELLVL	436	TYNAELLVL
NA	100	YSKDNSVRI	14	SIAIGIISL
	183	ACHDGINWL	61	WVNHTYVNI
	250	QASYKIFRI	100	YTKDNSIRI
	298	GSNRPWVSF	365	NRLRKGFEM
	418	IRPCFWVEL	418	IRPCFWVEL

The table shows a comparison of antigenic sites in the amino acid sequences of HA (segment 4) and NA (segment 6) of isolates A/California/08/2009(H1N1) and A/District of Columbia/WRAMC-1154048/2008(H1N1).

* Red colour indicates the differences between antigenic sequences of isolates A/California/08/2009(H1N1) and A/District of Columbia/WRAMC-1154048/2008(H1N1).

The evolution of H1N1 2009 by triple reassortment from three different hosts and co-infections with other influenza A viral strains is an alarming concern because it suggests that the virus is not only assorting in multiple hosts, but also getting more chances to reassort in humans. Along with antigenic shift and antigenic drift, H1N1 may evolve into a novel influenza A supervirus, which may be antigenenically unique. It may then transmit as well as infect and replicate in multiple hosts and may have resistance to known antivirals; therefore, future preparedness is mandatory. Long-term preventive measures should be considered along with short-term preventions [14]. Apart from viral factors, host factors may play an important role in influencing the dynamics of H1N1 infection [15]. Earlier serological evidence suggests that, due to immunity from prior exposure to the H2N2 influenza strains before 1900, the elderly were not affected severely in the 1957 epidemic [16], but because the present influenza A/H1N1/2009 is a novel strain and has not been reported earlier, similar antigenic protection is not anticipated. The lack of pro-human adaptive molecular markers in the currently circulating strain suggests the involvement of new determinants responsible for transmission of the virus to human and low infection [8]. This virus, therefore, can be used to study the involvement of new determinants, which may help us to develop effective vaccines against lethal H1N1 strains.

Collectively, our results highlight the need for studies on the evolution of H1N1 immunity, and for the first time, provide evidence that H1N1/2009 uses the same glycosylation sites as its predecessor H1N1/2008 and may have a potential to initiate a more seriously mortal pandemic, owing to its antigenic difference with H1N1/2008. Our study may facilitate the development of better therapeutics and preventive strategies.

Acknowledgments

The authors are grateful to the Council of Scientific and Industrial Research (CSIR), India, and Director, Centre for Cellular and Molecular Biology, for the encouragement and support for this work. We also thank Dr. Archana B. Siva and Ms Ira Bhatnagar for reviewing this manuscript. S.K.S. and M.P.N. are also supported by NIH (USA) MERIT Award R37DA025576. N.M. gratefully acknowledges a CSIR-JRF/NET research fellowship.

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Conflict of Interest: No conflict of interests is declared.