

Special features of the 2009 pandemic swine-origin influenza A H1N1 hemagglutinin and neuraminidase

VAVRICKA Christopher John^{1*}, LIU Yue¹, LI Qing^{1,2}, SHI Yi^{1,3}, WU Yan¹, SUN YePing¹, QI JianXun¹ & GAO George Fu^{1,2,3,4*}

¹ CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China;

² School of Life Sciences, University of Science and Technology of China, Hefei 230027, China;

³ Graduate University of Chinese Academy of Sciences, Beijing 100049, China;

⁴ Beijing Institutes of Life Sciences, Chinese Academy of Sciences, Beijing 100101, China

Received March 14, 2011; accepted April 11, 2011; published online May 10, 2011

Since the 2009 pandemic H1N1 swine-origin influenza A virus (09 S-OIV) has reminded the world about the global threat of the ever changing influenza virus, many questions regarding the detailed re-assortment of influenza viruses yet remain unanswered. Influenza A virus is the causative agent of the pandemic flu and contains 2 major antigenic glycoproteins on its surface: (i) hemagglutinin (HA); and (ii) neuraminidase (NA). The structures of the 09 S-OIV HA and NA proteins (09H1 and 09N1) have recently been resolved in our laboratory and provide some clues as to why the 09 S-OIV re-assortment virus is highly infectious with severe consequences in humans. For example, the 09H1 is highly similar to the HA of the 1918 influenza A pandemic virus in overall structure and especially in regards to its 5 defined antibody binding epitopes. For 09N1, its most distinctive feature is the lack of a 150-loop active site cavity, which was previously predicted to be present in all N1 NAs, and we hypothesize that the 150-loop may play an important role in the substrate specificity (α 2,3 or α 2,6 linked sialic acid receptors) and enzymatic mechanism of influenza NA. Combination of the HA and NA with special characteristics for the 09 S-OIV might contribute to its high increased transmissibility in humans.

2009 pandemic H1N1, hemagglutinin (HA), neuraminidase (NA), swine-origin influenza virus (S-OIV), reassortment

Citation: Vavricka C J, Liu Y, Li Q, et al. Special features of the 2009 pandemic swine-origin influenza A H1N1 hemagglutinin and neuraminidase. *Chinese Sci Bull*, 2011, 56: 1747–1752, doi: 10.1007/s11434-011-4517-9

In early 2009, severe cases of H1N1 influenza A virus broke out primarily in Mexico. On April 29, 2009, the World Health Organization (WHO) raised its alert level for this influenza outbreak to Phase 5, the highest level ever announced since the warning system was introduced in 2005 (WHO). By June 11, 2009, this pandemic H1N1 virus had officially been reported in 74 countries and the WHO alert level was then raised to Phase 6, the pandemic level (WHO). In 2010, reports of pandemic H1N1 infection significantly declined and on August 10, 2010 the WHO declared that the pandemic was over (WHO). As of August 6,

2010, the 2009 pandemic H1N1 had been attributed to the deaths of over 18449 individuals (WHO).

Previous influenza pandemics include the 1918 ‘Spanish Flu’ (H1N1), ‘Asian Flu’ of 1957 (H2N2), and ‘Hong Kong Flu’ of 1968 (H3N2) [1]. Like the 2009 pandemic virus, the 1918 virus was also an H1N1 influenza A virus and it is estimated to have led to the deaths of over 40 million people (WHO). The 1957 H2N2 Asian flu is estimated to have killed about 2 million people and the 1968 H3N2 Hong Kong flu is estimated to have killed about 1 million people, (WHO). Although the 2009 H1N1 virus was raised to the pandemic level by the WHO, only about 20000 deaths have been attributed to it, comparable to that of the seasonal flu

*Corresponding authors (email: chris@im.ac.cn; gaof@im.ac.cn)

(WHO). However, like the 1918 pandemic virus, the 2009 pandemic virus often leads to severe respiratory problems in young, pregnant and healthy individuals [2].

Influenza viruses can be categorized into 3 types: A, B and C. Influenza A virus is the causative agent of pandemic flu. The influenza A virus genome contains 8 RNA segments which encode up to 12 proteins: NP, NS1, NS2, M1, PA, PB1, PB2, PB1-F2, PB1-N40 and 3 surface proteins, M2, HA and NA [1,3]. HA (hemagglutinin) and NA (neuraminidase) are the principal influenza surface antigens and contain multiple serotypes based upon their antigenicity [1]. HA is responsible for sialic acid receptor binding, facilitating the process of virus and host endosomal membrane fusion and allowing the viral genetic materials to gain entry into the cytoplasm [1]. NA, which is a sialidase, catalyzes hydrolysis of terminally linked sialic acid receptors and functions as the receptor destroying element of influenza A and B viruses, thereby helping newly formed virions to break free from infected cells and to continue infecting new cells [1]. HA is grouped into 16 different known serotypes and NA is grouped into 9 [1,4]. Therefore influenza A virus strains are named based upon their combination of HA and NA (e.g. H1N1, H5N1, H3N2) [1]. Certain HA/NA virus types are highly specific for different animal hosts which is promoted, partially, by evolutionary differences in host immune systems and host restriction factors [4,5]. For example, H5N1 is highly common in birds whereas H3N2 is a common seasonal flu strain in humans [5]. Furthermore, human seasonal flu strains produce more variation in their HA/NA antigenic sites due to a higher selective pressure in the human immune system [6].

During infection of a host with multiple influenza A virus strains, reassortment of RNA segments from different viruses into new budding virions may occur [7]. The 2009 pandemic swine-origin H1N1 influenza A virus (09 S-OIV) is a reassortment with PB2 and PA derived from avian influenza, PB1 derived from human H3N2, HA, NP and NS derived from classical swine H1N1, and NA and M derived from a Eurasian avian like swine H1N1 influenza lineage [8–10]. Both the 1957 H2N2 pandemic and the 1968 H3N2 pandemic were also reassortments between avian and human viruses [11]. Highly pathogenic avian influenza viruses, such as H5N1 are often very deadly to humans, however infection of human with these avian influenza viruses is rare [12]. Recently it has been confirmed that multiple reassortments of avian H5N1 with human H3N2 can lead to increased virulence in mice [13]. It is therefore a serious concern that highly pathogenic avian influenza strains may gain higher transmissibility in humans through an intermediate mixing vessel; especially swine [14].

According to the current understanding of influenza virus interspecies transmission, swine may act as a primary mixing vessel between avian and human virus strains [14]. HA and NA receptor specificities likely play an integral role in determining the basis for influenza host specificity. In gen-

eral, avian hosts primarily contain α 2,3 linked sialic acid receptors and avian influenza virus preferentially binds the α 2,3 receptor, however the upper respiratory tract of humans contains primarily α 2,6 linked sialic acid receptors [15–17]. This may explain why human influenza viruses preferentially bind the α 2,6 receptor and is considered a major reason why avian influenza virus is not highly transmissible in humans. Swine contain both α 2,3 and α 2,6 linked sialic acid receptors in their respiratory system and possess a less complex immune system than humans, which may explain why they are more susceptible to infection by avian and human strains of influenza and why swine influenza viruses may easily evolve the ability to bind the human α 2,6 receptor [15–17]. The recent 2009 pandemic H1N1 is the first pandemic influenza re-assortment virus with a primarily swine-influenza origin, which is a major reason why it has caused great concern [18–21].

Although other influenza proteins like PB1 and PB2 also play an important role in pathogenicity, HA and NA are on the front line of the process of virus entry, fusion and release of progeny virions. Furthermore, these two viral envelope glycoproteins are the primary targets of the immune system and interact directly with the receptors, and therefore are critical elements in determining host cell specificity [1,4]. For these reasons, the unique properties of the 09H1 and 09N1 can provide many insights into the increased transmissibility and pathogenicity of the 09 S-OIV.

1 HA

HA binds primarily to α 2,3 or α 2,6 linked sialic acid receptors on the mammalian cell surface acting as the receptor binding element of type A and B influenza viruses [1,4]. The receptor binding function of HA is the first step of the influenza infection cycle and there has been a great deal of research into its function [22]. The sialic acid binding site is located in the head region of each HA molecule [22]. Influenza HA contains various *N*-glycosylation sites and is secreted as a homotrimer to the membrane/viral envelope where it is anchored via transmembrane helices [22,23]. Cleavage of each HA monomer into two polypeptides, HA1 and HA2, by cellular proteases is necessary for HA to facilitate host-virus membrane fusion [22].

Sequence analysis indicates that 09H1 has recently evolved and that two major subtypes of 09 S-OIV emerged, with a Mexico and New York origin [24,25]. The structure of 09HA has been reported by several research groups and the detailed structure is strikingly similar to 1918 pandemic H1 HA (18H1) [6,26,27]. The similarity is especially pronounced in regards to the 5 defined antibody binding sites [6,7]. This can explain why older generations which have been exposed to the 1918 pandemic H1N1 have resistance to 09 S-OIV [26]. In a similar manner, it has recently been

reported that survivors of the 09 S-OIV have acquired an effective immune response against many influenza strains [28]. Furthermore, neither 18H1 nor 09H1 contain conserved *N*-glycosylation sites in the head region which may interfere with receptor binding and the lack of *N*-glycosylation in the head region has been shown to increase their receptor binding affinities [29].

Another primary similarity of 18H1 with 09H1 is the sialic acid receptor specificity, which is the critical determinant of host range as demonstrated in numerous previous studies [30,31]. Most importantly, the 190 and 225 residues (all HA residues here are H1 numbered) have been identified to play a predominant role in determining binding specificity of H1 [32]. For 09H1, the Asp190/Asp225 pair is responsible for specifically recognizing only the α 2,6 human receptor (unpublished data), whereas other matching patterns such as Glu190/Gly225 and Asp190/Gly225 are regarded as crucial factors for binding α 2,3 receptors in avian hosts and both receptors in swine, respectively [22,33,34]. Moreover, recent analysis using glycan microarrays also has revealed that Asp225Glu/Gly substitution results in slightly or moderately enhanced binding affinity towards a broader range of different α 2,3 receptor analogs [35]. Based on the binding modes in 30H1 and 34H1 with the human and avian receptors [32], 09H1 with the Gly225 substitution may bind to both human and avian receptors [36]. Regarding the Asp225Glu substitution, it was hypothesized that the longer glutamate side chain might facilitate the interactions with Gal-2 of the α 2,3 avian receptor [36], however, in our recent unpublished studies, the Asp225Glu mutant of 09H1 preferentially binds to the α 2,6 human receptor due to a different mechanism.

09H1 contains additional special features worth mentioning that distinguish it from other HA proteins, including 18H1. For example, 09H1 contains larger basic patches relative to all other reported HA structures (Figure 1) [6]. The His/Lys basic patch is also relatively strong in the 18H1 structure and is hypothesized to be involved in facilitating membrane fusion [36,37]. Additionally, there is an extra *N*-glycosylation site in 09H1 close to this very basic patch (Figure 1) [6]. This *N*-glycosylation site may modulate the effect of the stronger basic patch or may play a role in masking antibody recognition. The precise effects of the basic patch and additional *N*-glycosylation site on the function of HA deserve further research and investigation.

2 NA

There are currently 9 different serotypes of NA, which can be further classified into two separate groups based upon phylogenetic sequence analysis. Group 1 consists of N1, N4, N5 and N8, and group 2 consists of N2, N3, N6, N7 and N9 [38]. Before the structure of 09N1 was released, all published group 1 neuraminidase structures contained an addi-

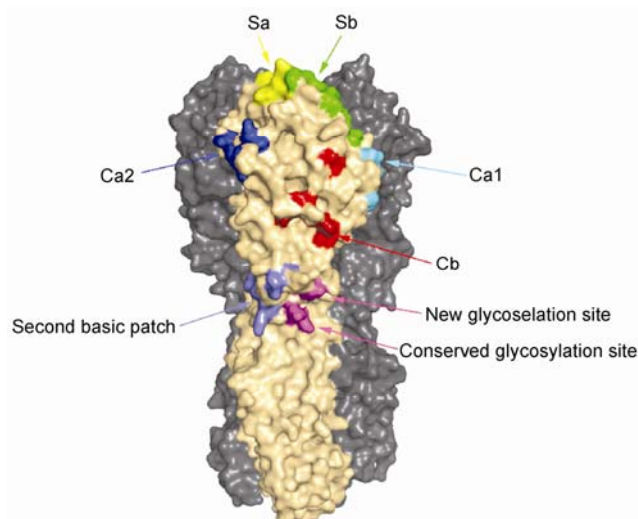


Figure 1 Overall structure of 09H1 with an emphasis on the classical antigenic sites, second basic patch and *N*-glycosylation sites adjacent to the second basic patch. Modified from Zhang et al. [6] (with permission from *Protein & Cell*).

tional 150-loop cavity, which is not present in all other currently reported group 2 neuraminidase structures [38]. This led to the correlation of this structural feature with the primary sequence of NA [38]. However, our recent work has revealed that the 09N1 crystal structure actually does not contain the 150-loop cavity (Figure 2) [39]. This indicates that the formation of the 150-cavity (at least in the crystal state) may possibly be influenced by a single amino acid at position 149 (Ile or Val, all NA numbering here is based on N2) and its coordination with the 430-loop, rather than entire NA amino acid sequence [39]. Nevertheless, in the case of N2 structures, a salt bridge between Asp147 and His150 appears to be the major factor in stabilizing the 150-loop in its closed conformation [39]. Still, the 150-loop is likely very flexible and should adopt various open and closed conformations in solution [40]. However, the 09N1 150-loop may be thought of as an intermediate between the typical group 1 NAs with the 150-loop cavity in their crystal structures and typical group 2 NAs with no 150-loop cavity.

There is a highly conserved network of electrostatic interactions between the NA active site and its ligand, sialic acid, consisting of residues Arg118, Glu119, Asp151, Arg152, Trp178, Arg224, Glu276, Glu277, Arg292, Arg371 and Tyr406. These amino acid residues are 100% conserved in all of the current available NA structures from both groups (although Arg292 is replaced by Lys in an artificial drug resistant Australia N9 strain) [41,42]. Most of these residues are involved in substrate binding or may stabilize the enzymatic transition state; however Asp151, which is in the 150-loop, and Tyr406 are likely to directly participate in the catalytic mechanism (based upon mechanistic studies in bacterial NA), although it is possible that water molecules may replace the function of these two residues leading to alternative mechanisms [41,43–45].

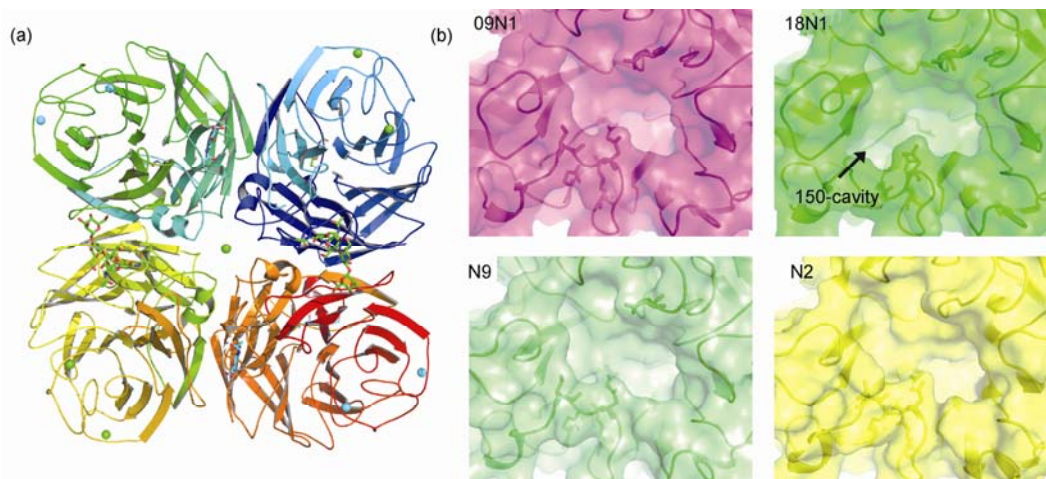


Figure 2 Overall structure of the 09N1 tetramer (a) and comparison of the active sites of 09N1, 18N1, N9 and N2, illustrating the 150-cavity deficient active site of 09N1 (b).

We hypothesize that the 150-loop is important for substrate binding and that the conformation of the 150-loop plays an important role in determining the specificity of NA for $\alpha 2,3$ or $\alpha 2,6$ linked sialic acid. The ability to obtain the first sialic acid complex structure with a group 1 NA using 09N1 may indicate the importance of the 150-loop in substrate binding (unpublished data). The 150-loop of influenza NA contains a strictly conserved Asp151 residue, which we also hypothesize may be directly involved in the sialidase mechanism of influenza NA. In the influenza NA-sialic acid complex structures, Asp151 participates in multiple hydrogen bonds with sialic acid, including the anomeric hydroxyl, which forms the glycosidic bond in the $\alpha 2,3$ or $\alpha 2,6$ receptor. Asp151 in the NA structures with an open 150-loop conformation is shifted over 1 Å from the sialic acid binding site relative to the structures with a closed 150-loop conformation (Figure 3). Based on these observations, we propose a 3 step model: (i) the flexible 150-loop may assist binding of $\alpha 2,3$ or $\alpha 2,6$ linked substrate in the open conformation; (ii) hydrolysis of the glycosidic bond may be facilitated by movement of the loop back toward the substrate via Asp151; and (iii) release of product sialic acid via movement of the 150-loop into the open conformation. In this way the 150-loop may act as a switch between substrate binding, subsequent NA activity and release of product sialic acid. We speculate that 09N1 may be like an intermediate state between the typical group 1 (150-cavity) and group 2 (no 150-cavity) structural types and this special feature of 09N1 may confer some advantage to the 2009 H1N1 pandemic virus which is highly transmissible in humans. Clearly, more detailed biochemical and structural studies are needed to test these hypotheses.

3 Discussion and perspective

Because HA and NA bind sialic acid in an independent

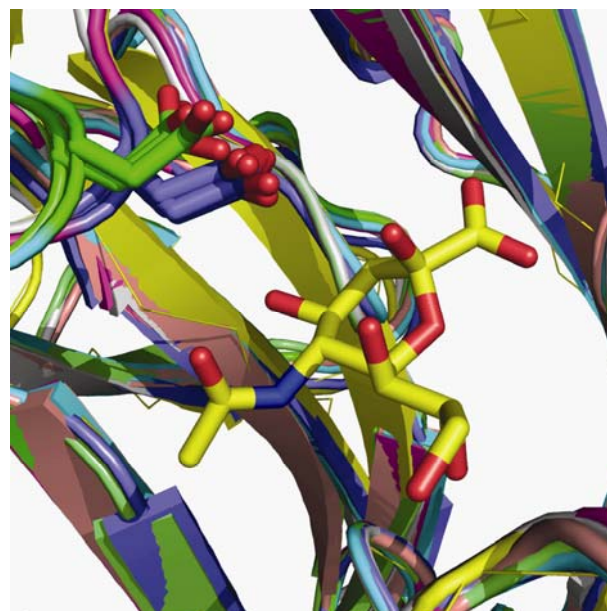


Figure 3 Comparison of 150-loop orientations and the position of Asp151 using structures from all the currently available NA serotypes in the protein data bank (PDB) (N4, N8, 18N1 and avian N1 with an open 150-loop conformation; N2, N6, N9 and 09N1 with a closed 150-loop conformation). The distance between Asp151 in the open and closed conformations is over 1 Å. Sialic acid (yellow) is included from the Tokyo N2 structure (2BAT). Conserved Asp151 residues from structures with an open 150-loop conformation are colored green and Asp151 from structures with a closed 150-loop conformation are colored blue. PDB IDs: 18N1 – 3BEQ, avian N1 – 2HTY, Tokyo N2 – 2BAT, N4 – 2HTV, N6 – 1VOZ, N8 – 2HT5 and Australia N9 – 1NCA.

manner, it should not be possible that NA cleaves the sialic acid glycosidic bond while it is bound to HA. Therefore, there must be a critical balance between the expression levels, substrate binding, specificities and activities of each HA-NA pair. If the affinity of HA to its receptor is too high relative to the NA affinity toward the same receptor, the virus will be efficient at infecting new cells, but ineffective

at release and vice versa. Although immunity is also an important factor, this may explain why certain HA-NA serotype combinations are more common than others and some are never observed. Recent data suggests that combination of 09N1 with 18H1 results in a higher infectivity, however the 09H1 with 18N1 (1918 pandemic N1) does not (Figure 4) [46]. Because 09H1 and 18H1 are highly similar, it is difficult to say the advantage of 18H1 in this system. Perhaps the stronger basic patch and extra *N*-glycosylation site of 09H1 exerts a significant effect on its function. Furthermore, the propensity of the 150-loop in 09N1 towards the closed state may be a major factor in determining the advantage over the 18N1 in regards to pairing with 18H1. One might speculate that if the 09N1 has higher activity than 18N1 than it is better balanced with 18H1 which may have better entry ability than 09H1 (or the opposite situation), however further experiments are necessary to arrive at any conclusion.

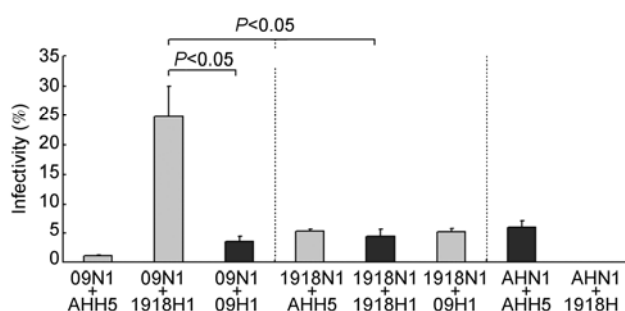


Figure 4 Infectivity of normalized influenza pseudotyped particles (pps) with various HA/NA combinations; infectivity is presented as the Mean±SD percentage of infected cells from 3 repeats. All pps were grouped by NA; 2009 H1N1, 1918 H1N1, and H5N1 (A/Anhui/1/2005) are abbreviated as 09, 1918, and AH, respectively. Reprinted from Zhang et al. [46].

There has been a great deal of interest and research into the α 2,3 and α 2,6 specificity of HA and the structural basis of substrate binding. Some studies have investigated the specificity of various NAs to α 2,3 vs α 2,6 linked receptor substrates [47,48], however no specificity rule for NA has been firmly established and the structural basis of the binding to α 2,3 or α 2,6 substrates is uncharacterized. The detailed characterization of NA specificity is essential to understanding HA-NA pairing. Further research regarding the receptor recognition of both HA and NA including not only specificity but relative affinity and covering a variety of prevalent mammalian sialic acid containing receptors will provide a strong basis for the mechanisms of HA and NA pairing. This will contribute to the comprehensive understanding of influenza virus re-assortment.

The authors thank Dr. Yoshihiro Kawaoka and Dr. Takeshi Noda for providing the electron micrograph image of influenza virions for our cover design. This work was supported by Chinese Academy of Sciences Research Fellowship for Young International Scientists (2010Y2SB12) and the National Natural Science Foundation of China for International Young

Scientists (31050110126) to Vavricka CJ, and the National Natural Science Foundation of China (81021003) to Gao GF.

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