

Influenza Surveillance: 2014-15 H1N1 'swine' derived influenza viruses from India

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Summary: The 2014-15 H1N1 outbreak in India has reportedly lead to 800 fatalities. The reported influenza hemagglutinin sequences from India indicate that these viruses contain amino acid changes linked to enhanced virulence and are potentially antigenically distinct from the current vaccine containing 2009 (Cal0709) H1N1 viral hemagglutinin.

Between 2009-2012, the 2009 pandemic H1N1 (2009pdmH1N1) virus is estimated to have caused over 18,449 deaths across 214 countries worldwide (Cheng et al., 2012). Since the initial outbreak, the 2009pdmH1N1 has replaced the prior seasonal H1N1, and established itself in the human population. This is largely due to sequence evolution of the hemagglutinin (HA) protein, whose activity critically governs the receptor binding, fusion and transmission properties of the virus (Smith et al., 2009).

The recent 2014-15 H1N1 outbreak in India has resulted in >8,000 cases with over 800 deaths, although remains unclear if this is an underestimation. Anecdotal reports indicate that the majority of these cases involve young adults - a trend that is similar to the 1918 Spanish Flu pandemic when 50-100 million people died worldwide. One recent news report from India indicates that influenza genes sequenced from patient swab samples revealed no new mutations in the virus (<http://indianexpress.com/article/cities/pune/silver-lining-no-mutation-of-h1n1-says-study/>). Additionally, it was suggested the 2009pdmH1N1 outbreak strain - A/California/04/2009 - was responsible for the outbreak in India.

Continuous surveillance of influenza viruses enables researchers to track viral evolution, understand amino acid sequences in key viral proteins governing their circulation, predict potential “outbreaks” and assist in the development of various “outreach” approaches to both treat as well as prevent further spread. Typically, influenza-genome data collected from field studies or research efforts are sequenced and submitted to GenBank and/or one or more specialized open-access databases. Open access databases such as National Center for Biotechnology Information (or NCBI) Influenza Virus Resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>), Influenza Research Database (<http://www.fludb.org/>) and EpiFlu (<http://platform.gisaid.org/epi3/start>) facilitate sharing of viral genome sequences and encourage collaborative research world over. In addition to providing access to nucleotide and amino acid sequences, these specialized databases provide researchers additional information such as genetic markers (e.g. drug resistance, increased virulence etc.) and form the basis of epidemiological and clinical data and tools for analyzing the genomic sequences e.g. sequence comparison and alignment, phylogeny tree construction, epitope prediction and mapping. An analysis of the publicly available influenza databases from the recent outbreak in India suggests that influenza monitoring has not yet reached sufficient levels to enable real-time surveillance.

Overall, there exists 15,173 H1N1 pdm HA sequences in the public sequence databases, out of which 4,213 represent full-length, non-redundant entries. Examination of the geography of the

isolated strains shows that the majority of the deposited influenza sequences come from US (38.4%), China (7.2%), United Kingdom (6.5%) and Singapore (6%). Unfortunately, India ranks low (14th) in this list, contributing less than 1.5% of sequences. Furthermore, despite the vastness of the Indian subcontinent, only two sequences have been deposited during 2014-15 from India, suggesting poor surveillance and potentially limiting the response to a deadly outbreak. Additionally, swine can also contribute to the emergence of novel H1N1 variants through the process of reverse zoonoses and thus should also be monitored. Despite the risk posed by these animals, the number of swine influenza sequences collected in the 2009-2015 period is insignificant with notably no swine influenza strains deposited from India. These numbers highlight the irregular, reactive nature of the influenza surveillance response.

Although there are limited Indian-origin sequences available in the public database to make any causal inference on the perceived increased fatalities in India, examination of the 2014 Indian H1N1 sequences shows traits with potential cause for concern. Amino acid changes in specific positions in the receptor binding site (RBS) of 2009pdmH1N1 have been shown to impact glycan RBS specificity and have been linked to increased virulence and disease severity. Among these changes, the Indian-origin strain A/India/6427/2014 contains amino acid changes T200A and D225N compared to the current vaccine 2009 H1N1 strain. The T200A amino acid change has been shown to improve human glycan receptor-binding of 2009pdmH1N1 HA (Xu et al., 2012b). The D225N mutation has been linked to increased virulence and disease severity in patients infected by the 2009 pdm virus (Ruggiero et al., 2013). Importantly, a previous study showed that the D225N mutation in the context of H1 HA affected receptor binding and also decreased susceptibility to NA inhibitors (McKimm-Breschkin et al., 2013). It should be noted that the D225N mutation was previously linked to serious illness resulting in hospitalization or death (L'Vov D et al., 2010). The high population density in India, ease of person-to-person transmission and lack of effective treatment options create ample opportunities for this variant to sustain and become dominant. The severity of the current outbreak seems to correlate with this observation.

Gene reassortment wherein segments of the genome are exchanged between different strains is another mechanism that drives rapid influenza evolution. Indeed, the previous three pandemics emerged as a result of gene reassortment. The 1957 H2N2 (Asian flu) pandemic emerged through reassortment between human H1N1 and avian H2N2. Similarly, the H3N2 (Hong Kong) pandemic was caused by a human-adapted H2N2 virus as it obtained avian H3 and PB1 genes through reassortment). The 2009 swine-origin H1N1 pandemic emerged as a result of reassortment between avian, human and swine influenza viruses. India houses billions of farmed birds and swine animals across the country. Combined with this, export of animals and challenges to farming infrastructure augment the risk of reassortment events. Although the 2014 Indian-origin strains appear to have not undergone reassortment, the involvement of gene reassortment in the current outbreak in India cannot be determined without full genome sequence information.

Since 2009, HAs of the 2009pdmH1N1 lineage have gradually acquired mutations in the H1 antigenic sites (Sa, Sb, Ca & Cb) (Caton et al., 1982) (**Fig. 1**). Notably, strains carrying 7 antigenic-site mutations appeared in 2013, which has implications for re-evaluation of the H1N1 vaccine component (A/California/04/2009). Importantly, the mutation K166Q at the 'Sa' antigenic site discriminated strains that circulated before 2013 from those that circulated during 2014-15. While the majority of strains that circulated before 2013 possessed Lys at 169, >80% of the strains that circulated after 2013, including the two 2014 Indian isolates, possessed a Gln. Importantly, a previous study showed that a variation at 166 could lead to escape from neutralizing antibodies elicited by the current H1N1 vaccine component A/California/07/2009 (Linderman et al., 2014). The K166Q mutation may also exert an influence on the receptor binding property of the HA due to its close proximity to the receptor binding site (**Fig. 2**). Additionally, while the antigenic residues N129 (Sa), G158 (Sa) and N159 (Sb) (**Fig. 2**) were observed at relatively lower, fluctuating frequencies before 2013, they continually increased in frequency and became more dominant after 2013. In summary, the set of mutations that characterize the 2014 Indian strain are T200A and D225N and K166Q. Additionally, as noted above antigenic residues (N129, G158, N159) are also important HA residue changes observed in the 2014 Indian derived HA. These extensive amino acid changes observed in 2014 Indian influenza HA contradict the recent reports by Indian news media on a lack of mutations observed in the H1N1 viruses in India.

Given the global reach of influenza, there is an urgent need to develop a comprehensive and at least somewhat standardized response to influenza epidemic outbreaks. Authorities and health officials should document outbreaks with limited delays. As was the case with the H7N9 outbreak, there needs to be genetic and phenotypic analysis of the virus and general dissemination of the data to ensure access to real-time information. For many strains, only the HA gene is sequenced leaving the rest of the genome incomplete. This is because many of these efforts are part of research studies that focus on receptor binding or immune response. Additionally the cost of sequencing is still prohibitively expensive for many. All the above factors pose challenges for real-time surveillance.

In the context of various outreach towards slowing or halting an epidemic outbreak, access to antivirals and vaccines both play a large part; thus a priority should be development of infrastructure to make, store, and distribute appropriate countermeasures. In this case, stockpiling of antivirals for targeted administration can buy time to allow for a widespread vaccination campaign. Additionally, the development of a universal vaccine can go a long way in mitigating the risk of high mortality.

One response to epidemic outbreaks is to identify vaccine strategies to abbreviate the time lag between advent of a novel virus strain and the manufacture of vaccine, such as through synthetic approaches (Dormitzer et al., 2013) or use of alternative vaccine formats, such as virus-like particles (Fries et al., 2013). An alternative approach may be through targeted use of prophylactic anti-virals. Modeling of such an approach for malaria has indicated that judicious use of artemisinin can affect spread (Peak et al., 2015). Similar epidemiological modeling of influenza epidemic outbreaks may

allow for such strategies, such as potentially targeting high risk populations such as health care workers with a prophylactic long-lived antiviral to possibly mute outbreaks, allowing time for implementation of vaccine strategies.

Even in the event of the absence of a successful vaccination strategy or the development of resistance to neuraminidase inhibitors, the use of antibody to treat influenza has some clinical experience to support efficacy. For example, a recent study evaluated the use of convalescent plasma in 93 patients with H1N1 2009 influenza in Hong Kong (Hung et al., 2011) In this prospective multi-center case control study, patients with severe influenza who were hospitalized and required intensive care unit support, were recruited and offered convalescent plasma containing influenza neutralizing antibody in addition to the standard of care with a neuraminidase inhibitor, either oseltamivir or zanamivir. Mortality was significantly lower in the treatment groups who received convalescent plasma compared to the controls (20.0% vs. 54.8%, $p=0.01$). To this end, there have been several reports of broadly neutralizing antibodies for influenza, which clearly neutralize influenza virus *in vitro*, provide complete protection after a single administration *in vivo*, and protect against multiple strains of influenza (Corti et al., 2011; Ekiert et al., 2009).

In summary, the influenza outbreak in India should be further examined to further determine the virulence and potential threat of this virus. Improved surveillance and monitoring of the influenza outbreak will significantly enhance the options of how best we can manage outreach to both treat as well as prevent spread of the virus.

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FIGURE LEGEND

Fig. 1. Phylogenetic tree of 1,000 representative H1N1 HA amino acid sequences belonging to the 2009 pdm lineage generated by Neighbor Joining method. Due to the sheer density of nodes and branches, only a handful of strains are labeled (by strain name and subtype). Clades are defined based on high bootstrap confidence values (>70%) at every node. Branches corresponding to the Indian H1N1 strains are highlighted in green. Scale bar indicates the number of nucleotide substitutions per site. The vast majority of the strains in clades I-III were isolated between 2009-2013 whereas the strains in clades IV and V were isolated between 2010-2015. The Indian H1N1 strains are clustered in clades IV and V except for a single strain that is part of clade II.

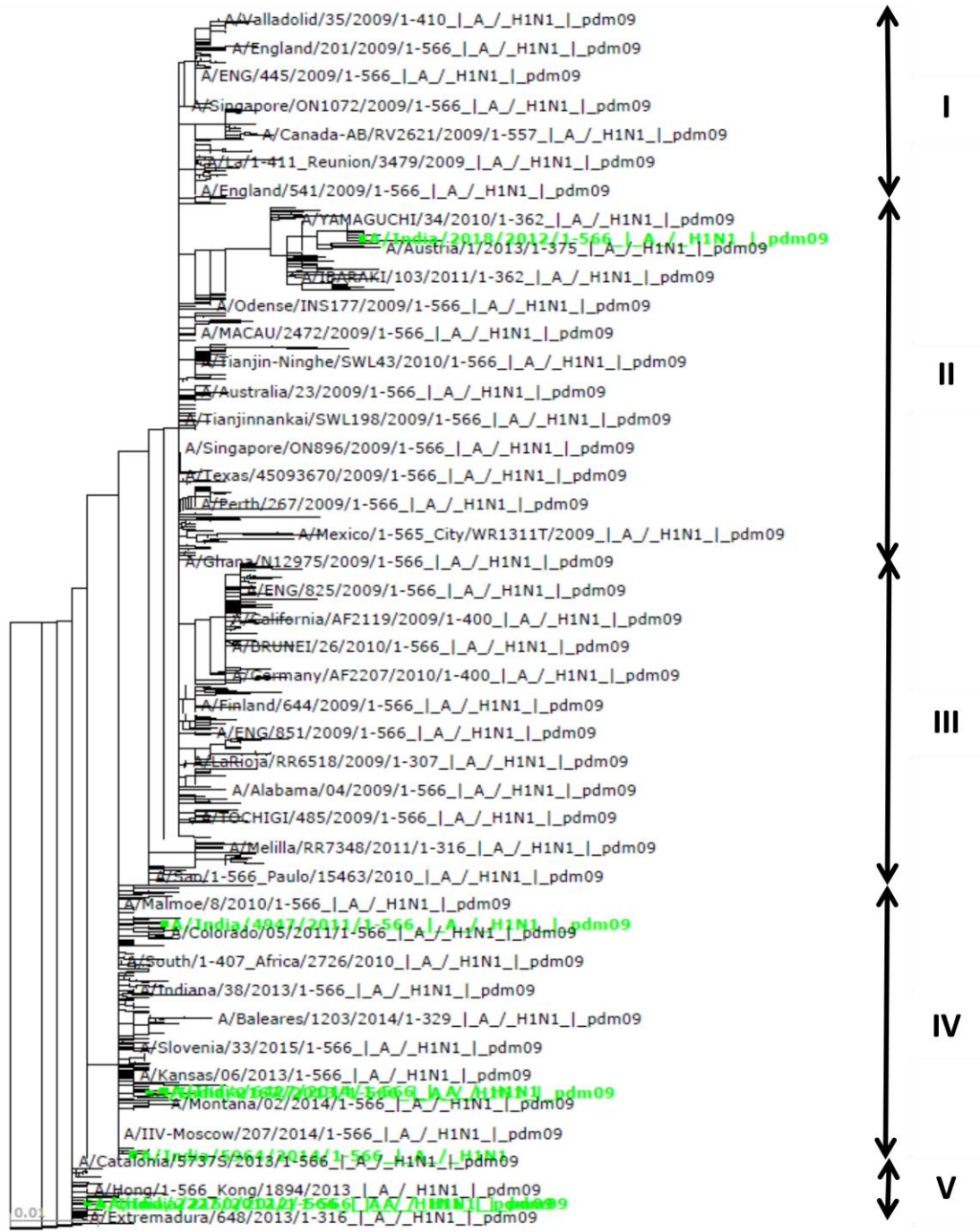
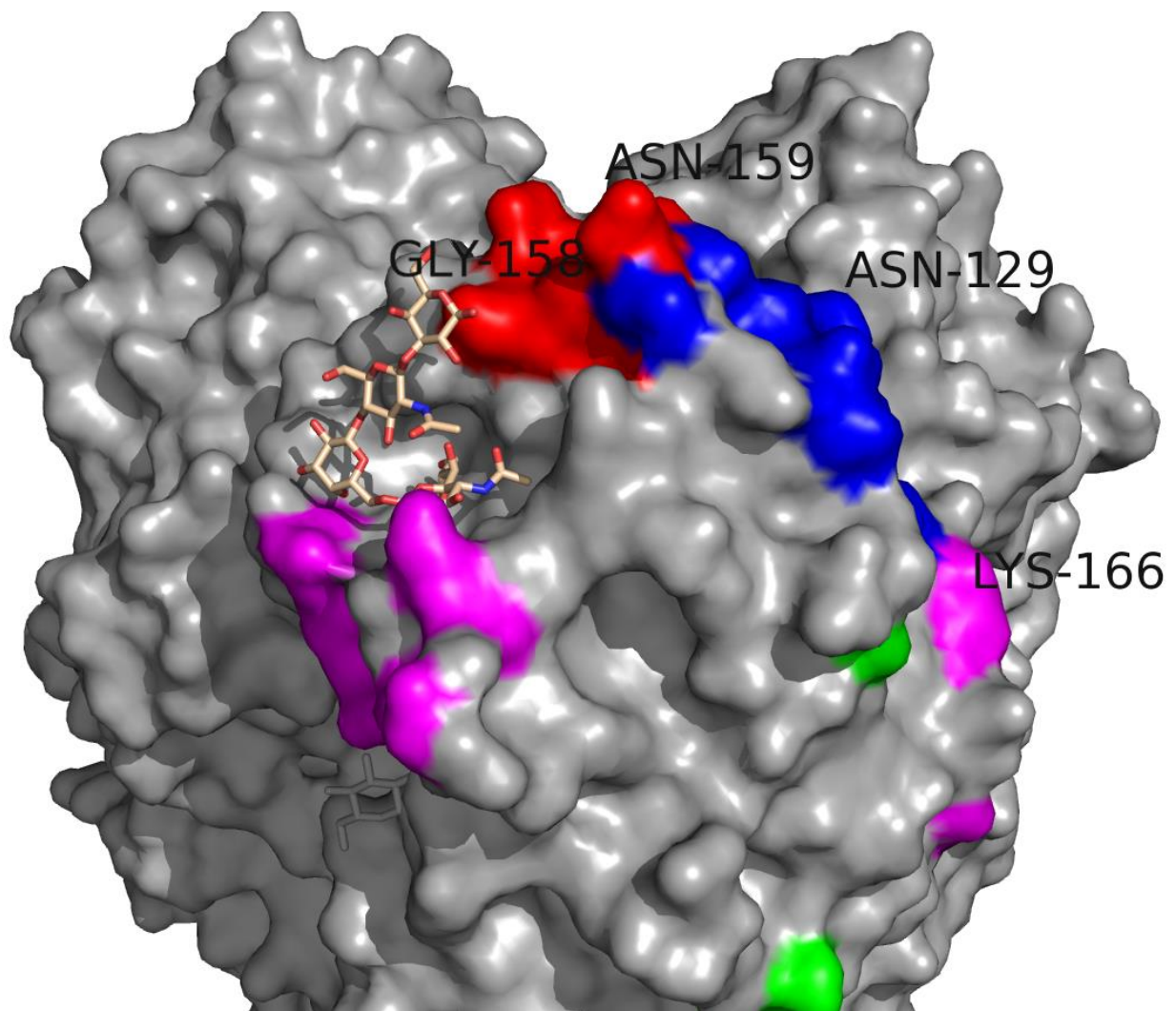


Fig. 2. Three-dimensional X-ray crystal structure of trimeric HA1 globular head of A/California/04/2009 in complex with human receptor LSTc (Xu et al., 2012a)(PDB: 3UBE). The view of the trimer is along axis perpendicular to 3-fold symmetry axis. The antigenic sites Sa, Sb, Ca and Cb are colored blue, red, magenta and green, respectively. The human receptor is represented in a stick format in tint color. The approximate locations of the antigenic sites mentioned in the text are marked on the structure.



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