Molecular character of influenza A/H1N1 2009: Implications for spread and control

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Abstract The world is experiencing a pandemic of influenza that emerged in March 2009, due to a novel strain designated influenza A/H1N1 2009. This strain is closest in molecular sequence to swine influenza viruses, but differs from all previously known influenza by a minimum of 6.1%, and from prior “seasonal” H1N1 by 27.2%, giving it great potential for widespread human infection. While spread into India was delayed for two months by an aggressive interdiction program, since 1 August 2009 most cases in India have been indigenous. H1N1 2009 has differentially struck younger patients who are naïve susceptibles to its antigenic subtype, while sparing those >60 who have cross-reactive antibody from prior experience with influenza decades ago and the 1977 “swine flu” vaccine distributed in the United States. It also appears to more severely affect pregnant women. It emanated from a single source in central Mexico, but its precise geographical and circumstantial origins, from either Eurasia or the Americas, remain uncertain. While currently a mild pandemic by the standard of past pandemics, the seriousness of H1N1 2009 especially among children should not be underestimated. There is potential for the virus, which continues to adapt to humans, to change over time into a more severe etiologic agent by any of several foreseeable mutations. Mass acceptance of the novel H1N1 2009 vaccine worldwide will be essential to its control. Having spread globally in a few months, affecting millions of people, it is likely to remain circulating in the human population for a decade or more.

Keywords Influenza · Swine flu · H1N1

Introduction

The world is currently experiencing a level 6 pandemic of influenza [1]. Within the first six months of the outbreak, the World Health Organization (WHO) reported 340,000 laboratory-confirmed cases and 4100 deaths in 191 countries around the globe, due to a novel strain of influenza virus that emerged in Mexico in March 2009 [2]. These figures are gross underestimates of the number of actual cases, considered by epidemiologists to be many millions. Even in the cases of deaths, there is still underreporting, though to a lesser degree. We are just entering the first major influenza season in the winter months in the Northern Hemisphere since the virus emerged. A major increase in influenza activity is expected that will dwarf what has occurred thus far. We are in the midst of a pandemic of acute respiratory disease unlike anything seen in decades.

The etiologic agent is a new strain of influenza type A virus, A/H1N1 2009. As we previously described in early May [3], this virus is about 6% different from any known influenza virus in nature, and 27.2% different from its predecessor, the 2008 “seasonal flu” strain of H1N1. The latter difference, which has been termed a “pseudo-shift” in viral protein sequence [4], gives this influenza strain great potential for widespread human infection.

Nevertheless, by the truly gargantuan standards of past influenza pandemics, this outbreak is still relatively mild. The severity of illness, measured as morbidity and mortality, is less than in past pandemics, as is the “attack rate”, the percentage of the population affected. However, the virus has the potential to better adapt to replication in humans, and to mutate over time into a more severe pathogen.

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The roots of this pandemic lie in the molecular characteristics of the genome and proteins of this novel strain of influenza, and the purpose of this article is to delineate those as they affect the prospects for continuance of the pandemic, as well as for its control.

Defining influenza, the virus

Influenza virus is an enveloped virus with an RNA genome, belonging to the family *Orthomyxoviridae* [5]. The RNA genome is segmented into 8 different RNA molecules, each with a nucleocapsid protein (NP) coat. Most segments code for only one protein of the virus, including all of those that code for the three principal structural proteins, the NP and the two surface glycoproteins. Influenza viruses are first classified into broad types by the antigenic properties of NP, into influenza A, B and C. The first of the glycoproteins, called the hemagglutinin (HA), is responsible for attachment and entry into susceptible cells, is the principal protective antigen of the virus, and, as such, is the principal target of influenza vaccines. The second glycoprotein, the neuraminidase (NA), is a mucus-digesting enzyme that releases nascent virus from the cellular surface and debris, facilitating spread of the virus through the respiratory tract. Antibody to NA also reduces the severity of influenza, and the importance of this enzyme to the virus is underscored by the fact that neuraminidase is the target of the two drugs currently licensed against influenza, Tamiflu (oseltamivir) and Relenza (zanamivir). Influenza viruses of type A are subtyped by the antigenic characteristics of the HA and NA glycoproteins. Widespread human infection has for over a century been limited to viruses with HA subtypes H1, H1 and H3, and with NA subtypes of N1 and N2 [6, 7]. Additional criteria used to identify an influenza virus include the source location, an identifying number, and the year of isolation. Thus, a compound designation is used for each isolate. For the current outbreak, the prototype isolate on 9 April 2009 is designated A/California/7/2009 (H1N1).

Three other RNA segments code for the three subunits of the influenza replicase, responsible for both replication of the genome and expression of viral messenger RNA. One of these, PB1, also codes for a second non-structural protein in reading frame 2. This protein, called PB1-F2, contains a pro-apoptotic peptide region [8–10]. Both of the two remaining genome fragments produce two proteins each, through alternate splicing. The segment 7 produces the matrix (M1) protein associated with the viral envelope. M1 and M2, present only in influenza A, a viroporin facilitating permeability of infected cells. Segment 8 produces protein NS1 that counters human interferon [11], and a nuclear export protein (NEP) that facilitates movement of influenza

genomes across the nuclear membrane early in influenza replication [12]. All of the 11 viral proteins are essential in infection even in cell culture, except PB1-F2, M2 and NS1.

The impact of influenza as an infectious disease is due entirely to two major and unique molecular features of the virus. The segmented genome allows for independent reassortment of viral genes. Positive mutations in one gene are genetically segregated from deleterious mutations in another. Also, the HA and NA proteins are capable of an enormous degree of variability, up to 50%, of their protein sequence while still remaining functional [13]. This molecular plasticity, coupled with the capability for frequent genetic reassortment, can generate an enormous level of virus variation in response to its host environment, most especially the antiviral antibodies raised in humans against it.

The natural host of influenza viruses is migratory waterfowl, and avian influenza spreads separately through each of the Western and Eastern Hemispheres through natural bird migrations [5, 14]. From this avian source, pigs become infected, and this serves as an intermediate host adapting the virus to mammalian host cells, from which most human influenza arises. Swine influenza is endemic except in commercial production operations where vaccine is routinely used. The virus evolves more slowly in swine than in humans at its antigenic epitopes. Swine live a maximum of 5 years as breeding sows, and only a few months when produced for human consumption, so they have little ongoing immunologic memory. Humans, on the other hand, present a highly selective immune environment for a lifetime exceeding 70 years. Crossover of swine influenza into humans is quite rare, and almost never leads to serial infection in humans. Before 2009, the biggest exceptions to this rule were the 1918 pandemic influenza virus (which may also have had a direct avian source), and 230 cases of the 1976 swine influenza outbreak in New Jersey in the United States [15, 16]. The H1N1 2009 virus thus represents a very rare event, the crossover of swine influenza into a human with secondary spread in the worldwide population. Once this event occurred, however it occurred, swine do not contribute to the further spread of the virus in humans at all.

Defining influenza, the illness

The actual term used to describe the disease is “influenza-like illness” (ILI), that is, as it directly implies, a rather uncertain diagnosis. Both the WHO and its US counterpart, the Centers for Disease Control and Prevention (CDC) have launched websites to monitor the pandemic and guide public policy [see <http://www.cdc.gov/h1n1flu/>]. Clinical influenza

varies greatly and overlies in symptoms a broad spectrum of acute viral respiratory infections. Classical influenza occurs during the winter months, November through March in the Northern Hemisphere, and May through September in the Southern Hemisphere, usually with a hiatus in influenza activity the other months of the year. This sharp seasonal appearance is ensconced in its name beginning in the 18th century, originally “*influenza di freddo*” in Italian, i.e. “influence of cold”, and is related to aerosol dynamics and low humidity in colder weather [17]. ILI refers to any febrile, prostrating respiratory disease of probable viral origin. Even during this pandemic, most cases of ILI will be identified on clinical grounds alone, without viral isolation or amplification of the viral genome, or even a specific antigen detection or serological diagnosis, and will thus not be definitively identified as a “laboratory confirmed” case of influenza.

The classic presentation of true influenza is an acute, febrile, respiratory disease of sudden onset, involving the throat and trachea. The onset of acute disease can be so sudden that the patient remembers the hour they fell ill. The virus is most effectively spread during the prodromal and initial clinical stages of infection in the upper respiratory tract, while the disease is maximal as a tracheitis. The virus infects the ciliated epithelium of the trachea, compromising the ciliary escalator that moves respiratory mucus upward and out of the lower respiratory tract. This produces a frequent and non-productive cough that is characteristic of influenza. Some degree of bronchitis is common, especially in those with asthma, but pneumonia is fortunately rare. Mortality is usually low, on the order of 0.5% of confirmed cases, and due to cardiopulmonary compromise such as asthma, heart disease, and other chronic lung disease. Secondary bacterial infection, especially with the pneumococcus, is much less a significant contributor to mortality with the widespread use of antibiotics. In the present H1N1 2009 pandemic, a higher incidence of diarrhea and vomiting have been noted [18], consistent with a younger age profile, and a greater severity in pregnant women has been a serious concern [19].

Abnormally severe influenza occurs in the case the avian influenza, so-called “bird flu”, of the H5 serotype, and also occurred during the catastrophic 1918 pandemic. This illness is quite different from classical influenza since it induces a “cytokine storm” of the acute phase non-specific immune response [20]. The current influenza in humans completely lacks the capabilities of this severe form of influenza, and is very unlikely to ever do so.

The WHO and CDC have issued specific guidelines for the detection of the novel H1N1 strain [21]. Specimens such as a nasopharyngeal swab, throat swab, tracheal aspirates are vital for a confirmed diagnosis. It is also important to acquire the sample before the administration of any antivirals

and within the first four days of symptoms. If the specimen needs transportation, it should be sent at 4°C in a viral transport medium. If the transportation is not possible for 24 h, they can be stored at –80°C. Serological testing of paired blood samples does not assist clinical decision-making and is only of epidemiological significance. The diagnostic tests include rapid tests that can be performed on spot within 10–15 min, with a low sensitivity but a specificity of >90%. The standard tests such as viral cultures in MDCK cells or egg inoculation and immunofluorescence antibody assay are time consuming. Real time PCR is now being widely employed for the detection of H1N1. Testing of samples with specific H1N1 primer probe sets should not be done if the patient does not meet the clinical and epidemiological criteria. The CDC guidelines indicate that the RT-PCR should be performed for Influenza A, B, H1 and H3. Unless new primer sets are available, the novel H1N1 virus would test positive for only influenza A given the differences in H1 from its predecessors

Impact of H1N1 2009 on India

The impact of H1N1 2009 on India obviously evolves daily as this is written. In the past, India has been a primary target of influenza, with great impact in its densely populated cities and river valleys especially in past pandemics [22].

The Ministry of Health and Family Welfare in India responded to the threat of H1N1 2009 by creating an elaborate network of monitoring stations at 22 international airports to interdict the arrival of passengers showing signs of ILI [<http://pib.nic.in/release/release.asp?relid=53224>]. Since entry into the Indian subcontinent is overwhelmingly by sea and air, this was a cogent strategy. Until about 1 August 2009, a high proportion of reported cases in India were interdicted imports rather than of indigenous origin. Eventually such quarantine efforts with influenza are doomed to fail, and since 1 August nearly all cases in India have been indigenous. However, it is important to recognize that this program was a great success, since the further back India is on the epidemic curve of H1N1 2009 infection before the availability of the H1N1 vaccine, the better off its population of 1.1 billion will be. Through 15 October, there have been 12,486 confirmed cases of H1N1 2009 in India, with 405 deaths [<http://pib.nic.in/release/release.asp?relid=53224>]. It is clear this is but the tip of a very large epidemiological iceberg, with most cases undetected or not counted. This is not unique to India, but a feature of virtually all countries attempting to keep up with this pandemic. In the past, prior to the recent popularity of specific viral testing, influenza activity was primarily monitored solely by reporting the number of deaths due to influenza/pneumonia, and this may

still be the most reliable indicator. Human beings have a predilection for numerical counts and statistics because they give the illusion of certainty, but it is clear that any numbers are only useful as an index of viral activity. However, given that the number of deaths is more accurate than the number of cases, control efforts in India have been relatively successful, given its huge urban population.

A key factor in limiting the impact of H1N1 2009 on India, as elsewhere, will be public health measures, many of them common sense in nature, to slow the passage of the virus through the population [see <http://www.cdc.gov/h1n1/flu/>]. Severe illness and deaths increase markedly if treatment centers become overwhelmed with cases and resources are stretched past the breaking point. Maintaining an ability to care for the sick reduces morbidity and especially mortality dramatically.

A single point of origin for H1N1 2009

Initial isolates of H1N1 2009 throughout the world from 30 March through 30 April showed an extraordinary level of homogeneity, differing among them by only 0.3%. The most recent common ancestor sequence (MRCA) has been calculated to have existed between August 2008 and January 2009, with most calculations favoring the latter date [23]. This indicates a virtually clonal source of H1N1 2009, when one takes into account that virus populations are rarely truly clonal, but rather represent a “quasispecies” of closely related sequences. Very many phylogenetic family trees of H1N1 2009 have been published [4, 23, 24]. Six of the 8 gene segments are from a so-called “triple-reassortant” swine influenza virus that appeared in North America in the late 1990s and subsequently appeared in Eurasia by 2003 (see [16] for summary of swine influenza). The other two segments, for the NA and M genes, are derived from Eurasian swine influenza. The consensus is that this virus emerged from a singular point of origin, after being sequestered from human observation for more than a decade. When, where and how during this sequestration, the “triple reassortant” and Eurasian segments reassorted into an ancestor of H1N1 2009 is unknown. However, neither the whole Eurasian swine influenza genome, nor the other six gene segments missing from H1N1 2009, have yet been found in the Western Hemisphere. The simplest explanation is that the “triple-reassortant” and Eurasian viruses reassorted years ago to form an ancestor of H1N1 2009, and that this reassorted influenza then became sequestered as a unit, and suddenly appeared in Mexico years later after a long period of replication independent of other swine influenza viruses.

Assigning a location for this point of origin is a dubious exercise in the midst of the pandemic. In the past, “Spanish

flu” or “Russian flu” were misnomers that did not reflect the virus’ true origin [25]. Fortunately, the scientific public has settled on a geographically neutral designation.

The earliest epicenter of the pandemic appears to have been in the state of Vera Cruz, Mexico [2], and particularly in the region surrounding Perote, a small community hemmed in by mountain ridges at 7700 feet above sea level. This region is the site of a very large piggery, producing nearly a million hogs per year, but H1N1 2009 has not yet been found either among the hogs in the piggery nor in local pigs, six months after the onset of the outbreak [http://granjascarroll.com/blog/2009/05/smithfield-swine-herd-in-veracruz-mexico-tests-negative-for-human-ah1n1-influenza_051409/]. It may not have arrived in Perote in a pig at all. Air travel in 2009 permits travel anywhere in the world well within the incubation period of a viral illness. Just as H1N1 2009 arrived in India carried by a human, and not a pig, so also might have been the case in Perote, Mexico. However it may have happened, it is clear that the data are consistent with a single source and single point in time in March 2009. The recognition of this outbreak as novel actually occurred in California where specific viral tests were more readily available [26]. The precise origin of H1N1 2009 remains a mystery, so it is important that the scientific community maintain a completely open mind on the subject until definitive evidence is obtained. It must be recognized that in ascribing a “natural” origin for H1N1 2009, that “natural” is a relative term. Emergence and spread of novel influenza is now inextricably linked with human behavior and globalization of commerce. In some way, shape or form, the current pandemic may well have human fingerprints on its origin.

Interplay of hemagglutinin protein sequence with the human dimension of the pandemic

Since influenza was first isolated in the 1930s, it has been axiomatic that the severity of a pandemic is proportional to the susceptibility of the human population, which is in turn directly related to the degree of change in the surface proteins of the virus, the H and N antigens [27]. The greater the change, the less that preexisting human antibodies to influenza can neutralize the virus, and the lower the “herd immunity” of the entire human population. Minor incremental changes in these antigens, denoted as “antigenic drift”, lead to mild outbreaks. Major, sudden changes in these antigens, denoted as “antigenic shift”, have led to the major pandemics of influenza in the 20th century. There has not been a major antigenic shift in human influenza since 1968.

The major component of influenza virus that determines its epidemiological dynamic is the predominant surface protein on the viral envelope, the H antigen. This protein serves as the hemagglutinin or HA1 attachment protein. It determines whether the virus is able to bind to cells of different species by its ability to attach to carbohydrate receptors on the cells [16]. The protein loops that determine the sites of binding for antibody dominate the immune response to the virus. Thus the H antigen is the principal component of any influenza vaccine and the efficacy of the vaccine or any naturally acquired immunity is measurable by determining the ability of the elicited antibodies to neutralize viral binding [23, 28, 29].

Table 1 shows an amino acid sequence alignment of Influenza H1N1 2009 with a number of H1 sequences obtained since 1918. A/California/08/2009 (CA08) is taken as representative of the earliest group of H1N1 2009 sequences in early April, and its sequence, in single-letter code, of the 327 amino acids of HA1 is shown in its entirety.

Other sequences are shown only insofar as they differ from H1N1 2009. Above CA08 are the two most closely related known swine influenza HA1 sequences [3, 23, 28], which each differ in sequence from H1N1 2009 by 6.1%. Below the H1N1 2009 sequence are listed a number of human isolates of H1 influenza. The first of these is A/New Jersey/8/1976, representing the swine influenza outbreak in Fort Dix, New Jersey, that represents the most closely related prior human influenza virus outbreak to H1N1 2009 [29]. Below that are, in reverse chronological order, a series of human isolates from 2008 back to 1918.

Several points can be made from this sequence alignment, each of which is related to the origin and profile of the current pandemic of H1N1 2009. First, the closest relative to the current human virus is found either in the United States, as A/swine/Indiana/P12439/2000 [3], or in Eurasia, as A/swine/Hong Kong/415/2004 [23], and both are swine viruses. However, 6.1% is a significant degree of evolution in swine influenza, that can be seen in the many phylogenetic trees of H1N1 2009 represented as a quite long branch, indicating an extended period of unsampled ancestry [4, 23, 24]. Clearly, the origin of the virus, at least that contributed by the “triple-reassortant” segments of the virus, may be from either Eurasia or North America.

Second, as has been stated beginning in early May [3, 23, 28], the peptide sequence of H1N1 2009 is quite different, 27.2%, from its 2008 predecessor and the prior “seasonal flu” vaccine. As for most human influenza viruses, these differences are concentrated in those regions of HA1 that define the antigenic epitopes of the protein. From this alone, one would predict no protective effect in humans of prior infection or immunization with the 2008 or similar H1 viruses, which has since been confirmed in serological

studies [28, 29]. Similar degrees of difference are seen in human influenza viruses extending back into the late 1940s. Prior to 1940, as human influenza was closer to the 1918 influenza virus, a closer degree of sequence similarity may be seen. Recent findings have shown that humans born prior to 1940, and especially those who experienced an influenza virus close in sequence to the 1918 virus, have some residual protection to H1N1 2009 [29]. This is reflected in a lower attack rate for H1N1 2009 for those born many decades ago, relative to younger adults and children who have never experienced a similar virus.

Third, there is a high degree of similarity among H1 swine influenza isolates, and swine-origin influenza viruses such as A/New Jersey/8/1976, and the differences are more randomly distributed across the sequence of HA1 rather than being concentrated in the area of the antigenic epitopes as in human pandemic strains. Even though A/New Jersey/8/1976 is 11% different in sequence from H1N1 2009, proportionally many fewer of these differences are in antigenic regions than the 27.2% between the 2008 and 2009 viruses. Thus, it has been noted that those who received the 1977–78 “swine flu” vaccine following the Fort Dix, New Jersey, outbreak, have residual immunity to H1N1 2009, especially if they had been “primed” by earlier experience with H1 viruses from 1918 through 1957 [29]. Protective levels of residual antibody (titer > 1/80) have been found in 54% of such individuals, versus 33% of individuals over 60 years of age who did not receive the vaccine. Unfortunately, the higher figure does not apply to India or other countries where the 1977–78 “swine flu” vaccine was not distributed to humans.

The sum total of these differences provide a ready molecular explanation for the fact that the current pandemic is concentrated in children and younger adults who have no experience with closely related H1 influenza viruses, and has largely spared the human population 60 and above, especially in the United States, who have immunologic memory of such viruses, albeit decades old.

H1N1 2009 is crippled, but could worsen clinically over time

It is a profound mystery to influenza virologists how H1N1 2009 is doing well enough in the human population to initiate a pandemic. Since 1918, novel influenza viruses have emerged by reassorting one or two gene segments, including the H gene segment, into the preexisting set of 6 or 7 gene segments that had long adapted to virus growth in human beings [30]. The pandemic H2 virus in 1957 and H3 in 1968 therefore had their root in the genetic lineage already successful in humans for decades. H1N1 2009 has broken

Table 1 Hemagglutinin HA1: Evolution in H1 from 1918–2009

A/sw/Indiana/P12439/2000	-----R-----	
A/sw/Hong Kong/915/2004	-----R-----	
A/California/08/2009	DTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLDKHNGKLCCKLRGVAPL	50
A/New Jersey/8/1976	-----R-----G-I---	
A/USA/WRAMC-1154048/2008	--I-----NS-----L-K-I---	
A/USSR/90/1977	--I-----S-----R-K-I---	
A/Fort Monmouth/1/1947	--I-----S-----R-K-I---	
A/South Carolina/1/1918	--I-----S-----K-I---	
A/sw/Indiana/P12439/2000	-----L-----F-----S-----N---	
A/sw/Hong Kong/915/2004	-----L-----F-----SN-----	
A/California/08/2009	HLGKCNIAGWILGNPECESLSTASSWSYIVETPSSDNGTCYPGDFIDYEE	100
A/New Jersey/8/1976	-----L-----L-L-V-----SN-----N---	
A/USA/WRAMC-1154048/2008	Q--N-SV-----L-ISKE-----K-NPE-----H-A---	
A/USSR/90/1977	Q-----FSKK-----A--N-E-----Y-A---	
A/Fort Monmouth/1/1947	Q-----LSKR-----A--N-E-A-----A---	
A/South Carolina/1/1918	Q-----L-----DL-L-----SN-E-----	
	Site C Site E	
A/sw/Indiana/P12439/2000	-----T-R-----Y-----R---	
A/sw/Hong Kong/915/2004	-----D-----R---	
A/California/08/2009	LREQLSVSSFERFEIFPKTSSWPNHDSNKGVTAAACPHAGAKSFYKNLIW	150
A/New Jersey/8/1976	-----D-ET-_------Y--N--R---	
A/USA/WRAMC-1154048/2008	-----E-----TVT_-S-S-S-N-ES--R--L-	
A/USSR/90/1977	-----ER--K-NVTR--S-S-K-KS--R--L-	
A/Fort Monmouth/1/1947	-----ER--K-NITR--S--KS-----L-	
A/South Carolina/1/1918	-----K-----ETT-----SY--S--R--L-	
	Site A	
A/sw/Indiana/P12439/2000	----E-----N-----	
A/sw/Hong Kong/915/2004	-----N-----	
A/California/08/2009	LVKKGNSYPKLSKSYINDKGKEVLVLWGIHHPSTADQQSLYQNADAYVF	200
A/New Jersey/8/1976	-----V-N-----P--T-----	
A/USA/WRAMC-1154048/2008	-TG-NGL--N---A-N-E-----V--PNIG--KA--HTEN--S	
A/USSR/90/1977	-TE-NG--N---V-N-E-----V--NIE--KTI--RKEN--S	
A/Fort Monmouth/1/1947	-TETDG-----V-N-E-----V--NIE--KT--RKEN--S	
A/South Carolina/1/1918	-T--S-----V-N-----V--P-GT-----S	
	Site B * Site B	
A/sw/Indiana/P12439/2000	-----A-----A--I-----	
A/sw/Hong Kong/915/2004	-----T-----A-----	
A/California/08/2009	VGSSRYSKKFKPEIAIRPKVRDQEGRMNYWTLPVPGDKITFEATGNLVV	250
A/New Jersey/8/1976	---K-NR-----A---G-A-----I---T-----	
A/USA/WRAMC-1154048/2008	-V--H--R--T---K-----I-----L---T-I--N---IA	
A/USSR/90/1977	-V--N-NRR-T---E---G-A--I-----L---T-I--N---IA	
A/Fort Monmouth/1/1947	-V--N-NRR-T---E---G-A--I-----L---T-I--N---IA	
A/South Carolina/1/1918	---K-NRR-T---A-----A-----L---T-T-----IA	
	Site D ** *	
A/sw/Indiana/P12439/2000	-----S-----S-----V---	
A/sw/Hong Kong/915/2004	-----IK--S-----S-----V--V---	
A/California/08/2009	PRYAFAMERNAGSGIIISDTPVHDCNTTCQTPKGAINSTSLPFQNIHPITI	300
A/New Jersey/8/1976	-----N-GS-----A-----K-----V---	
A/USA/WRAMC-1154048/2008	-----LS-GF---N-NA-MDK-DAK---Q---S-----V--V---	
A/USSR/90/1977	-WH---LN-GF---T-NASMDE-D-K---Q---S-----V---	
A/Fort Monmouth/1/1947	-W---LS-DF---T-NASMDE-D-K---Q---S-----V---	
A/South Carolina/1/1918	-W---LN-GS---T-DA-----K---H---S-----V---	
	Site C	

A/sw/Indiana/P12439/2000	-E-----M-----V-----	20 = 6.1%
A/sw/Hong Kong/915/2004	-E-----R--M-----V-----	20 = 6.1%
<u>A/California/08/2009</u>	GKCPKYVKSTKLRLATGLRNIPSIQSR 327	
A/New Jersey/8/1976	-E-----M-----	36 = 11.0%
A/USA/WRAMC-1154048/2008	-E-----R-A--MV-----	89 = 27.2%
A/USSR/90/1977	-E-----R-----MV-----	87 = 26.6%
A/Fort Monmouth/1/1947	-E-----MV-----	81 = 24.7%
A/South Carolina/1/1918	-E-----R-----M-----	57 = 17.4%

The amino acid sequence of the HA1 protein, shown in its entirety for the Influenza H1N1 2009 virus, is derived from the segment 4 sequence of the isolate A/California/08/2009(H1N1) submitted from the CDC by Shu et al. on 29 April 2009, as Genbank FJ971076. Only the sequence of the mature protein, after cleavage of the signal sequence, is shown. Standard single-letter abbreviations for the amino acids are used. The canonical sites for N-linked glycosylation are underlined. Above and below this reference sequence are shown the selected sequences derived from the segment 4 sequence of several other influenza isolates, insofar as they differ from H1N1 2009. The sequences were obtained from Genbank, and are, respectively, in the order shown: A/Swine/Indiana/P12439/2000 (AF455680); A/Swine/Hong Kong/915/2004 (GQ229269); A/New Jersey/8/1976 (CY044365); A/District of Columbia/WRAMC-1154048/2008 (CY038770); A/USSR/90/1977(DQ508897); A/Fort Monmouth/1/1947 (U02085); A/South Carolina/1/1918 (AF117241). The collinear sequences were hand-aligned and also confirmed by online use of ClustalW. After each sequence is shown the number of amino acid differences, and per cent difference, from H1N1 2009. Amino acid regions contributing to each of five antigenic sites are labeled Site A through Site E [13]. Selected Hamming distances were also determined from BLAST [<http://blast.ncbi.nlm.nih.gov/Blast.cgi>] between the entire segment 4 nucleotide sequences of the following: A/Swine/Indiana/P/2000 vs. A/Swine/Hong Kong/915/2004 = 2.88%; A/Swine/Indiana/P/2000 vs. H1N1 2009 = 4.76%; A/Swine/Hong Kong/915/2004 vs. H1N1 2009 = 5.76%.

this mold, being derived largely from animal influenza gene segments with no evidence of any adaptation to replication in humans. In several ways definable at the molecular level, it is a crippled virus in terms of human replication.

Within the PB2 polymerase molecule, all known prior human influenza, including the A/New Jersey/1976 virus, has had lysine (K) at position 627 [31]. Avian and swine influenza viruses generally have a glutamate (E) at that position, substituting an acidic for a basic amino acid. This affects the pH optimum and the temperature optimum of the PB2 protein, such that a virus with 627E only relatively poorly replicates at the lower temperature of 33°C. This represents a single point mutation that, if changed to K, would be predicted to significantly enhance viral replication in the upper respiratory tract of humans, potentiating the spread of H1N1 2009 from person to person, and perhaps also worsening the clinical syndrome as well.

In H1N1 2009, unlike nearly all prior human influenza viruses, one of the 11 viral proteins is not expressed at all. PB1-F2, a protein of 90 amino acids that contains a proapoptotic peptide at positions 66 through 75 [9], is completely missing, and with it, any effect this protein normally exerts in limiting immune clearance of the virus from the respiratory tract [10]. This cripples part of the virus' defense mechanisms against human immunity, and presumably weakens its pathogenesis. The nucleotide sequence in H1N1 2009 that would normally code for PB1-F2 represents a very unusual genetic construct, in that it contains three stop codons at positions 12, 58 and 88 in the amino acid sequence [32]. Detailed consideration of this unusual genetic element is beyond the scope of the discussion here, but suffice it to say that the stop at

position 12 can be circumvented by alternate initiation at a methionine at position 39, resulting in a functional fragment [10], such as that found in the A/New Jersey/8/1976 virus [see Genbank listing for CY039997]. The stop at position 88 may be too close to the carboxy terminus to exert any significant effect. Thus, the most significant element in preventing the expression of a functional fragment of PB1-F2 lies in the stop at position 58. Again, as with PB2, a single point mutation would restore the stop codon to one coding for tryptophane (W), and rejuvenate H1N1 2009 with a functional proapoptotic protein that could interfere with immune clearance of the virus from the respiratory tract.

Thirdly, the emergent sequence of the neuraminidase of H1N1 2009 was uniformly sensitive to the licensed drugs that inhibit the enzyme, giving us a powerful tool to combat the virus [28]. However, resistance to Tamiflu can be mediated by a single point mutation in the NA gene that results in a change from histidine to tyrosine (H274Y) close to the active site of the enzyme [33]. This H274Y mutation has already been noted a number of times independently around the world within the last few months, mostly in association with use of Tamiflu to combat infection [34].

Just three point mutations, strategically placed and all selectable, stand in the way of an "improved" and potentially more dangerous H1N1 2009.

Added to this scenario, which we would emphasize has not yet occurred except for the H274Y mutation, is the observation that H1N1 2009 is showing signs of genetic instability that may reflect rapid adaptation to its new human host. Multiple studies have shown that there is an abnormally high ratio of non-synonymous to synonymous mutations as H1N1 has evolved in humans over the last several months

[23, 24, 28]. Simply stated, the virus is changing in amino acid sequence faster than normally predicted by the overall mutational frequency. This is occurring in spite of the fact that the virus is not yet under immunological selective pressure – the overwhelming proportion of those it is infecting have never seen a similar H or N antigen. Only when the virus begins to encounter a significant proportion of humans immune to H1N1 2009 by virtue of natural infection or immunization should we expect accelerated evolution in the antigenic sites of H and N antigens seen in previous human influenza subtypes. H1N1 2009 therefore must be considered genetically unstable at present, and unpredictable in terms of the changes that will occur as it continues to adapt to a human environment.

The H1N1 2009 Vaccine is essential for control

There are three options in managing pandemic influenza. Option 1 is to use general public health measures, such as handwashing, face masks, quarantine, school or public event closures etc. to inhibit the spread of the virus through the population [<http://www.cdc.gov/h1n1flu/>]. These can produce temporary and localized effects, but ultimately the virus will reach a high proportion of the global population. Option 2 is mass prophylaxis by distributing antivirals such as Tamiflu to the population. This would only accelerate the eventual transition of the virus to Tamiflu-resistance within 18 to 36 months. Such general use of drug prophylaxis is no longer recommended by WHO and CDC [see <http://www.cdc.gov/H1N1flu/recommendations.htm>], although it is obviously still being pursued in many communities. Neither of these options prevents a novel strain of influenza from infecting a large fraction of the human population over time.

Option 3 is to massively immunize a large fraction of humanity with an appropriately formulated influenza vaccine. This not only slows the progress of the virus through the human population, but provides permanent protection against serious infection. This option is being actively pursued by governments throughout the world, and billions of doses of vaccine will eventually be made available as fast as current production schedules allow. It is our best hope of blunting the impact of H1N1 2009 globally.

There should be no doubt in anyone's mind that influenza virus is inherently dangerous, even deadly, and that the vaccine should be enthusiastically embraced. The formulations and methods being used to manufacture the vaccine have been safe for decades. Concerns derived from problems with the 1977 vaccine are not relevant 32 years later.

Influenza H1N1 2009 is here to stay

No influenza virus has achieved this degree of penetration into the world population and failed to remain a fixture in seasonal and epidemic respiratory disease for less than 10 years. Most have shown great staying power, causing periodic epidemics for over 30 years – as is true for each of the “seasonal” virus subtypes H1, H3 and B that have been in the formulation of the influenza virus vaccine for decades.

The most likely scenario is that H1N1 2009 will continue to propagate in humans for an indefinite period, undergoing periodic mutational drift, and continuing to be the etiology of periodic epidemics of influenza for a decade or more into the future.

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