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Molecular evolution of hemagglutinin gene of Influenza A virus

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TABLE OF CONTENTS

1. ABSTRACT

In the history of human civilization, influenza is the second most catastrophic killer disease for mankind with plague ranking first in the medieval times. The 1918–1919 'Spanish flu' killed 20–50 million people worldwide. According to a report from WHO, there have been four pandemics, several epidemics and recurrent seasonal outbreaks of influenza in different parts of the world. The virus is a potential bioterrorism threat with biological 'Chernobyl-like disaster' that occurred in Soviet Russia in 1977. Here, the author reviews the biology of the surface exposed hemagglutinin of the influenza virus, a gene under constant positive selection pressure to evade host immunity and vaccination. Global, local and seasonal outbreaks of influenza lead to a significant number of deaths both in humans and poultry birds.

2. INTRODUCTION

Human influenza virus was first isolated in 1933 from throat washings of convalescent patients (1). The virus belongs to the family; Orthomyxoviridae. Based on amino acid homology, phylogenetic convergence and divergence is the premise of classification, the US center for Disease Control and Prevention, classified influenza virus as types A, B, C and D (2).

Influenza is a negative stranded RNA virus having segmented genome. Influenza A virus (IAV) and Influenza B virus (IBV) share significant homology both at morphological and molecular level. The eight strands of IAV code for 12 proteins, viz., hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein M1, ion channel M2, nonstructural proteins NS1, NS2 and an array of polymerases labeled PA, PB1, PB1-F2, PB2, PA-X (3–5) (fig 1). The HA and NA glycoproteins are present on the surface of IAV and are responsible for viral entry into and exit from the host cell. Depending on the antigenicity, the hemagglutinin and the neuraminidase have been classified into 18 and 11 subtypes respectively, with the influenza A virus being characterized by the HA and NA subtypes such as H1N1, H3N2, H7N9, etc (6). It is to be noted that while it would have been possible for 18 subtypes of HA and 11 subtypes of NA to give rise to 198 subtypes of IAV, only a minuscule number of subtype combinations exist in nature. The reason for such paradox has been deciphered by mathematical descriptors, aided with graphical representation and numerical characterization (7). It is worthwhile mentioning that other viral genes have been characterized with mathematical descriptors (8–9).Unlike IAV, IBV is much more homogenous in that it does not have subtypes; on the other hand, IBV has diverged into two antigenically distinct lineages, viz., the Yamtagata and Victoria lineages, since 1970 (10–11).

Influenza A is a rapidly evolving virus exhibiting high variability in nucleotide and protein

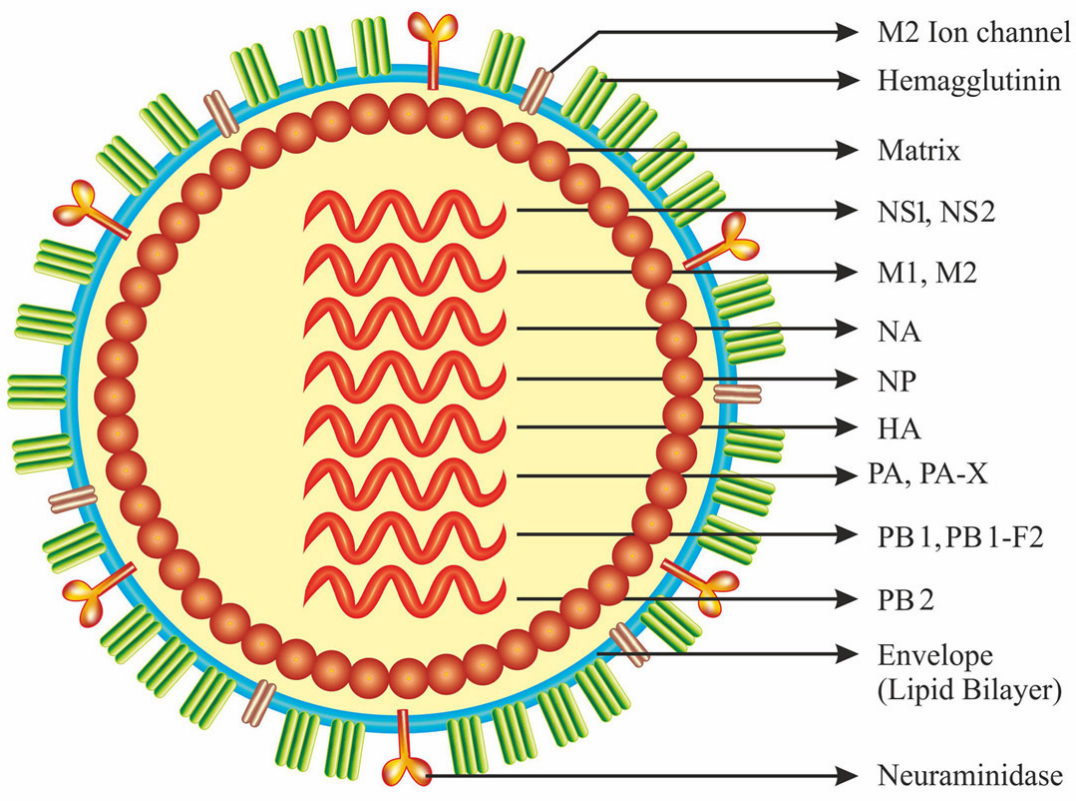

Figure 1. Schematic diagram of influenza A virus. The envelope is covered by three proteins i.e. HA, NA, M2. The proteins encoded by the genes are encoded by arrow.

sequences. The virus evolves by several mechanisms, of which two are well documented, viz., antigenic drift and antigenic shift. Antigenic drift occurs through replication errors since these RNA viruses do not possess error correction mechanisms. Antigenic shift occurs less often; when two or more influenza viruses infect the same cell, some viral gene segments may be exchanged and some of the progeny viruses may end up with new combinations of the HA and NA subtypes. A third mechanism, which is well known in mammalian genetics but is rather controversial in viral cases, is homologus recombination. If two different subtypes infect a single cell, it could have been possible for the RNA polymerase to switch the replication template from one viral strand to an equivalent strand of another virus. This mechanism of viral evolution, known as 'copy choice', though theoretically possible, is a highly debated issue in influenza virus research. Homologous recombination in IAV has been documented by some authors (12–13) but has been distinctly refuted by others (14–15). In a recent paper, using mathematical descriptors the authors have shown that 'homologous recombination-like events' may happen in IAV in 5% cases (16). In contrast to IAV, IBV undergoes slower rate of mutation (17). Antigenic drift occurs both in IAV and IBV. For long scientists have opined that antigenic shift only prevails in IAV (18), a fact which has been challenged by mathematical modeling in a recent paper (19). However, the mode of 'reassortment' in IBV is intriguing and complex. Not all genes in IBV can assort randomly. The genes coding for PB1, PB2 and HA exhibit strong 'linkage' while the other segments partially reassort between lineages. The reason for this kind of biased reassortmnet is unclear. It may be due to strong selection pressure which may prevail because the lineages have not reassorted for a long time.

Despite having different evolutionary dynamics, IAV and IBV co-circulate in nature producing recurrent viral outbreaks. However, there are certain differences. The two types diversified from common ancestor 4000 yrs ago (20). Primarily wild aquatic birds are natural reservoirs of influenza A virus. These viruses are of 'low pathogenic type' i.e. broadly asymptotic to cause any illness if any, in the rarest of rare occasion. IAV virus is contagious, exhibiting power of horizontal gene transfer causing recurrent infection in poultry birds, swine and human. Furthermore, they frequently mutate to 'high pathogenic type', priming life-threatening infection in humans and animals generating high mortality. In contrast, humans are the natural reservoirs of IBV, although the virus has exhibited reverse zoonosis and infected seals (21). Both the viruses infect respiratory systems; however IBV is less severe than IAV as the virus produces less amount of inflammatory cytokines (22). IBV has shorter

life span in human body compared to IAV. Nonetheless, the fact that IBV is less severe than IAV has been refuted (23), which may be due to shifting evolutionary pattern of IBV.

3. BIOLOGY OF INFLUENZA A VIRUS INSIDE THE HOST CELL

Entry of influenza virus into host cell is a receptor mediated process. Cell surface protein hemagglutinin binds to sialic acid (neuraminic acid) on the host cell membrane glycoproteins or glycolipids. Interaction of hemagglutinin with sialic acid is multivalent. Following internalization how is the virus processed by cell machinery?

Virus internalization occurs by a diverse array of mechanisms. Clathrin dependent process is the primary route of viral entry. Clathrin is synthesized *de novo* and encapsulates the viral population at the site of virus entry in the host cell (24). However, non-clathrin and non-caveola dependent processes do play role in the viral entry. The various mechanisms are not competing, but work with equal efficiency in the host cells. Micropinocytosis is also a presumed mechanism of influenza virus entry, though its role needs further understanding. It should be mentioned that all mechanisms of viral entry have been deciphered by live-cell imaging under fluorescent microscopy. Whatsoever the mechanism of viral cargo packaging is, they are finally destined to endosome having acidic pH. Endocytic vesicles are pertinent targets of nanomedicines, tailor made drugs may be directed right towards endocytic vesicles which may halt host-pathogen membrane fusion without affecting host cell viability (24–25). Nonetheless, no marketable drug against influenza has been successfully developed so far targeting the endocytic machinery.

HA plays a paramount role in fusion of viral cell with host cell endosomal membrane. Hemagglutinin is a trimeric protein with each monomer made of two subunits, HA1 and HA2 connected together with disulphide bond. The N terminal of the HA2 subunit has a stretch of hydrophobic amino acids known as fusion peptide. When the virus is destined to the late endosome, the fusion peptide undergoes conformational change from β loop to helix (26). This event is necessary for fusion of virus lipid membrane with host cell endosome membrane and leads to the release of viral genomic RNA associated with ribonucleoproteins in the host cell cytoplasm. Wet lab experiment has shown that Arbidol, an indole based biomolecule, prevents conformational change of HA and blocks fusion of virus and endosome membrane. The mode of interactions of HA with arbidol is hydrophobic interaction and salt bridges which have been deciphered by crystal structure of arbidol coupled with hemagglutinin receptors of H3N2 and H7N9 virus (27). M2, the ion channel protein on the envelope of influenza virus opens in the low pH of endosome. Opening of M2 channel enhances proton concentration inside the virus which in turn aids in virus-endomembrane fusion (28). Anti-influenza drug amantadine targets M2 channel and prevents viral uncoating in the endosome. However, people worldwide are becoming increasingly resistant to this drug (29–30).

One of the distinguishing features between avian and mammalian virus is that, the avian viruses fuse with early endosomes having low acidity, whereas the mammalian viruses fuse with late endosomes having high acidity (31). Once the viral RNP complex is released in the host cell cytoplasm, they are transported to the nucleus guided by nuclear localization signal (NLS). Transcription of viral genome takes place in the host cell nucleus. Synthesis of viral proteins takes place in the host cell cytoplasm or ER. Influenza being a negative stranded RNA virus; the viral genes have to be replicated to a positive stranded mRNA. This is achieved by viral RNA dependent RNA polymerases. M2 ion channel protein of IAV plays significant if not essential role in viral replication. *In vitro* experiment has shown that mutated viruses having loss of function of M2 ion channel exhibit 15 times reduced replication in cell culture media (32). The replicated viral genes have to be transcribed to negative strands of the viral genome. The virus achieves this by hijacking host cell's cellular machinery (33–34). Once viral RNAs are synthesized, they travel to the host cell cytoplasm via nuclear pore. They are packed with ribonucleoproteins. In the host cell cytoplasm, vRNP gets surrounded by matrix protein M1, which is synthesized in the host cell cytoplasm. Translation of HA, NA and M2 take place in the ER; they travel through the transgolgi network. The virus gets enveloped by host cell derived lipid membrane bearing HA, NA and ion channel protein M2. The mechanism of combinatorial clustering of three membrane proteins on the viral membrane is still a mystery and the mechanism is yet to be deciphered. However, the proteins get associated with lipid-rafts and trafficked to the apical surface of cell to exit from polarized cells (35). The virus buds off by 'breaking' sialic receptors by enzymatic action of neuraminidase (fig 2).

4. HA GENE, THE BASICS

HA is a type I membrane glycoprotein, secreted as precursor peptide which undergoes three types of post translational modifications. 1. Proteolytic cleavage- Removal of 14–18 amino acids at the N-terminal end. This signal sequence is used to transport HA protein to the destination. 2. Glycosylation- Attachment of carbohydrate residues at specific amino acid residues on HA. 3. Acylation- Palmitic acid residues are added to cysteine residues near the carboxy terminus of hemagglutinin. 4. Posttranslational cleavage- The final

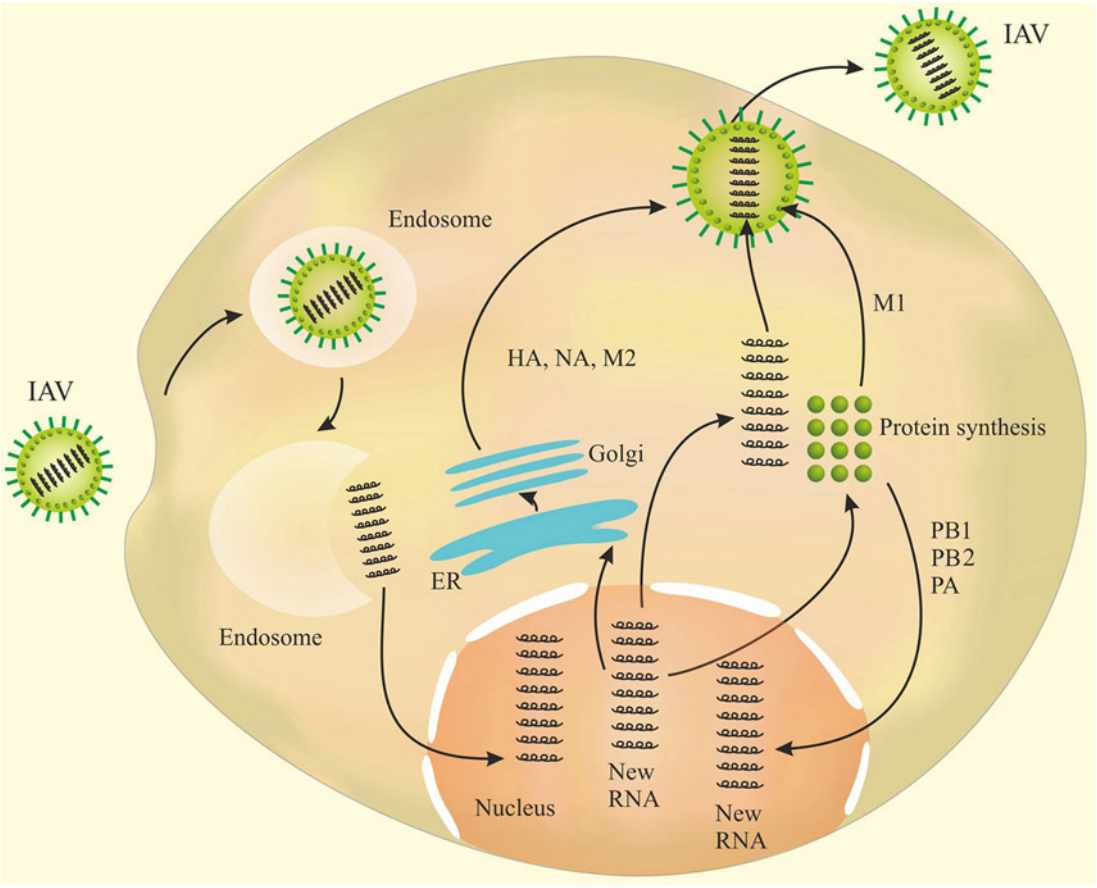

Figure 2. Schematic diagram of life cycle of influenza A virus in the host cell. After viral entry, it gets endocytosed. Viral RNA is released in the cytoplasm. New RNA and proteins are synthesized. Finally the virus buds off from the host cell.

processing is cleavage of hemagglutinin precursor peptide into HA1 and HA2 subunits which are linked by disulphide bond. The cleavage is mediated by host cell secreted serine proteases. The HA1 domain contains both receptor binding site (RBS) and antigenic sites; HA2 secures the HA protein on the lipid membrane of the virus and mediates fusion of viral and host cell endosomal membrane. The acid stability of the virus is important for viral fitness; the virus undergoes mutation to promote its acid stability and survival at low pH (36–38).

The classification of IAV into high and low pathogenicity is based on number of basic residues present at the cleavage site of HA. A highly pathogenic influenza virus (HPAIV) has polybasic residues at the cleavage site, whereas, a low pathogenic influenza virus (LPAIV) does not. However the cleaving enzymes of HA exhibit differential distribution. Highly pathogenic influenza subtypes i.e. H5 and H7 undergo cleavage by intracellular subtilin like enzymes. These proteases namely furin and proprotein convertase 6 (PC6) exhibit wide array of distributions. Therefore the highly pathogenic viruses exhibit broad spectrum systemic infection. Many of these enzymes are involved in post translational processing of growth factors (39). In contrast, low pathogenic influenza viruses, including H1N1 undergo cleavage by trypsin like serine proteases (40). These enzymes may be membrane bound (type II transmembrane serine protease) or extracellular. Trypsin like serine proteases have limited distribution, being present in the extracellular spaces of intestinal tract of birds, respiratory tracts of birds and mammals. The membrane bound serine proteases include human airway trypsin-like protease, (HAT), differentially expressed in squamous cell carcinoma protease (DESC), hepsin, transmembrane protease serine 2 (TMPRSS2), TMPRSS4 (41–42). The extracellular serine proteases include cell tryptase, tryptase clara secreted by Clara Cells of bronchiolar epithelium, plasmin, thrombolytic protease, trypsin, urokinase, plasma kallikrein, thrombin (43). Many of these proteases are secreted as zymogen granules and need further activation. It is worthwhile mentioning that classification of influenza A viruses on the premise of cleavage of hemagglutinin precursor peptide into a biochemically active protein is highly subjective. There is no hard and fast rule in biology, exceptions are always there (44). The paradox has been enumerated in the later section.

Another enigmatic feature is exhibited by the type II transmembrane protease, namely matriptase (TMPRSS6). The protein is expressed along the basolateral membrane of the cells of the respiratory tract and the catalytic domain of the protein is secreted in the extracellular space of the cells. Once secreted, the catalytic domain matriptase acts as extracellular protease and exhibits differential ability to cleave HA precursor peptide into biologically active form (45). The extracellular protease can activate H1 precursor protein but only in strain specific manner. Neither the enzyme can cleave H2 or H3; no doubt the cleaving property of matriptase enzyme needs further understanding. Also, matriptase has the ability to activate zymogen granules. It cleaves the proproteins of urokinase, plasma kallikrein, thrombin to their functional form, which in turn cleaves HA.

However, each cleavage site of HA must contain optimal number of basic residues. Addition or deletion of basic residues at the cleavage point curtails peptide cleavability and virulence of influenza virus (46). Nevertheless, the cleavage site of hemagglutinin exhibits high degree of heterogeneity (47–48). Highly virulent strains can arise from non-virulent strains with insertion of 54 nucleotide sequence of 28S ribosomal RNA.

As a general rule, it can be concluded that viruses harboring multibasic residues at the cleavage site can be propagated *in vitro* in absence of trypsin. However, trypsin activation is essential for viruses that have monobasic residue at the cleavage site. Nevertheless, the infamous H1N1 'Spanish flu' virus has monobasic residue at the cleavage site. The 'Spanish flu' virus does not require trypsin in cell culture.

5. EVOLUTION OF HA GENE

HA is the primary target of host immune system. It is the major antigen needed to be neutralized for successful design of a vaccine. HA undergoes rapid evolution to evade host immune system. Most mutations in HA occur in the RBS and antigenic site of HA1 domain (49). HA evolves three times faster than the non-structural genes (50). Inside the host cell, the hemagglutinin gene of influenza A virus is under selective pressure to undergo changes to evade host's immunity, which differs according to the polymorphism of host immunological genes, gender, age. It is debated whether the selection pressure on the HA1 segment is continuous or punctuated. However, study has proposed that positive selection does play a towering role, with non-synonymous mutations having greater value than synonymous mutations. Also, drift in the HA1 segment is more continuous that previously deciphered (51–52). The HA1 domain exhibits high variability, the HA2 domain, exhibits less variability. Genetic drift is more prevalent in HA1 segment in comparison to HA2. HA1 domain in H1N1 has five antigenic domains namely Sa, Sb, Ca1, Ca2, Cb; HA1 segment in H3N2 has five antigenic region namely A, B, C, D, E. These antigenic types in the two subtypes are not homologues, some regions in the same subtype exhibit linkage. Antigenic drifts in the HA1 region of the virus result in polymorphisms in the antigenic site, the substitutions are formidable enough to cause an epidemic or a seasonal outbreak (53). Influenza vaccines are needed to be redesigned every year to address such antigenic drifts and combat outbreaks. Paramount changes in the HA gene occurs due to antigenic shift, formidable enough to rise to a new subtype and a pandemic. Pandemic subtypes arise due to reasortment of segmented genes between human and non-human viruses from other animals, predominantly birds. Antigenic drift in the existing subtypes cannot give rise to a pandemic (54). 1957 Asian flu (H2N2) and 1968 Hong Kong Flu (H3N2) correlate with antigenic shift among various subtypes. Therefore, new strains and subtypes can be made due to drift and shift in the HA gene which contribute to the evolution of IAV. In a similar way NA gene can undergo drift and shift to promote evolution of influenza A virus. However, comparison of the amino acid changes among all proteins of any IAV subtype depicts that percentage change in amino acid in HA protein is highest (69%), followed by NA (61%) (55). HA protein predominates over NA by 5 times on influenza virus surface (56) .Therefore, from view point of evolution and clinical medicine cumulative changes in HA are more important than that of NA and all other viral proteins.

This review will give a brief overview of molecular biology and evolution of HA gene during the course of pandemics, major epidemics and recurrent season outbreaks.

6. MOLECULAR EVOLUTION OF HA GENE IN THE COURSE OF PANDEMICS AND EPIDEMICS

6.1. The pandemic influenza A viruses i.e. 1918–19 'Spanish flu', 1957 Asian flu, 1968 Hong Kong flu, 2009 'Swine flu'

The 'Spanish flu' was not a single thrust of flu. It occurred in three waves during the time period of 1918 to 1919. Scientists hold the data suggesting that comprehensive mortality due to 'Spanish flu' was higher than that of World War I. In contrast to many other diseases, the age group which suffered highest mortality was the young adults. Most people died of acute respiratory failure due to severe secondary bacterial infection caused by *Streptococccus pneumoniae*, *Staphylococcus aurues*, *Streptococcus pyrogenes, Haemophilus influenzae*. The age group

which was largely protected was the old people. They possibly had antibodies against the killer virus, may be due to an earlier infection about which we do not know much.

Jeffery Karl Taubenberger, virologist in the National Centre for Allergy of Infectious Disease, National Institute of Health Bethesda, USA sequenced the genes of 1918–19 H1N1 flu virus from frozen lung samples of pneumonia-influenza affected dead US soldier (57). He was able to isolate, amplify the viral sequences and did phylogenetic study. There cannot be any doubt that the viral genes were of avian origin. The avian virus was of 'low pathogenic type' as 1918–19 H1N1 lacks polybasic residues at the cleavage point of hemagglutin precursor peptide. Did the 'Spanish flu' virus directly originate from avian virus? We are not confirmed yet. However, results from two research papers are noteworthy. First, sequence comparison of H1 gene from 'Spanish flu' virus, avian virus isolated from preserved waterfowl of 1917 and present day avian influenza virus has shown that the H1 gene from wild water fowl was more similar to present day avian virus H1 gene than to 'Spanish flu' H1 gene (58). Second, a chimeric construct with insertion of LP avian H1 gene in 1918–19 'Spanish flu' backbone was as virulent as 'Spanish flu' virus. In fact 7 of the chimeric viral constructs having replacement of one segmented gene from LP avian virus in 1918–19 'Spanish flu' backbone were as virulent as 'Spanish flu' virus. The only exception was the chimeric construct having replacement with LP avian PB2 gene. This was the only chimeric virus which exhibited low pathogenicity in mouse model. The study may make a plausible conclusion that the 'Spanish flu' virus was of avian origin (59). However, several questions need further understanding. Was the 'Spanish flu' virus a direct descendent of an avian virus with 'gain of function' mutations in the PB2 gene? If not, what were the subtypes which made the unique gene constellation of the 'Spanish flu' virus? What was the 'reassortment vessel'?

The 'Spanish flu' virus genome was extraordinary in many respects. The genome had unusual number of silent nucleotide changes. Besides, there were two strains of flu simultaneously circulating in human populations (60). One strain had mutations at amino acid positions 190 (E190D) and 225 (D225G) in the HA1 segment of HA, rendering the virus to bind only at α-2–6 linked sialic acid receptors present in the upper respiratory tract (i.e. nasal lining, pharynx, tracheolar and bronchiolar lining of human) of humans. The other strain had mutation only at amino acid position 190 (E190D) in the HA1 segment of HA. This strain was capable of binding to both α-2–6 and α-2–3 linked sialic acid receptors. The α-2–3 linked sialic acid receptors are present in the deep linings of human alveolar epithelial cells. The flu virus exhibits interesting features regarding the cleavage of hemagglutinin precursor peptide. Recent research has shown that the hemagglutinin precursor peptide of the H1N1 virus undergoes cleavage by TMPRSS2, a type II transmembrane serine protease (TTSP) in human bronchiolar epithelium. Knock-out mouse having deletion in a single TMPRSS2 gene showcases almost no spread of disease, viral replication and pathogenicity. HA0 precursor protein undergoes highly reduced or no cleavage in the knockout mouse having deletion of TMPRSS2 gene. Therefore, it may be reasonable to conclude that TMPRSS2 is the predominant protease to cleave hemagglutinin precursor peptide of 'Spanish flu' virus (61). *In vitro* study has also demonstrated that plasmin, an extracellular serine protease has a potential role in cleavage. However, cleavage of HA0 is aided by NA, a protein which always counterbalances HA to make functional virions. The entire live cell experiments were carried out on a viral strain A/WSN/33 (H1N1), a putative successor of 1918–19 H1N1, as NA the gene of the 1918–19 was not sequenced. Due to atypical biology of the 'Spanish flu' virus scientists have postulated that the virus originated by genome adaptation and parallel evolution in the new human host (62).

A search in NCBI database shows that H1N1 is the most preponderant of all influenza virus sequences in web. The reason may be cumulative. It may due to wide interest among scientific community about this virus or easy availability of the viral data due to its recurrent outbreaks (7). The life realm of H1N1 'Spanish flu' did not go down in the nature with a stint for 2 years. The virus still roams in the nature under its own shadow (63).

There was another pandemic in 1957, the Asian flu, caused by H2N2. It was a reassorted virus between avian H2N2 virus and circulating H1N1 subtype. The HA, NA and PB1 genes of the pandemic virus were of avian origin, rest were derived from circulating human adapted H1N1. Study of pandemic H2N2 virus genome has shown that ratio of non-synonymous to synonymous mutation (d_N/d_S) is higher in avian derived gene compared to the gene constellations derived from human H1N1 (64). Therefore, it can be concluded that the pandemic virus was under intense selection pressure to get adapted to human. The virus acquired mutations in the RBS of HA gene to bind in the α-2–6 linked sialic acid receptors in the tracheolar epithelium of upper respiratory tract of human. The virus persisted for a decade and vanished in the wild. It only exists in wild aquatic birds. Present day human population has lost herd immunity against the virus, a fact fearful enough to cause another pandemic.

In 1968, a pandemic influenza virus H3N2, the Hong Kong flu emerged from circulating human adapted H2N2 and avian H3 virus by reassortment. Since 1968, H3N2 is evolving simultaneously with H1N1. However, rate of evolution H3 is higher than

H1. Since 1968, H3N2 has undergone paramount changes in its genes. Most changes have occurred in RBS and antigen binding site. Different strains of H3N2 are circulating in different parts of the world. The genetic diversity of the virus correlates with its size of the host population. A virus with small host size has less genetic diversity exemplifying 'bottleneck' effect and vice versa. Thus, H3N2 isolates from New York are highly diversified in comparison to the isolates from New Zealand (65). H3N2 virus continues to perpetuate worldwide, exhibits seasonal outbreaks due to antigenic drift in the viral genes.

Apart from devastating pandemic in 1918–19, H1N1 has struck human population several times. It caused the 1977 'Russian flu'and the only pandemic in of this century in 2009. H1N1 also causes seasonal flu in different parts of the world.

In 1977, there was an 'epidemic' outbreak of H1N1, scientifically documented as 'Russian flu' or 'Red flu' in electronic media. The virus was first identified in China but reported to WHO by Russia. 'Russian flu' is no doubt one of the bleak, cold events in the history of science. Theory, hypothesis and speculation loom large with the conjecture that viral outbreak was manmade, not ruling out release from biological warfare making factory (66). However, there is also a theory that the virus was as an accidental release from research laboratory or an unmindfully released strain from a failed vaccination trial (67). Whatever the source of origin of the virus was, there is high probability that the disaster was manmade, as the virus had high sequence similarity with another strain during a seasonal outbreak of influenza virus in 1950 (68–69). For the next two decades since 1950, H2N2 was preponderant followed by H3N2. Naturally, the question which baffled the scientific community was that, where from the virus originated from a purportedly dormant evolutionary phase? The virus showed considerable if not exact sequence similarity with its predecessor subtype of 1950. There was disparity between the rate of evolution of RNA and time of evolution (70). Most people who suffered from virus infection were of 26 yrs or under as they lacked protective immunity against the virus. Whatever, the source of origin is there is no doubt that the disaster was man made with an enigmatic pedigree.

H1N1 subtype reemerged in 2009; the virus was reported in USA, Mexico and gradually disseminated all over the world (71). The subtype was completely new to which people had no immunity (72–73). The virus bears various names i.e. 'Mexican flu, 'Swine flu' etc. According to standardization by WHO, the H1N1 virus of 2009 is documented as A(H1N1) pdm09 in research publications (74). The pandemic flu virus originated by reassortment, deriving genes from multiple hosts, i.e. bird, human and swine. Swine derived genes were from two different lineages namely North American and European. The hemagglutinin gene was from Northern American lineage whereas the neuraminidase gene was from European lineage. The virus did not directly originate from swine; however, swine did definitely work as a mixing vessel. The virus was much different from seasonal H1N1influenza virus, nevertheless very much homogeneous indicating a single event of ingression in human population or manifold attack of identical strains. The virus was unique in the sense that it did not exhibit many known molecular markers for human adaptation. Paradoxically the $^{Glu}PB2^{Lys}$mutation present in human adapted viruses, responsible for viral replication at 33º C was absent in the strain (75). Nonetheless, the virus exhibited human to human transmission by respiratory droplet, the most remarkable feature of any pandemic virus. Noticeable is that, reappearance of same subtype of virus does not mean the virus is under evolutionary stasis. Over the course of time a RNA virus undergoes antigenic drift, antigenic evolution and human population ceases to have herd immunity. According WHO report of August 2010, the A (H1N1) pdm09 is now in post-pandemic stage and looms large only as seasonal influenza virus in most part of the world (72). However, post pandemic emergences have been reported in India and New Zealand. India suffered a major resurgence of H1N1 in March 2015 (76), there was lingered festering of pdm09 H1N1 in India since 2009 pandemic (77). It is worthwhile mentioning that there are several clades of pdm09 H1N1 prevailing in different parts of the world. Also, there are several mutant strains among a single clade, originating by antigenic drift (78). Researchers have isolated three different strains among a single clade in India, all exhibiting different substitutions at different amino acid positions in the HA protein namely P83S, S203T, and I321V. Why does influenza show recurrent seasonal outbreaks? Apart from rapid antigenic drift which makes the virus unsusceptible to vaccination; scientists have discovered that several environmental factors i.e. temperature and relative humidity do play a role (79). Influenza virus shows efficient transmission in cold dry weather. In hot, humid weather it depends on salt and protein concentration of media.

Over the course of time the influenza A virus is exhibiting different dynamics of evolution. The whip of H1N1 virus has come down over years. Though the virus binds to α-2–6 linked sialic acid receptors, receptor binding assay has shown that avidity of HA for sialic acid has gone down. The H1N1 'Spanish flu' virus had broad range of avidity and specificity for α-2–6 linked glycan receptors. However, present day H1N1viruses have low or even very low avidity for its substrate and have become more precise in substrate selection (80). Since its inception H1N1 has developed new stratagems to trounce host population. H3N2, H1N1 and IBV are the most preponderant and

rapidly evolving influenza viruses in today's world. Due to breakthroughs in modern medicine, anti-influenza drugs and antibacterial drugs are available in market to counter viral and secondary bacterial infections. Nonetheless, over the course of past few decades the scourge of IAV infection has gone down throughout the world. No doubt it is due to advancement in molecular medicine and better surveillance programs. As follows the dogma of evolution, a parasite which annihilates its host is not going to perpetuate in nature.

6.2. The avian influenza viruses infecting human population

In the course of last few decades there are many media and scientific reports of avian influenza viruses infecting human. These viruses include H5N1, H7N3, H7N7, H9N2, H7N9 to mention a few are known as 'bird flu'. The disease spectrum is vast, encompassing flu like symptoms, pneumonia and allied respiratory diseases, conjunctivitis and even death. All these viruses have adroitly overcome species barrier. They jump from poultry birds to human with no aid of any intermediate host acting as 'mixing vessel'. Most of these viruses transmit via contact; people who are infected live close to hatcheries. Human to human transmission of these viruses require further research and understanding.

The foremost among the avian influenza viruses is no doubt the H5N1 subtype. The first notorious case of HPAIV H5N1 was among poultry birds in the farm houses of Guandong province in China (81). The virus first showed up in poultry firms, subsequently dispersed into human. In 1997, there was a major outbreak of H5N1 in Hong Kong poultry farms, leading to human infection and death (82). This was the first case of human infection of H5N1 virus. Both as regulatory and prophylactic measures there were mass cullings of poultry birds. Since then there have been several perennial outbreaks of HP H5N1 'bird flu' all over the world, predominantly in South Asia. Over the course of time 'bird flu' virus has become more virulent. In 2005, thousands of wild aquatic birds viz. bar-headed goose died due to HP H5N1 infection in Qinghai Lake, China, a place where migratory birds conglomerate. This was the first reported case of mass death of wild birds due to infection by highly pathogenic flu virus. The wild birds likely acquired HP H5N1 from local poultry farms rather than on their way of migration or at the wintering site. It may be plausible to conclude that H5N1 may have an atypical trait of reverse zoonotic potential, moving to and fro between different species of bird with poultry birds acting as intermediate host.

H5N1 virus exemplifies is one of the few rare cases of bird to human transmission. Analysis has showed that the HP virus of avian origin binds to sialic acid receptor linked to terminal galactose residues by α-2–3 linkage, a characteristic of avian receptor (83). This has broken down the long hold 'myth' that human infecting IAV must bind to the sialic acid receptors having α-2–6 linkage present in the upper respiratory tract. Also, receptor specificity may not restrict the virus to cross avian to human species barrier. Why is the H5N1 virus highly pathogenic? One reason is that the presence of polybasic residues of RRRKK at the cleavage point of HA0 peptide renders the precursor peptide better cleavable by ubiquitously distributed subtilin like intracellular endopeptidases. The substrates having the polybasic residues are hold tightly in the catalytic cavity of the endopeptidases forming greater number of hydrogen bonds and excluding water (84). How does a virus acquire polybasic residues at the cleavage site? It may happen during viral replication due to slippage of RNA polymerase.

Nonetheless, presence of polybasic residues at the cleavage point of HA gene may not make a virus highly pathogenic. Existence of H5 virus has been reported, having polybasic residues at the cleavage site of hemagglutinin gene, but the virus is of low pathogenic type (85). *In vitro* experiment has reported that a low pathogenic H5 virus having polybasic residues at the cleavage site may became highly pathogenic by serial passage, rendering the virus to gain 12 nonsynonymous mutations including accumulation of R293K mutation in the NA gene. The mutation attenuated enzymatic property of NA. Nonetheless, the virus became resistance to oseltamivir. Virulence of IAV is ascertained by an array of gene constellations making functional balance of several genes. Dropping of the receptor destroying potential of NA must have been counterbalanced by receptor-recognition activity of HA, making the IAV highly pathogenic.

Since the emergence of highly pathogenic H5N1 virus scientists have searched for molecular markers associated with its virulence. It has been deciphered that, over years the stalk length of NA has gone down in the highly pathogenic H5N1 with attainment of additional glycosylation in the HA1 domain of hemagglutinin (86–87). Paradoxically, shortening of stalk enhances virulence but reduces virus elution. However, while studying virulence of IAV in the context of glycosylation few points are needed to be kept in mind. Glycosylated sites in the HA1 domain act as antigenic sites. A motif bearing Asn-Xaa-Thr/ Ser may be glycosylated. Glycosylation protects a virus from proteolytic digestion and stabilizes 3D conformation of HA molecule. Nonetheless, glycosylation close to cleavage site hinders cleavage, making the virus less virulent (88). Also glycosylation needs to be studied in the backdrop of length of basic residues at the cleavage site. Loss of glycosylation may be synonymous with loss

of virulence, nonetheless, that can be counterweighed by addition of basic residues at the cleavage site (89). What are the molecular markers in the viral genome which have made the highly pathogenic virus to cross species barrier from chicken to human? Whole genome viral sequencing has deciphered that chicken and human genes of highly pathogenic H5N1 virus have a quantum of greater than 99% homology. Sequences of the amino acids in the HA protein of chicken and human are identical (90*).* Therefore it may be prudent to conclude that there is least likelihood that HA gene has played evolutionary role in crossing the species barrier from chicken to human. The virus must have a conglomeration of genes which have aided in crossing the species barrier. Apart from $^{Glu}627^{Lys}$ mutation at the amino acid position 672 in the PB2 polymerase protein derived from antigenic drift (91*),* which boosts virus replication at 33°C in the mammalian upper respiratory tract (92), the human adapted virus bears an array of mutations in its PB2 gene. These mutations no doubt act in biochemical coordination to promote human adaptation of the highly pathogenic H5N1 virus (93). None doubt it will be a challenging task to decipher the other molecular markers which aided the virus to cross species barrier.

Cross human dissemination is always a contentious topic in HPAIV. A virus can only become pandemic if it can undergo sustained transmission via respiratory droplet in nature. Research in animal model has shown that Q222L and G228S mutations (H3 numbering) in the RBS site of HA may render HP H5N1 to bind α-2–6 linked sialic acid receptors (94). Garnering two additional mutations viz. H107Y and T160Y close to the RBS of HA enhance the avidity of the virus for both avian and mammalian receptors. The four mutations and the E627K mutation in PB2 may cause respiratory droplet transmission of HP H5N1 among mammals. It stands to reason that in the course of time, in case the virus garners five selective mutations it may cause a pandemic. No doubt, HP H5N1 may be exploited for the purpose of bioterrorism. Has there been any report of isolation of HP H5N1 strain which has amassed any of the forenamed mutations apart from E627K mutation? To the knowledge of the reviewer there may be not, however, the highly crafty virus is accumulating mutations for sustained human adaptation (95).

In February 2003, the poultry firms in Netherlands were attacked by highly pathogenic avian flu H7N7. It was the first case of HP H7N7 to cause massive morbidity and mortality in poultry birds (96). The virus subsequently attacked human population. As a prophylactic measure, millions of chickens were culled. Most people suffered from mild to moderate conjunctivitis with one exceptional case of a veterinarian, who died of acute respiratory distress syndrome. In majority of the cases, HPAIV H7N7 exhibited tropism in ocular tissue with partial preference for α-2–6 linked sialic acid receptors. Though cases of respiratory illness have been reported, viral load and replication was always high in ocular tissue in comparison to respiratory tract (97). Nevertheless, HP H7N7 is not a unique virus among all IAV to exhibit eye infection. Research paper has reported cases of conjunctivitis due infection by H5N1and H1N1–2009 pandemic virus (98).

During the 2003 outbreak in Netherlands, there were several strains of H7N7 circulating in the nature exhibiting high level of genetic diversity. Presence of considerable diversity among viral strains made it perplexing to decipher human adaptation markers. Scientists found 37 amino acid differences between avian and human strains; 11 of which were in HA, 14 and 12 amino acid differences were found between avian and human NA and PB2 respectively. Two significant markers in the HA were V223A/I in the RBS and A143T in the HA1 domain, which may have played a role to alter viral receptor binding potential and cause high pathogenicity (99). It is noteworthy that a previous study has found high homology between HA gene of H7N7 to that of H7N1 and H7N3. The H7N1 and H7N3 strains in the study were confined to poultry with no evidence of crossing species barrier. Pathogenicity is a polygenic trait. It may be reasonable to conclude that H7N7 must have developed an array of cryptic mutations in its gene constellations which made it highly pathogenic across dual species spectrum (97). The impact of NA stalk in respect to pathogenicity of H7N7 needs further understanding; reduction of stalk length was not definitely a universal marker though it exhibited high level of nucleotide diversity. Nonetheless, unlike H5N1 which has sagas of global outbreaks, HP H7N7 infection is rare apart from a report of serendipitous discovery of a new strain in China in 2013 (100).

In March 2013, there was another massive outbreak of H7N9 virus in Chinese poultry firms which subsequently lead to human infection and death (101–102). The H7N9 ‘China flu’ in 2013 was the first report of H7N9 virus to cause human mortality. H7N9 was a triple reassortant avian virus; HA gene was derived from H7N3 virus of ducks, NA gene was from H7N9 virus of wild birds and rest of the genes were derived from H9N2 virus of bramblings (101). Genetically the virus was of ‘low pathogenic type’ i.e. it did not have polybasic residues at the cleavage site of hemagglutinin precursor peptide. The virus was isolated from lower respiratory tract of humans, most people had secondary bacterial infection followed by acute respiratory failure. Subsequent research has shown that H7N9 can bind and replicate in mammalian upper and lower respiratory tracts having preponderance of α-2–6 and α-2–3 linked receptors respectively (103–104). The virus has retained its affinity for α-2–3 linked avian sialic acid receptors, yet displays avidity

for human receptors having α-2–6 linkage (105). Nonetheless, scientists have concluded that the differences in avidity for α-2–3 linked and α-2–6 link sialic acid receptors are not significant (106). Yet two points are significant. First, the X-ray crystallographic structure of receptor-substrate complex of human adapted H7N9 virus is different from pandemic viruses coupled with human receptor. Second, high resolution X-ray crystallographic data has shown that structure of receptor-substrate complex of either avian or human H7N9 viruses complexed with avian or human receptors or vice-verse are different having different conformations (106).

During the 2013 outbreak, there were several strains of H7N9 viruses circulating having different amino acids at the critical sites of the viral genes. The initial isolates of H7N9 human viruses (i.e. A/ Shanghai/ 1/ 2013) had glutamine (Q) at the amino acid position 226 in the 220 loop of HA RBS. Subsequent isolates showed Q226L (i.e A/ Shanghai/ 2/ 2013, A/ Anhui/ 1/2013) or Q226I mutation (A/ Hangzhou/ 1/ 2013). The three isolates from Anhui and Shanghai had glycine (G) at position 228 in HA. All the sequences underwent T160A mutation close to hemagglutinin cleavage site (107) and deletion of 5 amino acids in the NA stalk. A/ Shanghai/ 1/ 2013, A/ Shanghai/ 2/ 2013, A/ Anhui/ 1/2013 sequences had E627K mutation in the PB2 peptide. What are the biological significances of the aforementioned mutations?

It is well documented that both the pandemic H2N2 and H3N2 viruses had leucine (L) and serine (S) at amino acid 226 and 228 respectively at the RBS of HA1 domain. Q226I mutation is found in seasonal influenza virus (107). All the viruses had strong avidity for human sialic acid receptors and exhibited transmission via respiratory droplet. The T160A mutation in the HA globular domain results in loss of glycosylation at the amino acid position 158 concomitant with enhanced binding affinity of HA for sialic acid receptor. Deletion of stalk length results in enhanced replication kinetics in the upper respiratory tract with concomitant change in tissue tropism. However, reduction of stalk length results in less efficient release of virus from host cells. The significance of E627K mutation in PB2 polymerase is well established. It is noteworthy that, HA and NA have opposing roles and must work in balance. A cell has to balance onerously the contending functions of HA and NA. The trade-offs may be required for stability and adaptability of an avian virus in mammalian host in the process of natural selection due to evolutionary pressure (108).

There is an apprehension that H7N9 may be a new pandemic threat. The virus has differentiated into two clades. One clade may cause the next pandemic of the century. H7N9 has attained an array of gene constellations and mutations which may have promoted its human adaptation (107,109–110). The strains of H7N9 virus having leucine (L) at amino acid 226 in the RBS site of HA1 domain have higher affinity for mammalian receptor in comparison to that of avian sialic acid receptor. But, the strains have to undergo 'biased' mutation at amino acid position 228 (G228S) in the RBS of HA1 domain to attain satiated binding potential for mammalian receptor. That may be one crucial evolutionary step in the trajectory towards prospective pandemicity. Nonetheless, evolution of H7N9 provides a vision about the dynamics of human adaptation of an avian virus. It may be prudent to conclude that the virus is still measuring the 'water-depth of human adaptation' (111).

All the above influenza viruses are rapidly evolving viruses which have been reported from different corners of the world. Besides, there are several subtypes of avian viruses which have killed poultry birds, human or may be both. H6N1, H10N8 and H9N2 are to name a few (112–115). H9N2, an endemic virus in Asian poultry is undergoing reassortment with H7N9 in the live poultry birds in China (116). H5N8 is a highly pathogenic virus which has reportedly attacked poultry birds in South Korea and Europe. Though the risk of human with H5N8 is reportedly low, the virus is another subtype which has undertaken reverse zoonosis and attacked wild birds following its evolution to high pathogenicity (117).

7. WHAT ARE THE DIFFERENCES BETWEEN PANDEMIC AND SEASONAL VIRUSES?

Seasonal flus may be controlled by annual vaccination. There is no vaccine against a pandemic influenza virus; it takes months to develop a vaccine against a pandemic subtype. According to the rule of US government, vaccines have to undergo three phases of clinical trials before being released in the market. Pandemic strains exhibit severe health effects; induce swift antibody response in comparison with seasonal strains. Pandemic strains secrete large amount of proinflammatory cytokines, cause fever for long duration. However, seasonal strains may exhibit high level of ephemeral effect. A comparative study has revealed that among all the present strains circulating in nature, H1N1pdm2009 is the most dreadful followed by seasonal H3N2, seasonal H1N1 and influenza B virus (118). This is not only due to their surface exposed proteins but total gene constellation. However, it has been deciphered that previous infection with seasonal subtype may produce cross protective immunity against a pandemic strain of same subtype having different gene constellation (119).

8. CONCLUSION

Research in the past few decades has made considerable progress in understanding the intriguing

biology of pandemic and seasonal influenza virus strains. One paradoxical feature of IAV evolution is that the prospective markers for human adaptation of the highly pathogenic viruses have even been found in the poultry birds. Early detection of molecular markers for human adaptation will no doubt render better surveillance program. Nonetheless, we need better understanding of few more aspects of influenza virus biology.

1. Pathogenicity of influenza virus should not be studied only in the context of molecular markers present in the hemagglutinin gene, though it plays a big role in pathogenicity. It has been studied that NA stalk length has scaled down over time from 1450 nt to 1310 nt. We need a better understanding of this molecular marker in the context of pandemics, epidemics, and seasonal outbreaks of IAV.
2. Why the virus is not virulent in its natural reservoir but becomes so only by interspecies and cross species transmission?
3. What made H7N7 virus to change its tissue tropism from respiratory tract to ocular tissue? Was it solely associated with molecular markers in the viral genome or polymorphism of immunological genes in human population?

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