Molecular events leading to the creation of a pandemic influenza virus

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Abstract Influenza A virus is a potent pathogen of annual respiratory illness with huge potential of causing occasional pandemics of catastrophic consequences. In April 2009, a novel, swine-origin influenza A H1N1/09 virus was identified in Mexico which continued to spread globally. This unique virus emerged from an avian, human, Eurasian swine viral strain and a North American swine strain belonging to the lineage of the 1930 swine virus. Till date H1N1/09 pandemic has been relatively mild and lacks the previously described molecular markers of influenza A pathogenicity and transmissibility. In this review, we will discuss the molecular and antigenic determinants of this virus and its designation as a low pathogenic strain, which carries the potential to develop into a devastating strain with subsequent mutations and reassortments.

Keywords H1N1 · Pandemic · Antigenic determinants · Pathogenicity

Introduction

The more we share space the more we share disease. Appropriately nature takes its revenge to restore a balance among different species by introduction of communicable diseases and viruses. Viruses are the most mutable and rapidly evolving human parasites. They are the cleverest of the whole lot and have the capability to defy all rules of nature. These organisms are more intelligent than humans and display great intelligence and skills to survive and evolve at the same time. Therefore, it is not very surprising to see, in the present century, a number of zoonotic diseases re-emerge in different parts of the globe and also witness more pathogenic viral reassortants.

For past many decades influenza virus has caused pandemics at irregular intervals and has been successful in killing millions of humans globally and still threatens to continue its devastation. Recently, cases of a novel H1N1 influenza virus of first human infection were identified in April 2009 in California and soon thereafter in Texas and Mexico, killing as many as 100 people. Since then, the virus has spread throughout the globe across Europe, Asia, South America, Australia and has caused 4,735 deaths, impelling the World Health Organisation (WHO) to raise its pandemic alert level to Phase 6, widespread onset of an influenza pandemic. It is very essential to develop a global surveillance system for the early detection of a virus having a pandemic potential.

Biology and epidemiology of influenza virus

Influenza viruses are, extremely abominable but very agile and rapidly mutating. They form a unique group of viruses

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classified into A, B and C types depending upon their matrix and nucleoprotein. Influenza A viruses, members of the Orthomyxoviridae family, genus influenza virus A, are an ingenious group of viruses endemic to wild aquatic birds, pigs, horses, ferrets and seals [1–12]. Influenza A virions contain 8 RNA genome segments of negative polarity encoding a total of 11 proteins. Enveloped virions are 80 to 120 nm in diameter, 200 to 300 nm long, and may be filamentous [13, 14]. They consist of spike-shaped surface proteins, a partially host-derived lipid-rich envelope, and matrix (M) proteins surrounding a helical segmented nucleocapsid. Mutations resulting from the error-prone replicative machinery of the virus (antigenic drift) and an ability to produce reassortants by the mixing of gene segments from two viruses (antigenic shift) in the event of a dual infection, both contribute to the self-sustenance of these viruses [15]. The influenza A virus can be further classified on the basis of antigenically different surface glycoproteins HA and NA with 16 types of HA and 9 types of NA. The virus undergoes antigenic drift with progressive continual changes in the external glycoproteins to evade the innate immune system. The new pandemic strains of influenza that have emerged have used a similar mechanism of antigenic shift.

Initially it was thought that the influenza virus cannot break the interspecies barrier but the myth was cleared when the prototype strain of SIV was recovered in 1930, which was genetically and antigenically similar to the 1918 pandemic H1N1 virus [16]. Influenza viruses are endemic to wild aquatic birds, waterfowl and ducks, hence exposure to them gives more opportunities for the introduction of an influenza virus into a swine herd, which acts as the much needed carrier in nature to bridge the gap between the two species – human and avian. Pigs are considered to be a potential source for novel influenza viruses, because they are the mixing vessels having receptors on their tracheal epithelial cells for both mammalian and avian influenza viruses to bind, thus increasing the opportunity for the recombination of genetic segments in vivo among various strains of viruses [17, 18].

Influenza in pigs was first detected in 1930 and was considered of same lineage as 1918 human pandemic strain [16]. Termed as classical swine H1N1 virus it remained antigenically stable and continued to circulate in pigs of Asia and North American in 20th century and until 1980s in Europe, where it was later replaced by a novel avian origin swine H1N1 virus [19]. In Europe this avian-like swine H1N1 reassorted with human H3N2 to give rise to swine H3N2 and H1N2 separately. Until 1997 in North America swine influenza was almost exclusively of H1N1 lineage, then a new swine influenza virus subtype H3N2 appeared with gene segments acquired from human, swine and avian influenza viruses leading to an epidemic in immunologically naive pigs which were only immune to the H1N1 strain [20].

Further reassortment between H3N2 and classical H1N1 resulted in the appearance of H1N2.

Swine influenza of subtypes H1N1, H1N2 and H3N2 has been variously reported as zoonosis in humans. In 1976, 13 cases of classical H1N1 swine influenza were observed in human with 1 death in USA. During 2005–2009, 11 human cases of triple reassortant swine H1N1 and H1N2 were recognized. In addition variants of human H3N2 have established in swine population where they show minimal drift, with this herd acting as a reservoir after these strains have disappeared from human population only to re-emerge later to infect humans.

Past influenza pandemics

The past century has witnessed around three devastating influenza pandemics and the most catastrophic of the three was the 1918 Spanish flu pandemic which resulted in approximately 40 million deaths worldwide [21–23]. The 1918 H1N1 virus was undoubtedly the most unique, lethal and highly virulent till date. The origins of this influenza variant still remain elusive, because of the unavailability of techniques to isolate the virus. It was thought to have originated in China in a rare genetic shift of the virus. The sequence of the 1918 HA resembled closely to A/sw/Iowa/30, the first influenza virus isolated from swine [24].

In 1957 a new Asian influenza pandemic virus emerged from the reassortment of the H1N1 virus and other influenza A virus which contained the HA, NA, and the gene for polymerase proteins (PB1) from an avian source, along with the remaining five RNA gene segments from the human H1N1 influenza virus which killed approximately 1 million people. With time the H1N1 virus circulation decreased and halted in humans. In 1968 another reassortant appeared in the form of the Hong Kong pandemic strain H3N2 carrying the HA and PB2 gene segments from H3 avian isolate and the remaining gene segments from a H2N2 human virus [25].

The Russian influenza outbreak in 1977 was benign and emerged from a H1N1 strain circulating in humans. The virus was restricted to immunologically naive individuals and caused an epidemic disease in children and adults below 25 years of age worldwide. The antigenic and molecular characterization of this H1N1 virus showed that HA and NA were remarkably similar to the H1N1 strain circulating in 1950s [26].

Swine flu outbreak

On 17 April 2009 CDC confirmed two cases of novel swine-origin influenza A (H1N1) virus (S-OIV) in the region of

California, also found in samples obtained from Mexico by a Canadian public health agency. WHO confirmed the Mexico and California cases of the same viral origin and issued the outbreak notice on 24 April (<http://www.who.int/csr/disease/swineflu/updates>). Since then this H1N1/09 virus has caused an influenza pandemic all over the globe. On 11 June WHO issued pandemic alert level of 6 (<http://www.who.int/mediacentre/news/statements/2009>). As of 17 October 2009, worldwide there have been more than 414,000 laboratory confirmed cases of pandemic influenza H1N1/09 with nearly 5000 deaths. In India, the first case of pandemic H1N1/09 was reported from a person travelling from USA on 13 May. Until 22 October total reported cases in India rose to 13,142 cases with 431 deaths, within a period of five months.

A nucleotide sequencing and phylogenetic analysis from samples obtained, identified the virus to be H1N1 that was antigenically and genetically unrelated to human seasonal influenza isolates and genetically related to viruses known to circulate in swine [27]. Samples collected from Mexico and USA reveal that this novel strain of swine origin is a result of reassortment between closely circulating Eurasian Swine and North American triple reassortant swine H1N2 virus. The gene segments encoding the proteins NA and M are genetically similar to the Eurasian swine isolate which was reported to enter the Eurasian swine population through wild ducks in 1979 [28]. It has since been confined to Eurasia and never been reported in North America. The triple reassortant swine virus H3N2 first reported in 1998 USA

swine population, was the result of reassortment between the North American human H3N2, the classical swine H1N1 virus and an unknown North American avian influenza isolate. A further reassortment of the 1998 isolate with the classical H1N1 swine resulted in North American swine H1N2 in 1999 [29, 30] (Fig. 1). Thus, the gene segments NP, NS and HA are derived from the classical H1N1 swine virus, the PB1 gene from the human H3N2 virus and the PB2 and PA genes from the avian lineage which entered the swine population in 1998.

Virus transmission

The pandemic H1N1 strain has acquired the ability to be transmitted human to human, even though the individual is not in direct contact with the infected swine. The virus has adapted itself in human population and the reassortments that had occurred in its genome have conferred efficient replication, transmission and sustenance in humans. As mentioned earlier, pigs serve as a mixing vessel for such reassortment between various strains of influenza due to the presence of both avian and human receptors in its tracheal epithelium [31]. Human influenza viruses preferentially bind sialic acid linked α -2,6 galactose receptors whereas, avian influenza viruses bind sialic acid linked α -2,3 galactose receptors [32]. So, the direct transmission of avian viruses to humans is restricted, as the co-infection of both viruses in pigs, in the same cell, will lead to recombination of their genomic segments.

Fig. 1 The reassortment between the avian, classical swine and human influenza virus resulted in the emergence of the triple reassorted North American H3N2 and H1N2. This triple reassortment virus then further reassorted with Eurasian swine flu lineage which lead to the emergence of the current pandemic H1N1 2009.

The H1N1/2009 isolate is not as transmissible as the 1918 H1N1 pandemic strain, but comparatively it is more transmissible than the seasonal influenza. The incubation time of the H1N1/2009 pandemic strain ranges between 2–7 days, which is longer than that of seasonal influenza A viruses (approx 1.4 days) [27]. Individuals in direct contact with influenza infected pigs are likely to contract infection. This strain is transmitted from infected to healthy individuals through dissemination of droplets (coughing/sneezing), infected saliva, nasal secretion, fecal matter and blood [33, 34]. The symptoms of swine flu in humans are similar to those of regular seasonal influenza and include fever, lethargy, lack of appetite and coughing. Some people with swine flu also have reported running nose, sore throat, nausea, vomiting and diarrhea. Signs of a more serious swine flu infection include pneumonia and respiratory failure. Individuals at high risk of getting afflicted are pregnant women, people with chronic medical problems, such as chronic lung disease, asthma, cardiovascular disease, diabetes, and immunosuppressed patients.

Viral pathogenicity

The pathogenesis of the virus is defined by its ability to enter and replicate within the host cell, its capability to combat host defense mechanisms and its ability to produce temporary or permanent damage. All these factors are determined by its genetic and antigenic composition. The host factors that decide the pathogenicity and virulence of a virus include the presence of target receptors on the host cells and availability of proteases in host cells which are essential for viral entry and replication.

Molecular characterization of influenza viral proteins

HA cleavage sequence

The hemagglutinin (HA) glycoprotein of influenza A viruses have two important functions that are required for the virus to be infectious. The HA protein is responsible for the attachment of a virus particle to the host cell and also the subsequent fusion of the host and viral membranes in the endosome for the introduction of viral genetic material into the cell.

Cleavage of HA into HA1 and HA2 by host proteases [17] such as furin in the trans-golgi network is a prerequisite for viral infectivity. HA cleavability is determined by the amino acid sequence at the cleavage site. The cleavage site has a

basic amino acid in 7 of the 8 positions (RERRRKRR) in the wild type influenza [35–37] (Table 1). The HA proteins of highly pathogenic H5 and H7 viruses and the HA of the 1918 influenza virus strain possess multiple basic amino acid residues at the HA1–HA2 cleavage site, which is eventually cleaved by ubiquitous cellular proteases. On the other hand, low pathogenic variants have a single Arg at the cleavage site which is only recognized by extracellular proteases secreted by cells in the respiratory and intestinal tract [38]. The low pathogenic strain can evolve into a highly pathogenic one if it acquires the necessary mutations at the cleavage site facilitating their processing and activation by the host proteases.

HA receptor specificity

Changes from leucine to glutamine and a change from serine to glycine at position 226 and 228 respectively have been reported to be involved in determining binding specificity to host cell receptors and changing the specificity from avian to human cell receptors [39–41]. For H1 HAs, amino acids at position 190 and 225 determine receptor-binding specificity. HA-Asp 190 and HA-Asp 225 confer binding to human type receptors, whereas HA-Glu 190 and HA-Gly 225 confer binding to avian-type receptors [42]. The recently emerged swine originated strain possesses the human type amino acid substitution at position 190 and 225, thus facilitating its adept transmission in humans.

NP, PB1 and PB2 as genetic determinants of high pathogenicity and virulence

There is an increasing evidence that the viral polymerase plays an important role in host adaptation. In a comparative study of the avian strain SC35 (H7N7) and its mouse-adapted variant SC35M it was shown that adaptation to mice was the result of seven mutations in the polymerase proteins, six of which (L13P and S678N in PB1, D701N and S714R in PB2, K615N in PA, and N319K in NP) were responsible for enhanced polymerase activity in mammalian cells [43]. In avian cells, replication and transcription of SC35M was reduced, whereas both activities were increased with SC35. PB2 mutation D701N has also been implicated in the adaptation of H5N1 viruses to mammalian hosts [44, 45] but other mutations notably PB2 mutation E627K has been found in most influenza viruses pathogenic to humans and also influences virulence in mice. It has been proved to be one of the host range determinants [46, 47].

Therefore, viruses with Lys at position 627 in PB2 are more pathogenic in mice and those with Glu at the same position are less pathogenic. The recently emerged SOIVs possess Glu and Asp at position 627 and 701 (Table 1) thus

classifying them as a low pathogenic strain however more comparative data on the other polymerase genes is not available.

PB1-F2: Pandemic protein

A recently identified and characterized influenza virus protein called PB1-F2 encoded by the PB1 gene from the +1 reading frame is a potential virulence factor that could play a role in pathogenesis of pandemic influenza viruses [48]. The influenza virus PB1-F2 is a 90-amino acid protein that is associated with the induction of cell death. PB1-F2 has been implicated in the causation of severe symptoms and high death rate associated with the 1918 virus [49]. All classic swine sequences have a truncated PB1-F2 (in-frame stop codons after 11, 24 and 35) (Table 1). Human H1N1 from 1918 through 1947 contains full-length PB1-F2, whereas human H1N1 beginning in 1956 has a truncated PB1-F2. The C-terminal domain of PB1-F2 contains the mitochondrial signal and can trigger apoptosis in specific cells. In a study, an influenza virus was engineered to express a version of PB1-F2 identical to that in the 1918 pandemic strain which was virulent in mice and led to more severe bacterial pneumonia, explaining the high virulence and high incidence of fatal pneumonia during the pandemic [50]. The above study suggested that the expression of full-length PB1-F2 may contribute to disease severity in mice and humans.

Role of NS-1

The non-structural protein 1 (NS1) of influenza A virus is a potent antagonist of the cellular antiviral interferon (IFN) response [51, 52]. It is a multifunctional protein with two domains, a dsRNA binding domain (RBD) and an effector domain (ED) that interacts with various host cellular proteins. NS1 is shown to be a virulence determinant, especially in the highly pathogenic H5N1 viruses. Thus, the ability of NS1 to counteract interferon activity and RNA

silencing suppression activity contribute to the virulence and pathogenicity of the virus.

The C-termini of highly pathogenic H5N1 viruses have a PDZ-binding motif that has been implicated in causing increased pathogenicity in mice [53]. The SOIVs lack the 11 C-terminal amino acids of NS1, and hence lack the PDZ domain motif therefore making this strain a low pathogenic one (Table 1).

The molecular and functional analysis of virulence determinants can be utilized for the development of molecular-based vaccines. Amid fears of a second wave of this potentially lethal virus, many countries have developed vaccines which would be administered to people at the most effective and safest dose to generate a strong immune response to the 2009 pandemic strain.

Conclusion

Ever since 1918, this tenacious virus has developed tricks to survive in one form or another. The virus evolves in a harmless reservoir making it hard to predict how and when it will exploit its gene pool to re-emerge. The possibility of re-assortment of this virus with seasonal influenza viruses and more importantly with avian (H5N1) viruses is a serious concern.

So far the virus is not that dangerous. However, it cannot be predicted whether it will stay mild or become serious. It is known that the 1918 virus was relatively mild in its first wave and it had acquired virulence when it came back in the second wave in winters.

The currently circulating H1N1 virus is a new strain of the flu virus, and because the human population has no immunity to it the potential to causing harm is greater. Unlike the various strains of animal flu that have emerged in the past, the latest pandemic strain is capable of spreading from human to human. The impact of any pandemic would vary in different countries, as it is mild in countries with developed health facilities but may be devastating in developing

Table 1 Comparison of molecular determinants of swine origin influenza virus and highly pathogenic avian influenza virus

Protein	Residues in highly pathogenic avian influenza virus	Residues in swine origin influenza viruses
PB 2	Lys 627	Glu 627
PB1	Leu 13, Ser 678	Pro 13, Ser 678
PB1-F2	90 amino acids, full length expressed	Truncated 11, 24 and 35 amino acids, expressed
NP	Asp 319	Asp 319
HA	Multiple basic amino acids at the cleavage site	Single basic amino acids at the cleavage site
NS-1	Presence of PDZ ligand domain motif at C-terminal	Absence of PDZ ligand domain motif at C-terminal

nations lacking adequate health systems. Understanding the molecular determinants responsible for pathogenicity and virulence of this virus is essential to develop drugs and vaccines to combat its lethality.

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