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Review

Influenza Virus Evolution, Host Adaptation, and Pandemic Formation

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Newly emerging or "re-emerging" viral diseases continue to pose significant global public health threats. Prototypic are influenza viruses that are major causes of human respiratory infections and mortality. Influenza viruses can cause zoonotic infections and adapt to humans, leading to sustained transmission and emergence of novel viruses. Mechanisms by which viruses evolve in one host, cause zoonotic infection, and adapt to a new host species remain unelucidated. Here, we review the evolution of influenza A viruses in their reservoir hosts and discuss genetic changes associated with introduction of novel viruses into humans, leading to pandemics and the establishment of seasonal viruses.

Introduction

Zoonotic infections, in which a pathogen adapted to another animal species causes disease in a human, may be sporadic, dead-end infections without adaptation to humans (e.g., Ebola and Hanta viruses). Other zoonotic infections can stably adapt to humans, leading to sustained person-to-person transmission (e.g., HIV and SARS). Influenza A viruses (IAVs) fall into this latter category, in which multiple stable host switch events have been reported. The mechanisms by which viruses stably adapt to new host species, often distantly related, are still largely unelucidated. Like IAVs, many of the viruses that have demonstrated an ability to cause zoonotic infections and/or transmit between host species have RNA genomes (Holmes, 2010).

With a low-fidelity viral RNA polymerase lacking exonuclease proofreading capability and inherently high error rate, IAVs exist as populations of quasispecies (Domingo et al., 1998). Random mutations can be rapidly selected for or against, depending upon the evolutionary pressures applied, including novel host environment (Landolt and Olsen, 2007), response to pre-existing immunity leading to antigenic drift (Smith et al., 2004), or antiviral drug pressure leading to resistance (Ong and Hayden, 2007).

The major natural host species of IAVs include wild aquatic waterfowl and shorebirds (Webster et al., 1992). IAVs have been able to adapt stably to a wide variety of animals, including avian and mammalian species, and novel human-adapted IAVs have emerged to cause pandemics several times in the last 100 years (Taubenberger and Morens, 2009). Each of the viruses causing these pandemics has emerged in a different way, making generalizations about zoonotic IAV adaptation to humans difficult. As data have accumulated, it has become clear that IAV host switch events are polygenic and represent different solutions to the common problem of replication and transmission in a host (Taubenberger and Morens, 2009). The relative paucity of stable IAV host switch events from the avian reservoir to humans and domestic animals further supports the complexity of the process and severely limits our ability to predict future emergence. Host switch/adaptation, transmissibility, and pathogenicity/virulence are each complex multifactorial interactions between virus and host and are likely under independent selective pressures (Taubenberger and Morens, 2009).

Overview of Influenza Pandemics

Influenza viruses are among the most common causes of human respiratory infections and among the most significant because they cause high morbidity and mortality. Influenza outbreaks have apparently occurred since at least the Middle Ages, if not since ancient times (Taubenberger and Morens, 2009). In the elderly, infants, and people with chronic diseases, influenza is associated with especially high mortality. In the United States, influenza results in approximately 200,000 hospitalizations and 36,000 deaths in a typical endemic season. In addition to annual winter outbreaks, pandemic IAVs occasionally emerge, as they have every 8–41 years for at least several centuries. IAV pandemics are global outbreaks due to viruses with novel antigenic subtypes. Up to 50% of the population can be infected in a single pandemic year and can be associated with a dramatic increase in number of deaths.

In the last 500 years, since 1510, there have been approximately 14 IAV pandemics; in the past 120 years, there were pandemics in 1889, 1918, 1957, 1968, 1977, and 2009 (Taubenberger and Morens, 2009). In 1918, the worst pandemic in recorded history caused approximately 675,000 total deaths in the United States and killed up to 50 million people worldwide (Johnson and Mueller, 2002). The 1957 and 1968 pandemics caused approximately 70,000 and 34,000 excess deaths in the United States, respectively (Noble, 1982). While it is too early to assess the full impact of the 2009 pandemic, in its first year, there have been approximately 12,000 deaths in the United States, of which the vast majority have been in individuals under age 65.

Overview of the Biology of IAVs

Influenza viruses (family *Orthomyxoviridae*) are enveloped negative-strand RNA viruses with segmented genomes containing 7–8 gene segments. The five genera in the family include influenzavirus A, influenzavirus B, influenzavirus C, thogotovirus, and isavirus (infectious salmon anemia virus) (Palese and Shaw,

2007; Wright et al., 2007). The three influenzavirus genera differ in host range and pathogenicity and are likely to have diverged evolutionarily at least several thousand years ago. Influenza A and B viruses have a similar structure, whereas influenza C is more divergent. Influenza A- and B-type viruses contain eight discrete single-stranded RNA gene segments, each encoding at least one protein. Only IAVs pose a significant risk of zoonotic infection, host switch, and the generation of pandemic IAVs.

IAVs are enveloped with a host cell-derived lipid membrane. The eight gene segments encode at least 11 open reading frames (ORFs) (Figure 1). IAVs are covered with projections of three proteins: hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2). HA is a glycosylated type I integral membrane protein with functions both as the viral receptor-binding protein and fusion protein. The matrix 1 (M1) protein is found under the membrane and interacts with cytoplasmic domains of the surface glycoproteins and also with the viral ribonucleoprotein (RNP) complexes. HA recognizes sialic acid (SA) (N-acetyl neuraminic acid) bound to underlying sugars on the tips of host cell glycoproteins. IAVs have HAs with different specificities for the disaccharide consisting of SAs and the penultimate sugar (galactose or N-Acetylgalactosamine [GalNAc]) with different glycosidic bond isomerization. IAVs adapted to birds have an HA receptor-binding specificity for α2-3 SA, while HAs from IAVs adapted to humans have higher specificity for α2-6 SA (see below). After binding its receptors, the virus is internalized, and the acidic pH in the endosomal compartment leads to a conformational change in HA, mediating fusion of the viral and endosomal membranes, allowing release of viral RNPs into the cytoplasm. Mature HA is a trimer, and each monomer undergoes proteolytic cleavage to generate disulfide-bonded HA1 and HA2 polypeptide chains prior to activation. IAV requires exogenous serine proteases (trypsin-like enzymes) for activation, recognizing a conserved Q/E-X-R motif found at the HA cleavage site (Chen et al., 1998). In humans and other mammals, this is likely to be the tryptase Clara produced by cells of the bronchiolar epithelium. Cleavage activation of HA likely requires similar proteases in avian intestinal and/or respiratory cells. IAV of H5 and H7 subtypes can acquire insertional mutations at the HA cleavage site, changing their protease recognition site to a furin-like recognition sequence, R-X-R/K-R. This polybasic cleavage site broadens protease specificity, allowing intracellular cleavage activation and systemic replication, resulting in a highly pathogenic avian influenza (HPAI). Prototypic is the Eurasian lineage of H5N1 HPAI, which has circulated widely in the last decade, causing high mortality in domestic poultry as well as causing human infections and death.

NA is a type II integral membrane glycoprotein with sialidase enzymatic activity required for cleavage of both host cell SAs, allowing release of newly produced virions, and SAs on viral glycoproteins to prevent aggregation of nascent viral particles. The complementary functions between SA binding by HA and SA removal by NA likely require evolutionary coadaptation. Both HA and NA are the major antigenic targets of the humoral immune response to IAV, and NA is the target of the antiviral drugs oseltamivir and zanamivir. The small protein M2 is a proton channel necessary for viral replication and is the target of the adamantane class of antiviral drugs.

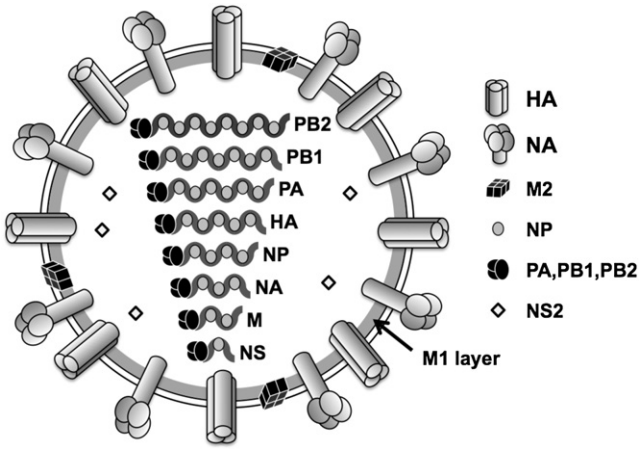

Figure 1. Diagrammatic Representation of an Influenza A Virus
The two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), along with small numbers of the matrix 2 (M2) ion channel protein, are embedded in a lipid bilayer. The matrix 1 (M1) protein underlies the envelope and interacts with the surface proteins and also with the ribonucleoproteins (RNPs). RNPs consist of the eight negative-stranded RNA segments and nucleoprotein (NP) and the polymerase complex heterotrimer (PB2, PB1, and PA). The nuclear export protein (NEP, or nonstructural protein 2, NS2) is contained within the virion, but the nonstructural protein 1 (NS1) is not.

Each IAV RNA segment is encapsidated by nucleoprotein (NP). In virions, viral RNA are wrapped around NP monomers and packaged into viral RNPs, along with the three polymerase proteins (polymerase basic protein 2 [PB2], PB1 polymerase basic protein 1 [PB1], and polymerase acidic protein [PA]). The polymerase proteins form a heterotrimer bound to a short hairpin structure formed by the partially complementary terminal 5′ and 3′ untranslated regions (UTRs) of each RNA segment. PB1 serves as the RNA-dependent RNA polymerase. PB2 functions in mRNA synthesis by binding host mRNA caps. While PA is necessary for a functional polymerase complex, including endonucleolytic cleavage of host RNAs, its biological roles remain poorly understood. It may have additional proteolytic activity and may also act as an elongation factor during RNA synthesis. NP acts primarily as a single-strand RNA-binding protein and serves as the structural protein in the RNPs. In addition, it plays an important role in transcription and in the trafficking of RNPs between the cytoplasm and nucleus. IAV RNA transcription and replication occur in the host nucleus, since IAV is dependent on the RNA processing machinery of the host cell. The processes of being imported into the nucleus, exported back out to the cytoplasm, and then prevented from re-entering the nucleus also all appear to depend on the interaction of NP with host proteins.

The nonstructural 1 (NS1) protein has multiple functional domains, including N-terminal dsRNA binding (1–73) containing a nuclear localization signal (NLS), a central effector domain (73–207) containing a nuclear export signal, and a C-terminal region (207–230) containing a PDZ domain (Hale et al., 2008). NS1 has pleiotropic functions, including dsRNA binding, enhancement of viral mRNA translation, inhibition of host mRNA processing, and type I interferon antagonism (Palese and Shaw, 2007). The NS2 protein (also referred to as nuclear export protein, NEP) is found in virions and facilitates nuclear export of viral

RNP complexes. Another small viral protein, PB1-F2, is variably encoded within the PB1 gene by an alternative reading frame, targets the mitochondrial inner membrane, and may play a role in apoptosis during IAV infection. Recently, the PB1 gene has also been reported to encode a third polypeptide expressed via differential AUG codon usage, termed N40 (Wise et al., 2009).

Type B and C influenza viruses are adapted to and isolated almost exclusively from humans, although influenza B viruses have been isolated from seals and influenza C viruses have been isolated from pigs and dogs (Wright et al., 2007). In contrast, however, IAVs infect a wide variety of warm-blooded animals, including birds, swine, horses, and humans. Avian IAV in aquatic birds likely serves as the predominant natural reservoir for all known subtypes and probably is the ultimate source of all human pandemic IAV strains (Webster et al., 1992).

IAVs are subdivided by antigenic characterization of the HA and NA surface glycoproteins. Sixteen HA and nine NA subtypes are known, all of which have been isolated from avian hosts. Theoretically, therefore, 144 possible HA-NA subtype combinations are possible, and IAVs expressing at least 116 of these subtype combinations have been isolated in birds (Krauss et al., 2004; Munster et al., 2007). World Health Organization guidelines for the nomenclature of influenza viruses are as follows. First, the type of virus is designated (A, B, or C), then the host (if nonhuman), place of isolation, isolation number, and year of isolation (separated by slashes). For IAV, HA (H1–H16) and NA (N1–N9) subtypes are noted in parentheses. For example, strains included in the most recent trivalent vaccine for the 2010–2011 season in the United States are: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008.

Antigenic Drift and Antigenic Shift

IAVs are evolutionarily dynamic viruses and have high mutation rates (ranging from approximately 1×10^{-3} to 8×10^{-3} substitutions per site per year) (Chen and Holmes, 2006). Mutations that change amino acids in the antigenic portions of the surface glycoproteins HA and NA may produce selective advantages for viral strains by allowing them to evade pre-existing immunity. This is especially important in IAVs adapted to humans, which are subjected to strong population immunologic pressures. Antibodies against the HA protein prevent receptor binding, can be neutralizing, and are effective at preventing reinfection with the same strain (Murphy and Clements, 1989). Such selective mutation in the mapped antigenic domains of HA and NA has been termed antigenic drift.

Because the IAV genome consists of eight discrete RNA segments, coinfection of one host cell with two different IAVs can result in progeny viruses containing gene segments of both parental viruses. When this process of genetic reassortment involves the gene segments encoding the HA and/or NA genes it has been termed antigenic shift. There are theoretically 256 (2^8) possible combinations of the eight gene segments from reassortment between two parental viruses. Reassortment has been shown to be both common and important in IAV evolution (Dugan et al., 2008; Holmes et al., 2005) and host switch events (Garten et al., 2009; Scholtissek et al., 1978). Homologous recombination is not common in negative-sense RNA viruses like IAV (Boni et al., 2008), but recombination by template switching has been described and has played a role in changing the virulence or fitness of some IAVs (Wright et al., 2007).

IAVs in Wild Birds

Genetically and antigenically diverse IAVs are widely distributed in wild avian species around the world. They are maintained predominantly by asymptomatic infections (termed low pathogenic avian influenza [LPAI]), most frequently documented in aquatic birds of the orders *Anseriformes* (ducks, geese, swans, etc.) and *Charadriiformes* (gulls, terns, etc.). At least 105 species of wild birds have been identified as harboring IAVs (Munster et al., 2007). IAVs in wild aquatic birds tend to be predominantly transmitted via a fecal-oral route and to infect epithelial cells in the lower intestinal tract, where they cause little to no apparent disease.

The distribution of HA and NA subtypes is not uniform among wild bird IAV isolates, with some HA subtypes being more common than others. While most HA subtypes have been isolated from the *Anseriformes*, HA subtypes H13 and H16 have been isolated predominantly from *Charadriiformes* (Munster et al., 2007). IAVs have been isolated from other orders of birds (e.g., the large order *Passeriformes*, containing over half the bird species), but most surveillance efforts have focused on *Anseriformes* (aquatic waterfowl) and to a lesser extent *Charadriiformes* (shorebirds) because of the high prevalence of IAV infection in these species, especially in dabbling ducks. There are little available data to assess whether particular IAV subtypes or strains possess adaptations to particular wild bird host species outside of the observation that IAVs with H13 and H16 HA subtypes are isolated predominantly from gulls. Ecological differences, like differences in feeding behavior, geographic localization, and migratory patterns, likely all play roles in the complex ecobiology of IAV in birds.

Two evolutionary models can explain the global pattern of IAV diversity in wild birds, analogous to the allopatric and sympatric models of speciation, and it is likely that both have played roles in IAV evolution in wild birds (Dugan et al., 2008). Phylogenetic analyses demonstrate that while all IAV HA subtypes had a common ancestor, the HA subtypes did not originate in a single radiation and include higher-order clustering. Intersubtype genetic diversity is high, but intrasubtype diversity is quite low. Hence, the genetic structure of avian IAV HA is characterized by highly divergent subtypes that harbor relatively little internal genetic diversity. This is also the case for the evolution of the nine NA subtypes. Interestingly, analyses suggest that this diversity reflects a very recent origin, with ranges for the most recent common ancestors (TMRCAs) of the different HA subtypes in the period of the last several hundred years (Chen and Holmes, 2010). The NS gene segment in bird IAV is characterized by a deep divergence between the A and B alleles, strongly suggesting that the two alleles are subject to some form of balancing selection (Dugan et al., 2008). Far less genetic diversity is observed in the five remaining IAV gene segments in wild birds (PB2, PB1, PA, NP, and M). Phylogenetic analyses also reveal a clear separation of avian IAV sequences from eastern and western hemispheres, supporting allopatric evolutionary pressures (Dugan et al., 2008; Munster and Fouchier, 2009). Mixed infection and reassortment has also been shown to be extremely common in IAVs in wild birds (Wang et al., 2008) with little

evidence of genetic linkage among specific segments. The large number of different HA-NA subtype combinations recovered also highlights the frequency of reassortment in avian IAVs and provides little evidence for the elevated fitness of specific HA-NA combinations. In contrast to the extensive genetic diversity seen in HA, NA, and NS, the five remaining internal gene segments encode proteins that are highly conserved at the amino acid level, indicating that they are subject to widespread purifying selection. The fitness landscape for these genes is therefore not determined by cross-immunity but by functional viability, with less selective pressure to fix advantageous mutations. Given such strong conservation of amino acid sequence, large-scale reassortment likely involves the exchange of functionally equivalent segments, with little impact on overall fitness. Dugan et al. hypothesized that IAV in wild birds exists as a large pool of functionally equivalent and so often interchangeable gene segments that form transient genome constellations, without the strong selective pressure to be maintained as linked genomes (Dugan et al., 2008).

IAVs maintained in wild birds have been associated with stable host switch events to novel hosts, including domestic gallinaceous poultry, horses, swine, and humans, leading to the emergence of viral lineages transmissible in the new host. Adaptation to domestic poultry species is the most frequent (Wright et al., 2007). Stable host switching likely involves the acquisition of a number of mutations, depending on the virus and the species, that serve to separate an individual clonally derived IAV strain from the large wild bird IAV gene pool. Because adaptation to a new host likely limits the ability of these viruses to return to the wild bird IAV gene pool (Swayne, 2007), these emergent viruses must evolve as distinct eight-segment genome constellations within the new host (Dugan et al., 2008; Taubenberger and Morens, 2009).

IAVs in Domestic Galliform Poultry

Domesticated birds of the order *Galliformes* (e.g., turkeys, chickens, quails, etc.) are not a reservoir host of avian IAV, but are nonetheless susceptible to infection with wild-bird-derived IAV after adaptation. Once IAVs are adapted to gallinaceous poultry, they rarely circulate in the wild bird IAV viral pool (Swayne, 2007). A dramatic exception to this trend is the recent Eurasian-lineage HPAI H5N1 viruses that have been isolated in wild bird populations in Europe and Asia.

The molecular features of host adaptation to domestic gallinaceous poultry are not yet fully elucidated, but include positive selection for mutations in both HA and NA (Campitelli et al., 2004; Perez et al., 2003) and viral RNP proteins (Wasilenko et al., 2008). IAVs isolated from domestic poultry generally maintain an HA receptor-binding specificity for α2-3 SA (Wright et al., 2007). Another characteristic feature of poultry-adapted IAV is an in-frame deletion of approximately 20 amino acids from the stalk region of the NA (Blok and Air, 1982). NA stalk deletion has been associated with reduced enzymatic activity of the NA (Baigent and McCauley, 2001) and may be a compensatory adaptive change to reduced HA receptor-binding activity of wild bird IAV adapted to replicate in the respiratory tract of poultry.

Sporadically, strains of poultry-adapted H5 or H7 IAV evolve into HPAI, usually through acquisition of an insertional mutation, resulting in a polybasic amino acid cleavage site within the HA (Wright et al., 2007). The current Asian-lineage HPAI H5N1 panzootic appears to be unique in the era of modern influenza virology (Webster et al., 2007), resulting in the deaths of millions of poultry in 64 countries on three continents, either from infection or culling. There are also significant zoonotic implications of this panzootic, with 498 documented cases in humans, resulting in 294 deaths in 15 countries since 2003 as of May 2010.

IAVs in Mammals

IAVs have been isolated from numerous mammalian host species, including humans, domestic pigs, horses, and dogs, as well as such diverse hosts as pinnipeds (seals), cetaceans (toothed whales), mink, and anteaters, among others (Landolt and Olsen, 2007). Phylogenetic evidence suggests that all mammalian IAV strains ultimately derive from the avian IAV pool (Wright et al., 2007). IAV strains have been consistently isolated from pigs and horses, but only sporadically from other domestic and wild mammals. It is unclear if there are stable wild mammalian IAV hosts or if occasional zoonotic infections leads to localized outbreaks that do not persist. Only additional surveillance can answer this question.

IAV in Swine

Swine IAV was first clinically detected in 1918 in association with the 1918 pandemic (Taubenberger et al., 2001), and it is unclear if IAV infections in swine occurred prior to that time. Since then, swine IAV has been continuously recognized and is a disease of major economic and public health importance (Landolt and Olsen, 2007). IAVs of a number of subtypes have been isolated from swine globally, a few causing enzootic infections and many causing only limited outbreaks without continued circulation. Since the isolation of the first IAV by Shope from pigs in the 1930s, these ''classical'' swine H1N1 viruses, likely derived from the 1918 pandemic IAV (Taubenberger et al., 2001), caused enzootic seasonal disease in pigs in North America and worldwide (Reperant et al., 2009). From 1998, several different lineages of ''triple'' reassortant viruses of H3N2, H1N2, and H1N1 subtypes, containing genes from the classical swine H1N1, human H3N2, and avian IAVs, emerged to cause enzootic disease in pigs in the U.S. and globally (Olsen, 2002). In Europe, a novel lineage of H1N1 emerged in the late 1970s by adaptation of an avian IAV to swine, leading to enzootic disease in Eurasia (Dunham et al., 2009; Pensaert et al., 1981). Other fully avian or fully human IAV-derived isolates or reassortant viruses containing genes of avian, swine, and/or human IAV origin have been associated with less widespread disease in swine (Reperant et al., 2009).

Since swine have been shown to be susceptible to infection with both avian and human IAV strains, this host species has been considered a prime ''mixing vessel'' or intermediate host for the generation of IAVs of pandemic potential to humans (Webster et al., 1992). This dual susceptibility has been associated with the presence of both α2-3 and α2-6 SA linkages on the glycocalyx of epithelial cells lining the pig trachea, but recent lectin histochemistry studies have shown little α2-3 SA in the nasal, tracheal, and bronchial epithelium (Van Poucke et al., 2010), with a SA receptor pattern not unlike the human respiratory tree (Nicholls et al., 2007). These data, along with evidence of limited replication and transmission of avian IAV in swine,

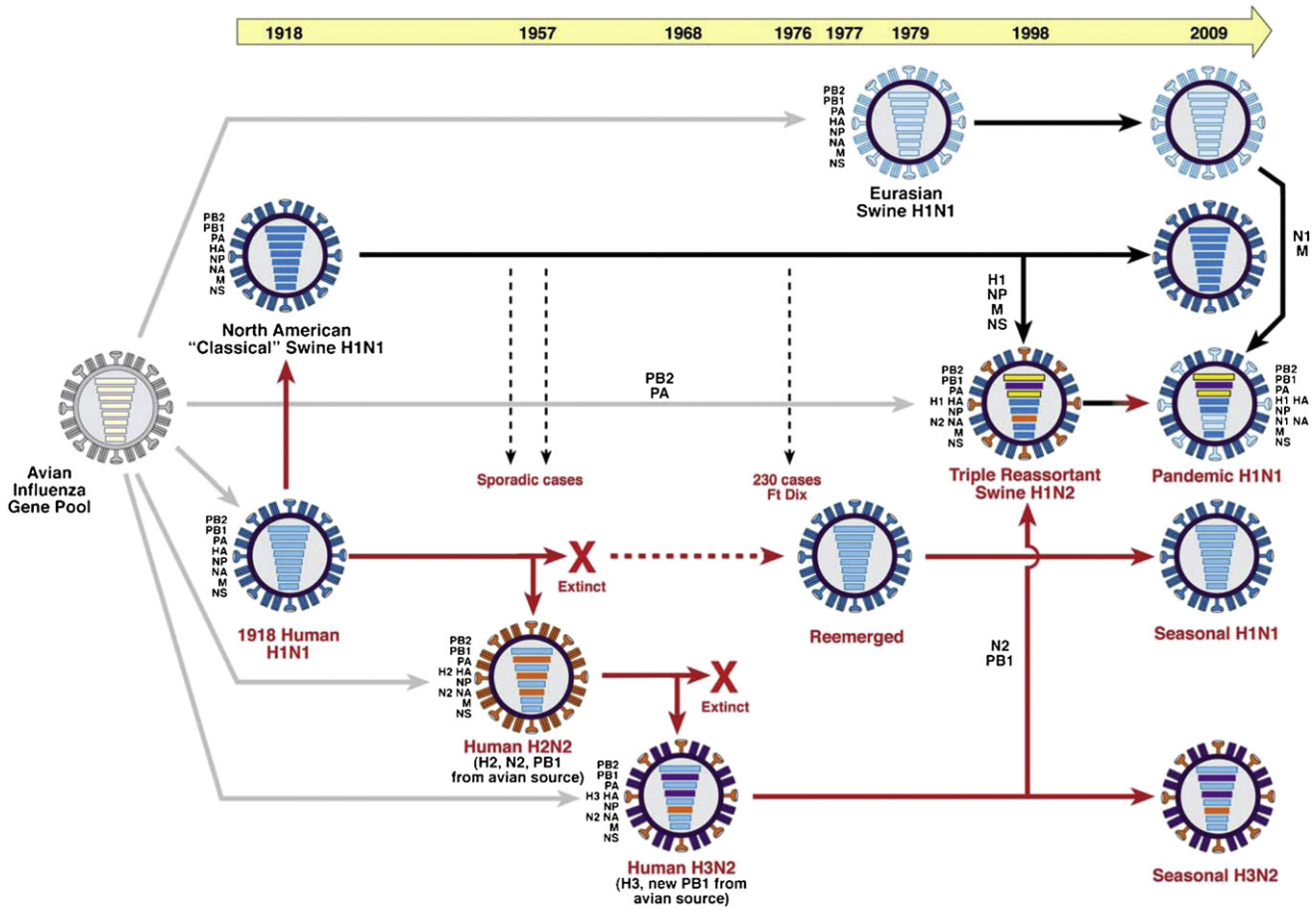

Figure 2. Genetic Relationships between Human and Relevant Swine Influenza Viruses, 1918–2009
Gray arrows reflect derivation of one or more gene segments from the avian IAV gene pool (although the timing and mechanism of emergence in each case remains unknown). The dashed red arrow indicates a period without circulation of H1N1 in humans. Solid red arrows indicate the evolutionary paths of human IAV lineages; solid black arrows, of swine IAV lineages; and the black-to-red arrow, of the swine-origin 2009 human H1N1 pandemic IAV. IAVs contain eight gene segments (as shown in Figure 1). The dashed, descending black arrows reflect human zoonotic infections with swine IAVs. Figure is modified from (Morens et al., 2009).

further complicate our understanding of the mechanisms of IAV avian-to-mammalian host switch events. Clearly, changes in HA receptor-binding specificity play a significant role, but the constellation of genes that control efficient replication and transmission in the new host is also vitally important.

IAV in Horses

Unlike swine IAV, equine IAV has been recognized for hundreds of years (Taubenberger and Morens, 2009): interestingly, often in close association with human IAV epidemics or pandemics. In the virologic era, the first IAV isolated from horses occurred in an epizootic (an outbreak in animals) in 1956. This lineage of equine H7N7 is now likely extinct, but an H3N8 equine IAV lineage first detected in the early 1960s continues to circulate enzootically (endemically in animals) and cause a high disease and economic burden on the horse industry. In 1989, an independent, avian-like H3N8 virus was isolated following an epizootic in horses in China, but this lineage has not persisted. Available phylogenetic evidence suggests that all these equine lineages were derived from avian IAV (Horimoto and Kawaoka, 2001).

IAVs in Humans

Our understanding of pandemic IAV at the viral level is limited. Pandemic viruses have been isolated from 1957 onward, and the 1918 pandemic virus was reconstructed using an "archaevirologic" approach (Taubenberger et al., 2007), but we do not yet know the subtypes or genetic makeup of the pandemics before 1918. All the pandemic viruses since 1918 contain gene segments derived from the 1918 virus (Figure 2), and consequently, it may be considered that the past 91 years are part of a larger "pandemic era" (Morens et al., 2009).

The 1918–1919 Pandemic Virus

The "Spanish" influenza pandemic killed an estimated 50 million or more people (Johnson and Mueller, 2002). Reconstruction of the viral genome from the tissues of several victims has demonstrated that the causative agent was an avian-descended H1N1 virus (Rabadan et al., 2006; Taubenberger et al., 2005). As no human pre-1918 IAV sequences are currently available, the origin of the pandemic virus, including timing of its emergence in humans and whether an intermediate host was involved, remains unresolved (Smith et al., 2009a). The high mortality

associated with the 1918 virus appears to have been a result of bacterial pneumonia, but the copathogenic mechanisms responsible for such fatal bacterial diseases remain unknown (Morens et al., 2008). Epidemiological features of the pandemic were also unprecedented, including its appearance in up to three waves within the first year and a "W-shaped" (trimodal) age-specific mortality curve that featured an unexplained peak in healthy young adults.

By about 1920, the virus began to circulate in a pattern of seasonal endemic recurrences, and it remained so as it "drifted" antigenically for nearly 40 years. When the next pandemic appeared in 1957, the H1N1 virus disappeared from circulation. However, 20 years later, in 1977, it returned to circulation and caused a (low-grade) pandemic that disproportionately affected persons of less than 20 years old. The 1918 H1N1-lineage IAV continues to cocirculate globally today, along with H3N2 IAV descended from the 1968 pandemic and. since 2009, with the new H1N1 pandemic virus. It is remarkable not only that direct (all-eight-gene segment) descendants of the 1918 virus still circulate in humans as epidemic H1N1 viruses and in epizootic form as classical swine H1N1 viruses, but that for the past 50 years the original virus and its progeny have continually donated genes to new viruses to cause new pandemics, epidemics, and epizootics (Figure 2). The novel H1N1 virus associated with the ongoing 2009 pandemic is a fourth-generation descendant of the 1918 virus (Morens et al., 2009).

The 1957–1958 Pandemic Virus

The H2N2 "Asian" pandemic virus that emerged in 1957 was a lineal descendant of the 1918 H1N1 pandemic virus that acquired three novel gene segments by reassortment with an unknown avian virus. The gene segments encoding HA and NA were replaced by an avian-like H2 subtype HA and an N2 subtype NA (Scholtissek et al., 1978), respectively, with the other five gene segments retained from the 1918-derived H1N1 lineage. The gene segment encoding the PB1 polymerase was also replaced with an avian-like gene segment (Kawaoka et al., 1989). Even though this pandemic occurred in an era when experimental influenza virology was active, identity of the host in which reassortment event(s) occurred remains unknown. It is also not known how long it took from the initial reassortment event(s) for the virus to evolve into the efficiently transmissible, human-adapted IAV that caused the pandemic. Its pathology and clinical appearance were similar or identical to those of the 1918 virus, although the unusual epidemiologic features of the 1918 pandemic noted above were not seen in 1957. As was true for the 1918 pandemic, after about two years the virus became seasonally endemic and sporadic, disappearing entirely within 11 years. To date, it has not returned.

The 1968 Pandemic Virus

Like the pandemic that preceded it, the 1968 H3N2 "Hong Kong" pandemic was caused by a reassortment event between a circulating human H2N2 virus and an avian IAV, acquiring novel HA (H3 subtype) and PB1 gene segments (Kawaoka et al., 1989; Scholtissek et al., 1978). The other six gene segments, including the NA gene segment, were retained from the 1957 H2N2 virus (including five segments—PB2, PA, NP, M, and NS—retained from the 1918 H1N1 lineage). Antibodies to NA, while not preventing infection, have been shown to reduce the duration and severity of illness. It has been suggested that the relative mildness of the 1968 pandemic was the result of the retention of the previously circulating N2 NA (Kilbourne, 1997). The 1968 pandemic was so mild in its mortality impact that in some locations, fewer deaths occurred than in certain nonpandemic years (Morens et al., 2009). As had been the case in 1957, the virus quickly became endemic and seasonal in its appearance and has now circulated globally for 42 years. Paradoxically, the morbidity and mortality burden of antigenic drift variants of the H3N2 lineage over the past decades has been very high (Morens et al., 2009). With the emergence of the novel H1N1 pandemic in 2009, little evidence of H3N2 circulation was noted. However, as of May 2010, H3N2 continues to be isolated at low levels around the world.

The 1977–1978 Pandemic Virus

The re-emergence in 1977 of a descendant of the 1918 H1N1 virus that had been absent from circulation for 20 years constitutes a pandemic by definition, but it is usually regarded as a "technical" pandemic that represents an unusual coda to the 1918 pandemic. It is curious that the same virus that disappeared on its own in 1957 has, after reintroduction, been able to survive for over 30 years in the face of immunity pressures thought to be as great or greater than those associated with its disappearance (i.e., high population immunity from natural infection and additional immunity from annual vaccination, which is much more common now than it was in 1957). It is considered unlikely that human IAVs could have been maintained in nature for 20 years without accumulating mutations, suggesting that the 1977 epidemic resulted from the release of a frozen strain from the 1950s. Molecular genetic analyses confirmed that the 1977 strain was very similar to early 1950s H1N1 strains in all eight gene segments (Nakajima et al., 1978). The H3N2 and H1N1 viruses cocirculated endemically for over 30 years in the face of high population immunity (Rambaut et al., 2008). Coinfection with both subtypes has been reported, together with the circulation of occasional reassortant H1N2 viruses. Like H3N2, seasonal H1N1 also continues to be isolated at low levels (as of May 2010) despite the emergence of the novel 2009 H1N1 virus. Whether seasonal H3N2 and/or H1N1 viruses will continue circulate along with derivatives of the 2009 pandemic H1N1 virus is unknown.

The 2009–2010 Pandemic Virus

The current pandemic, caused by a novel H1N1 virus derived from two unrelated swine H1N1 viruses (Garten et al., 2009), one of them a "classical" swine derivative of the 1918 human virus and the other the European avian-like H1N1 lineage (Dunham et al., 2009), adds to the complexity of pandemic virus emergence (Figure 2). The current H1N1 pandemic derived its NA and M gene segments from the European avian-like H1N1 lineage and its remaining six gene segments (PB2, PB1, PA, HA, NP, and NS) from the North American swine H1N2 "triple" reassortant lineage. The HA, NP, and NS gene segments of this lineage are derived from the classical swine H1N1 (1918 origin) lineage, while the polymerase gene segments have different origins: PB2 and PA were derived from an avian IAV source and PB1 from a human seasonal H3N2 virus when the "triple" reassortant swine lineage emerged in the late 1990s (Smith et al., 2009b). Despite the recent explosion in IAV surveillance and gene sequencing, we do not know when and where the novel pandemic virus originated or the species in which the

reassortment event took place, although evolution in swine is a favored hypothesis. TMRCA analyses suggest that the pandemic virus was circulating in humans for at least several months prior to its detection in early 2009 (Smith et al., 2009b).

IAV Host Switch Events

Avian-to-Mammalian Host Switch Events

The mechanisms by which avian IAVs cross species barriers to infect humans or other mammals, either causing dead-end infections or leading to subsequent transmission in the novel mammalian host, are unknown. Moreover, the properties of IAVs that have the greatest medical and public health relevance, such as human infectivity, transmissibility, and pathogenicity, appear to be complex and polygenic and are poorly understood (Parrish et al., 2008; Taubenberger and Morens, 2009).

Avian IAV Infections in Swine and Horses

Although a number of fully avian IAVs (without reassortment with a human or swine IAV) of various subtypes have been isolated from swine populations globally (Brown, 2000), most of these transmission events have been self-limited and not led to stable, long-term circulation of novel swine-adapted IAVs. The European avian-like lineage of swine H1N1 is an important exception, having circulated in swine since the late 1970s (Dunham et al., 2009) and also playing a role in the emergence of the 2009 pandemic H1N1 lineage (Garten et al., 2009). Both of the stable equine IAV lineages are thought to have derived from avian sources, and an epizootic of an avian-derived H3N8 virus was detected in horses in China in 1989.

Avian IAV Infections in Humans

In the past decade, a large number of documented zoonotic avian IAV infections of humans have occurred, predominantly in association with HPAI epizootics of H5N1 in Eurasia and Africa (Peiris et al., 2007) and in smaller epizootics with H7N7 HPAI in the Netherlands (Fouchier et al., 2004), H7N3 HPAI in Canada (Tweed et al., 2004), and LPAI infections with H9N2 (Lin et al., 2000), all without evidence of stable adaptation or sustained human-to-human transmission. Prior to this, there was limited evidence of direct avian-to-human IAV exposure, based on a small number of experimental human volunteer infections with LPAI (Beare and Webster, 1991) or by serological surveillance (Malik Peiris, 2009).

H5N1 Infections

The continuing spread of H5N1 HPAI viruses into poultry populations on several continents, associated with a growing number of human zoonotic infections, has been associated with intense public health interest and concern for a future pandemic (Wright et al., 2007). H5N1 HPAI viruses initially caused a 1996 poultry epizootic in southern China, followed within a year by an epizootic in Hong Kong that produced 18 human cases and six deaths (Claas et al., 1998; Subbarao et al., 1998). H5N1 strains continued to circulate thereafter in China, and they reappeared in epizootic form in 2003 and spread widely thereafter. Evidence suggests that H5N1 viruses are evolving rapidly; however, the direction of this evolution, which is driven by incompletely understood selection pressures, is unclear. While current strains of Southeast Asian H5N1 HPAI viruses are descendants of the 1996 Chinese epizootic virus, significant genetic and antigenic evolution has since occurred (Guan et al., 2004), involving antigenic drift in the H5 HA, mutations in other genes, and reassortment with other avian IAVs (Chen et al., 2006). It is not yet clear which of these many changes are associated with lethality in wild birds or with pathogenicity and transmissibility in poultry or other species. At the same time, adaptation of H5N1 HPAI strains associated with asymptomatic, endemic infection of domestic ducks is probably contributing to continuing spillover into poultry, leading to the maintenance of a pool of pathogenic viruses to which humans will be continually exposed (Sturm-Ramirez et al., 2005). Nevertheless, there are limited data relating to whether or not any H5N1 IAV strain is evolving in the direction of human adaptation.

Avian H7 and H9 Subtype Infections

Although overshadowed by the spread of H5N1, during the past decade at least eight other major poultry epizootics have occurred, caused either by emergence of novel H5 or H7 subtype HPAI viruses unrelated to Asian H5N1 viruses or, in one case, by an H9N2 LPAI virus (Alexander, 2007). Some of these epizootics have featured human infections with few human deaths. Since the mid-1990s, strains of H9N2 LPAI have become enzootic in domestic poultry populations on several continents (Alexander, 2007), leading to a small number of human infections. Like H5N1, different genetic lineages of H9N2 have been established, some of which share with H5N1 viruses closely related internal gene segments. Some H9N2 viruses have even acquired enhanced specificity for the human form of the HA receptor (Matrosovich et al., 2001). In 2003, an H7N7 HPAI virus caused a poultry epizootic in the Netherlands and spread regionally. Before the epizootic was contained, at least 86 poultry workers and three of their contacts had become infected and developed conjunctivitis with or without an influenza-like illness; one of them died (Fouchier et al., 2004). Similarly, two persons developed influenza conjunctivitis during an outbreak of H7N3 HPAI in Canada in 2004 (Tweed et al., 2004). The H5N1 epizootics are unique, however, in causing infections and deaths in a large number of wild bird species (Alexander, 2007), occasional infections in wild and domestic mammals, severe and fatal human spillover infections (Peiris et al., 2007), and in rare instances, limited human-to-human transmission.

Mammalian-to-Mammalian Host Switch Events

The historical medical literature frequently described equine IAV epizootics in relation to IAV epidemics in humans (Taubenberger and Morens, 2009) but also to IAV infections in dogs. Recently, equine H3N8 viruses have adapted to dogs in a stable host switch event (Crawford et al., 2005), where they are now evolving by antigenic drift. Equine H3N8 viruses were also recently isolated from pigs in China, without evidence of stable host switch (Tu et al., 2009).

Swine-adapted IAVs have also been associated with zoonotic infections in humans, with 37 documented cases between 1958 and 2005 with several reported fatalities (Myers et al., 2007), including an outbreak of classical swine H1N1 infection in soldiers at Fort Dix, NJ, in 1976 (Gaydos et al., 2006) with one death. The ability of a swine-adapted IAV to result in a stable host switch event to humans was proven only with the emergence of the 2009 H1N1 pandemic virus (Garten et al., 2009; Smith et al., 2009b). It is not known why zoonotic infection with previous swine-adapted IAV strains, including the 1976 Fort Dix strain (Kilbourne, 2006) in which human-to-human transmission was demonstrated, did not result in a stable host switch and

emergence of a novel pandemic IAV (Shinde et al., 2009; Van Reeth and Nicoll, 2009).

Molecular Correlates of Human Adaptation

Despite significant research, fundamental questions about how IAVs switch hosts remain unanswered. Also poorly understood are the viral genetic changes that underlie human adaptation, human-to-human transmissibility, and pathogenesis. Biological barriers to the fitness of viruses with various gene segment combinations are still poorly understood; however, virulence/pathogenicity, host adaptation, and host-to-host transmissibility are likely independent properties that are associated with different and possibly competing mutations. The role of virulence and pathogenicity in virus-host relationships is therefore unclear; pandemic viruses of comparatively low (e.g., 1968 and 2009), intermediate (e.g., 1889 and 1957), and high (e.g., 1918) pathogenicity have all adapted to humans and exhibited efficient transmissibility.

Another complicating factor stems from the fact that 5 of the 8 gene segments introduced with the 1918 IAVs (PB2, PA, NP, M, and NS) were retained in the reassortment events leading to the 1957 H2N2 and 1968 H3N2 pandemics (Figure 2) and thus have circulated continuously in both pandemic and seasonal human H1N1, H2N2, and H3N2 IAVs from 1918 to 2010 (Morens et al., 2009). A number of groups have performed comparative analyses to propose mutations that are signatures of human IAV adaptation (Finkelstein et al., 2007; Taubenberger et al., 2005) that relate back to this single host switch event in 1918. Examination of independent avian-to-mammalian host switch events, however, suggests a lack of parallel evolution (Dunham et al., 2009). The unexpected emergence of the 2009 H1N1 pandemic virus further supports the independent and polygenic nature of these host adaptation events in IAV (Herfst et al., 2010; Jagger et al., 2010; Mehle and Doudna, 2009).

Changes in IAV Genome GC Content

Rabadan and colleagues have shown that avian-origin IAVs have a higher GC content than IAVs adapted to humans (Greenbaum et al., 2008; Rabadan et al., 2006). Gene segments from the 1918 pandemic demonstrated a nucleotide composition and guanine-cytosine (GC) content similar to avian IAV, supporting an ultimate avian origin for this pandemic virus (Rabadan et al., 2006). Similar changes in nucleotide composition with a decline in GC content were also shown for swine-adapted IAV (Dunham et al., 2009). The biological basis for these observations is not yet clear, but hypotheses include core temperature differences (with higher core temperatures in avian hosts), a mutational bias in the cellular components needed for RNA replication (Rabadan et al., 2006), a human defense mechanism against RNA viruses operating functionally analogous to the Apobec family of deaminases against HIV infection, or avoidance of immune detection by mimicry of host mRNA composition (Greenbaum et al., 2008).

HA Receptor-Binding Specificity

IAV infection is initiated by viral attachment mediated by HA proteins binding to SA-bearing cell surface receptors. The binding interactions are very weak, and high avidity is achieved by binding interactions between multiple HA molecules on the virus and the SA receptors on the target cell (Matrosovich et al., 2009). HA glycoproteins bind to certain SA isomers on the tips of host glycoproteins, strongly supporting a role for HA binding to SA in determining host range (Nicholls et al., 2007; Taubenberger, 2006).

The HA receptor-binding domain (RBD) is conserved in avian HAs, but those IAVs adapted to humans have mutations in several key residues of the RBD, including sites 138, 190, 194, 225, 226, and 228 (H3 numbering) (Wright et al., 2007). It is thought that mutations at these sites increase binding from α2-3 to α2-6 SA. For H1-subtype IAV adapting to humans or swine, E190D and G225D (H3 numbering) were shown to be crucial changes for enhanced α2-6 binding, while for H2- and H3-subtype HAs, Q226L was shown to be crucial (Wright et al., 2007). IAV with H13 and H16 HA subtypes have been isolated from gulls rather than ducks, and this may reflect a host adaptation, as both H13 and H16 HAs bear mutations in the RBD (Matrosovich et al., 2009). The HA RBD requirements for host switch have been further complicated by recent studies. The majority of H5N1 virus infections have occurred in viruses with RBD specificity for α2-3 SA, although some isolates have acquired mutations giving them enhanced α2-6 SA binding (Wright et al., 2007). Some avian IAV of H7N2, H7N3 (Belser et al., 2008), and H9N2 subtypes (Matrosovich et al., 2001) have shown enhanced specificity for α2-6 SA and yet have only caused occasional zoonotic infections in humans. Sequence analysis of 1918 pandemic HA (directly from clinical material) has shown that isolates differed at HA residue 225 (with three cases having the human aspartic acid and two cases retaining the avian glycine) (Reid et al., 2003). Glycan array and structural analyses (Chandrasekaran et al., 2008; Stevens et al., 2006) showed that the 1918 variant with a glycine at 225 had a blended α2-3/α2-6 SA-binding specificity while those sequences with an aspartic acid at 225 had a α2-6 SA-binding specificity, yet there was no appreciable difference in clinical course or autopsy findings between the cases (Reid et al., 2003). Clearly, enhanced binding to α2-6 SA is not in itself adequate for host switch of an avian-adapted IAV to humans, nor is it a requirement for human infections. IAVs bearing the 1918 HA with different RBD configurations (α2-3, α2-6, and blended α2-3/α2-6 specificities) were all shown to be virulent in mice (Qi et al., 2009) and ferrets, but viral constructs with the 1918 HA having only α2-3 specificity did not transmit between ferrets (Tumpey et al., 2007). Interestingly, 2009 H1N1 pandemic viruses with variable amino acids at the 225 site have also been isolated.

Clearly, a number of fundamental questions remain unanswered. Structural studies have shown that it is inadequate to consider only the linkage of the terminal SA in regard to determining the specificity of different IAV HAs. What is the distribution of these diverse glycans on the respiratory tree? How much variability in expression patterns is observed between individuals, or are there changes in glycan expression with age or other physiological states? What about HA subtypes other than H1–H3? Several mutations have been reported to enhance the binding of H5 to α2-6, but fixation of such mutations has not occurred after at least 11 years of exposure of thousands of humans to H5N1, suggesting that changes in HA RBD during host adaptation are extremely complex and must differ from subtype to subtype (Ayora-Talavera et al., 2009).

The HA of H5 or H7 HPAI viruses with a polybasic cleavage site serves as a primary virulence factor in poultry. The HA of the

1918 pandemic virus does not have a polybasic cleavage site, but nonetheless has been shown to be a virulence factor in mammalian models (Kash et al., 2004, 2006; Kobasa et al., 2004). There is no evidence, however, linking HA virulence in a mammalian model either with or without a polybasic cleavage site with host adaptation.

IAV RNP Complex

Changes in the constellations of gene segments as well as mutations in the RNP genes have been long implicated in the adaptation of avian IAV to humans (Wright et al., 2007). The PB1 gene segment was shown to be very avian IAV-like in the 1918 virus (Taubenberger et al., 2005) and was replaced by reassortment with an avian IAV in the 1957 and 1968 pandemics (Wright et al., 2007). These reassortment events suggest that an avian IAV-derived PB1 segment can function in an otherwise mammalian-adapted IAV without prior adaptation, as has been shown experimentally. The PB1-F2 protein encoded from a +1 ORF within the PB1 ORF (Chen et al., 2001) is highly variable in coding sequence in wild bird IAV (Holmes et al., 2006) and is also variably encoded in human and swine IAV. Mutations in PB1-F2 have been mapped as virulence factors in the 1918 virus and in some H5N1 HPAI viruses (Conenello et al., 2007), but there are no clear data linking PB1-F2 with host adaptation (Taubenberger et al., 2005). The PA and NP proteins have been associated with host restriction, and a number of mutations have been proposed for human adaptation (Wright et al., 2007). However, further experimental data mapping the host range restriction associated with specific mutations are needed.

The PB2 gene segment, and in particular PB2 residue 627, has been identified as an important determinant of host range restriction (Subbarao et al., 1993) and virulence in animal models. Avian IAVs generally encode glutamine at this site, while human isolates from 1918–2009 typically encoded lysine. Residues 701–702, residing in a region of PB2 implicated in nuclear localization (Gabriel et al., 2008; Tarendeau et al., 2007), have similarly been identified as a host-adaptive locus (Gabriel et al., 2005), with the D701N mutation increasing both replication in mice (Li et al., 2005) and transmission in guinea pigs (Steel et al., 2009). Independent examples of selection of the PB2 D701N mutation have also been observed, for example, in the nonpandemic avian-origin European swine H1N1 viruses (Dunham et al., 2009), as well as in some HPAI H5N1 viruses. Several other PB2 mutations have been proposed to play a role in human adaptation (Taubenberger et al., 2005), but notably, the 2009 H1N1 pandemic virus PB2 (derived independently of the 1918 host switch event) shows little evidence of parallel evolution. For example, Finkelstein et al. proposed ten amino acid changes in PB2 as persistent human IAV host markers, but the 2009 pandemic virus only shares one, T271A. This change was, however, not present in the 1918 pandemic virus and thus was likely not required for human adaptation.

There has been concern that if the 2009 pandemic virus acquired some of these previously identified human IAV-associated changes, it might increase its virulence or transmissibility (Herfst et al., 2010; Jagger et al., 2010; Mehle and Doudna, 2009). Herfst et al. and Jagger et al. recently showed no enhanced replication or virulence when the E627K or D701N was added to viruses bearing the 2009 RNP complex. Mehle and Doudna recently proposed an alternate strategy for PB2 human adaptation, the so-called "SR" polymorphism, involving residues 590–591. Both strategies may operate by preserving a large, positively charged PB2 surface domain, implying that this region is involved in interactions with undetermined host-specific factors (Mehle and Doudna, 2008).

Remaining IAV Genes

Much fewer experimental data exist to evaluate the significance of proposed human-adaptive changes in IAV in the NA-, M-, and NS-encoded genes. Similarly, mutations associated with host adaptation in the NS gene are unclear. Although birds have two NS alleles (A and B), mammalian viruses contain only allele A, and substitution of an allele A NS with an avian allele B NS significantly restricted viral replication in nonhuman primates (Treanor et al., 1989). The NS gene from the 1918 virus has been reported to be a very potent inhibitor of antiviral and type I IFN responses in cultured human lung cells (Geiss et al., 2002), but has >98% identity with NS1 from a number of LPAI sequences. Moreover, the 1918 NS gene attenuated a lethal mouse-adapted virus (Basler et al., 2001); yet the 1918 virus is extremely virulent in mice (Tumpey et al., 2005). Several H5N1 NS1 mutations have been associated with replication and increased virulence, but their role in host switch is unclear. Seo et al. reported that increased virulence in pigs of a human H1N1 virus containing the NS gene of an H5N1 HPAI required substitution of aspartic acid for glutamine at amino acid 92 (Seo et al., 2002), and this mutation has been associated with increased resistance to interferon (Lipatov et al., 2005). Additional mutations have been observed in H5N1 HPAI viruses that have been associated with increased virulence, but their significance for host switch to humans remains unclear (Hale et al., 2008).

Conclusions

Host switch events in IAV, i.e., the formation of pandemic IAVs or the emergence of novel swine IAVs lineages, have been shown to be independently evolving polygenic processes. Evolutionary analyses have demonstrated little evidence of either parallel or convergent mutations in such events, suggesting a strong role for historical contingency in the origination of any particular host switch genotype. Instead, mutations identified as important in one host switch event may or may not be observed in other such events, depending on the viral and host genetic context of each event. The utility of identifying sets of mutations as proxies to define whether a future IAV is acquiring changes important in mammalian adaptation might thus be limited. Future progress will require a much better understanding of the structure-function relationship of all the IAV proteins.

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