Emergence of Influenza Viruses and Crossing the Species Barrier

ZEYNEP A. KOÇER, JEREMY C. JONES, and ROBERT G. WEBSTER
Department of Infectious Diseases, Division of Virology, St. Jude Children’s Research Hospital, Memphis, TN 38105

ABSTRACT Influenza A viruses are zoonotic pathogens that infect a variety of host species including wild aquatic birds, domestic poultry, and a limited number of mammals including humans. The error-prone nature of the virus’s replication machinery and its ability to transmit among multiple hosts lead to generation of novel virus variants with altered pathogenicity and virulence. Spatial, molecular, and physiological barriers inhibit cross-species infections, particularly in the case of human infection with avian viruses. Pigs are proposed as a mixing vessel that facilitates movement of avian viruses from the wild bird reservoir into humans. However, the past decade has witnessed the emergence of highly pathogenic and virulent avian H5 and H7 viruses that have breached these barriers, bypassed the pig intermediate host, and infected humans with a high mortality rate, but have not established human-to-human transmissible lineages. Because influenza viruses pose a significant risk to both human and animal health, it is becoming increasingly important to attempt to predict their identities and pathogenic potential before their widespread emergence. Surveillance of the wild bird reservoir, molecular characterization and documentation of currently circulating viruses in humans and animals, and a comprehensive risk assessment analysis of individual isolates should remain a high priority. Such efforts are critical to the pursuit of prevention and control strategies, including vaccine development and assessment of antiviral susceptibility, that will have a direct impact on the well-being of humans and animals worldwide.

INTRODUCTION
Influenza is a respiratory disease affecting humans, a limited number of other mammals, and birds. Of the three genera of orthomyxoviruses affecting humans, influenza viruses B and C established permanent lineages in ancient times, whereas influenza A viruses continue to emerge from zoonotic reservoirs and cause annual epidemics and, at irregular intervals, pandemics. Aquatic birds of the world are natural reservoirs for 16 of the 17 known influenza subtypes, and 1 subtype has been characterized from bats. Only three hemagglutinin subtypes (H1, H2, and H3) have caused pandemics in humans; the H1 and H3 subtypes are currently circulating in swine; and the H3 subtype is currently the only remaining epidemic subtype in horses and appears to be establishing a permanent lineage in dogs. All 16 subtypes of influenza viruses in the aquatic bird reservoir occur as low-pathogenicity strains that replicate predominantly in the intestinal tract and cause limited symptoms of overt disease. Subtypes H5 and H7 have the unique ability to evolve into highly pathogenic strains that are lethal in gallinaceous poultry and have the propensity for transitory interspecies transmission to mammals.

The available evidence indicates that all of the influenza pandemics in humans and other mammals have originated from the aquatic bird reservoir. Such events are relatively rare in humans; the occurrence of four pandemics in the past century indicates that there is a barrier to the free exchange of influenza viruses between aquatic bird reservoirs and humans. In this article, we discuss the nature of the proposed species barrier, the intermediate hosts, and the properties of influenza
viruses that make them a threat to both veterinary and human public health. The applicability of the concept of “One World—One Health” to influenza is incontrovertible. Characterization of the 2009 H1N1 unequivocally shows that each of the eight gene segments comprising the pandemic viruses in humans can be traced back to the wild bird reservoir and that these viruses were transmitted to humans via swine and subsequently transmitted back to swine. Thus, the early detection and control of influenza requires the collaborative effort of an integrated team comprising virologists, ecologists, veterinarians, and human public health officials. The present challenge in pandemic preparedness is to identify the influenza viruses in natural reservoirs that have the potential to cause the next lethal disease in domestic poultry or cause a pandemic in humans or other mammals.

**GENOMIC FEATURES OF INFLUENZA A VIRUSES**

Influenza A viruses are enveloped, single-stranded, negative-sense RNA viruses belonging to the family Orthomyxoviridae. The 13.5-kb genome consists of 8 segments coding for 12 proteins (1, 2) of which are surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), required for host cell recognition via receptor binding and the release of virus from the host cell, respectively. Influenza A viruses are further divided into 17 HA and 10 NA subtypes based on the antigenicity of the 2 surface glycoproteins. The remaining proteins, known as internal proteins, are required for the synthesis and packaging of new virions. Viral RNA is replicated via an RNA-dependent RNA polymerase encoded by a viral polymerase complex (PB1, PB2, and PA). PB1-F2 is a small protein that is transported to the mitochondria and nucleus and induces apoptosis in the host cell (2). PA-X protein is expressed from a second open reading frame on the same segment and is obtained by ribosomal frameshifting. PA-X modulates the host response by decreasing viral pathogenicity (3). The other major proteins are nucleoprotein (NP), matrix (M), and nonstructural (NS) proteins, which are involved in the transport of viral RNA from the nucleus to the cytoplasm and in encapsidation before the virus progeny leaves the infected cell (Fig. 1). The virus acquires its envelope from the host cell membrane via a budding process (4).

Of the three types (A, B, and C) of influenza viruses found in nature, influenza A viruses are the most prevalent and are the principal type that infects both avian and mammalian species, including humans. Thus, they represent a unique risk in terms of zoonotic transmission among a variety of hosts. Influenza A viruses are believed to have been in evolutionary stasis with their natural host reservoir for many centuries (5). Nevertheless, rapid genetic changes in the virus genome are inevitable due to virus-driven and host-driven forces. The changes caused by virus-driven forces are due to the segmented nature of the genome and error-prone nature of RNA polymerase. Host-driven forces include neutralizing antibodies produced by the host immune system and receptor specificity. Genetic variants resistant to antiviral drugs also contribute to the evolution of influenza A viruses.

Although some gene sequences retain a higher degree of conservation, mutations can occur in any gene segment of influenza A viruses. Mutant variants of influenza A viruses arise in each replication cycle, as the RNA-dependent RNA polymerase cannot proofread the newly synthesized gene segments. The majority of mutations are not tolerated in the viral population, but a few may confer a fitness advantage by facilitating the establishment of a virus in a new host (adaptation, transmission, modulation of host response, etc.). Genetic variants with mutations at the antigenic sites on HA and NA genes are usually well tolerated, as they provide a selective advantage for the newly produced mutants to escape recognition by the host immune system. The mutation rates at the antigenic sites are significantly higher (6.7 × 10⁻³ substitutions per nucleotide per year for HA and 3.2 × 10⁻³ substitutions per nucleotide per year for NA) than at other genes (6). These point mutations (nucleotide substitutions, insertions, or deletions) result in amino acid substitutions that affect the antigenicity of the virus, a process known as antigenic drift. Only a few point mutations can be sufficient to make the surface glycoproteins unrecognizable by the host immune system, resulting in the circulation of new antigenic variants in host populations.

Genetic variants also arise by antigenic shift, which occurs when a host cell is coinfected with more than one strain of influenza A viruses. In this case, it is likely that a virion packaged in the host cell contains segments from viruses of different origins. Antigenic shift occurs mainly in influenza A viruses, with which coinfection is more likely due to the broad range of hosts affected. Antigenic shift results in the emergence of new HA-NA combinations and antigenic variants by reassortment of antigenic sites from different virus strains. Reassortment of other gene segments is also likely during coinfection, which results in viruses consisting of mixed gene
constellations from different origins. True recombination is much rarer than antigenic drift or antigenic shift; to date, it has been reported in only a few cases, as it can occur only within and between gene segments of certain strains of influenza A viruses (4).

Viruses produced by point mutations, reassortment, antigenic drift, and antigenic shift may have greater selective advantage than parental viral strains because newly produced genetic variants can escape recognition by the host immune system or can develop resistance to antiviral drugs. Furthermore, mutant variants might confer a selective advantage in zoonotic transmission of viral strains among different host systems. Therefore, the viral evolutionary process can have dramatic consequences for public and veterinary health due to lack of existing population immunity, especially in the absence of available vaccines or antivirals. The continuous but independent evolution of influenza A viruses due to biological, ecological, and geographic barriers is a major concern from the One Health perspective, considering the zoonotic potential of the disease. Thus, understanding the genomic nature of influenza A viruses will

FIGURE 1 Influenza A virus structure and molecular determinants conferring pathogenesis and host range. Influenza A is an enveloped, negative-sense RNA virus with an eight-segment genome. The virion is studded with surface glycoproteins HA and NA and the M2 ion channel. Pathogenesis and transmission are mediated by multiple genes. Predominant molecular characteristics conferring these traits are diagrammed on each gene product above and as follows. HA: Sialic acid binding restrictions are partially mediated by residues 226 and 228 at the receptor binding site. The presence or absence of a multibasic cleavage site influences the cleavability of the virus by a broader range of enzymes, which leads to high pathogenicity of the virus in host species by causing a systemic infection (13). NA: Sialidase activity is specific to the binding restrictions of the HA protein and cleaves sialic acid residues, permitting release (14); deletions in the stalk region may confer adaptation to domestic poultry (15). PB2: Positions 627 (76) and 701 (22) are associated with enhanced replication in mammals. The immunomodulatory potential of changes or expression of the following proteins may contribute to early and productive replication in a new host. NS1: Position F92E/D confers cytokine resistance in mammalian hosts (77). PB1-F2: Position N66S is associated with increased virulence and cytokine dysregulation in mice (78). PA-X: Expression of this protein may lessen immunopathology during viral infection (3). doi:10.1128/microbiolspec.OH-0010-2012.f1
RESERVOIRS FOR INFLUENZA A VIRUSES

Influenza A viruses are known for their ability to infect a variety of host species, including humans, swine, mink, horses, marine mammals, cats, dogs, and wide range of wild and domestic birds. However, the Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, sandpipers, and waders) are considered the major reservoirs for influenza A viruses in nature. All 16 HA and 9 NA antigenic subtypes of influenza A viruses and their numerous HA-NA combinations have been detected in aquatic bird reservoirs (5, 7). The recent identification of a new antigenic subtype (H17) of influenza A virus from bats (8) indicates that there could be yet-undiscovered reservoirs for influenza A viruses (Fig. 2).

Ducks are the major host species in terms of sustainability, evolution, and spread of influenza A viruses. According to surveillance studies conducted in the Northern Hemisphere, low-pathogenicity avian influenza viruses (LPAIVs) are highly prevalent in juvenile birds, with the prevalence peaking before the birds migrate from north to south in early fall and then gradually dropping to low levels in spring (5, 7). This year-round presence of influenza A viruses in duck populations confirms that ducks are the major reservoir for influenza A viruses. Although most HA subtypes have been isolated from ducks, the predominant subtypes are H3, H4, and H6.

Shorebirds, including gulls, sandpipers, and terns, are susceptible to infections by LPAIVs. The majority of the HA subtypes, including H13 and H16, but not H14 and H15, have been isolated from shorebirds during active surveillance studies at Delaware Bay on the northeast seaboard of the United States. H1, H3, H7, H9, H10, and H11 are the predominant subtypes isolated from shorebird species (9). Influenza A viruses appear to be more diverse in shorebirds than ducks.

The isolation frequency of influenza A viruses from passerine birds is not as high as that for waterfowl and shorebirds. Although passerine birds can be infected and spread the virus to other birds (especially seen in high-pathogenicity avian influenza virus [HPAIV] outbreak areas), the evolutionary role of passerine birds in influenza sustainability remains unresolved. The presence of influenza A viruses in passerine birds is a controversial topic, as it has been almost impossible to isolate the viruses from field samples by known culture techniques. However, the number of positive samples determined by molecular detection methods has been significantly high in the Passeriformes (particularly starlings and sparrows) in the United States (10). The infectivity of an influenza virus isolated from starlings and the viral shedding via feces after experimental infection (11) raised the concern that passerine birds might transmit the disease to domestic poultry. In addition to wild waterfowl, shorebirds, and passerines, both LPAIVs and HPAIVs have also been isolated from captive birds, zoo animals, and domestic and commercially raised poultry species.

On the basis of geographic and ecological separation of host species and major migratory flyways around the world, two main lineages of avian influenza A viruses have been identified: North American and Eurasian lineages. Although these two lineages are separated by geographic barriers, limited mixing of the lineages occurs via the migratory birds moving through overlapping flyways between Alaska and Far East Russia (7); however, mixing of the viruses from two lineages on a complete-genome level is a very rare event (9). The isolation of H13N9 from gulls in Argentina suggests that a separate lineage of influenza A viruses evolved in South America independently of the other two known lineages (12).

Many waterfowl and shorebirds migrate because of seasonal changes. The migration distance can be short (e.g., around the same region) or long (e.g., intercontinental). Migratory birds contribute significantly to the spread and transmission of LPAIVs, which generally do not cause symptoms in birds and are excreted in large amounts in the feces. Therefore, the virus is carried along the flyway and can be spread to other wild and domestic birds on the way. The breeding and nonbreeding areas, wintering sites, and all other stopover locations along the flyway are convenient places for epizootics to launch, as bird populations reach a high density at some time points.

With the changing environmental and geographic conditions of the planet, the emergence of new strains and new reservoirs is inevitable. Influenza A viruses will also take action to stay in the game and ensure the survival of the fittest, like all other organisms in the evolutionary process. From the perspective of the One Health initiative, extensive global surveillance studies are essential to comprehend the ecology and evolution of influenza A viruses in wild and domestic birds as well as other host species, including possible new reservoirs. Thus, monitoring the distribution of current subtypes around the world, identifying new antigenic variants and/or reassortants, and studying changes in geographic conditions...
and ecological barriers between the host species will facilitate the urgent actions that need to be taken to ensure public and veterinary health.

**INFLUENZA VIRUSES: CROSSING THE SPECIES BARRIER**

Although all HA and NA subtypes have been isolated from migratory waterfowl, the number of viruses that move from this reservoir into other animals is relatively small. The idea of a species barrier that prevents the movement of viruses from the reservoir into other hosts is an important concept in influenza biology. Viruses that replicate in avian species often display reduced or inefficient replication in mammals, and the barriers that inhibit transmission between these groups are spatial, physiological, and molecular in origin (13). Spatially, the physical separation of host species from the wild bird reservoir may prevent initial contact and infection, but the continual growth and spread of human populations and their domesticated animals has narrowed this division. This is particularly evident in developing countries where local markets house domesticated and wild birds with other species in a constrained area (14).

Physiological differences between birds and mammals serve as a second barrier. In birds, influenza viruses replicate primarily in the enteric tract at temperatures of 40 to 41°C. In contrast, mammalian viruses replicate...
most commonly in the upper respiratory tract (32°C) and the bronchioles (37°C). Thus, avian viruses may be impaired in the ability to establish productive infection in mammalian airways due to the lower temperatures in these tissues (15).

Molecular characteristics, such as receptor specificity of the virus, present a final barrier to successful interspecies transmission. Influenza HA binds the terminal sialic acid residues on host cell carbohydrates. Avian influenza viruses preferentially bind sialic acids with α(2-3) linkages to the underlying galactose, whereas human viruses bind sialic acids with α(2-6) linkages. Other viruses, such as those that infect or are isolated from pigs, may bind both receptors (13). In humans, epithelial cells of the upper airway predominantly express α(2-6) linkages and may restrict replication of avian viruses, whereas swine airways possess both receptor types and allow infection of both mammalian and avian viruses (16). Virus NA activity also has specificity to cleave sialic acid linkages that corresponds with the HA receptor specificity (14). The combination of these factors represents a major obstacle the virus must overcome to recognize and enter the cells of a new host. Specific residues and glycosylations in the receptor-binding domain of the HA protein may further complicate this interaction (17, 18). Even if the virus gains the ability to bind to the host cell of a different species, changes in internal proteins such as the PB2 polymerase and immunomodulatory activities of NS1, PB1-F2, or PA-X may further influence its ability to replicate and cause disease in a new host (17) (Fig. 2). Despite the hurdles that exist for viruses attempting to emerge from a wild bird reservoir, the high mutation rate inherent in the replication process facilitates the genesis of novel variants with potential to transmit to a wide variety of poultry and mammals.

**From Wild Birds to Poultry**

Influenza A viruses infect a wide variety of domesticated gallinaceous birds including chickens, turkeys, quail, and pheasant. They can be divided in two categories: LPAIVs and HPAIVs. The vast majority of viruses exhibit an LPAIV phenotype, and the most prevalent HA subtypes are H3, H6, H9, and H10 (19). To date, only two subtypes (H5 and H7) have evolved to become highly pathogenic. LPAIV forms of these subtypes are currently circulating in poultry populations, while HPAIV forms cause severe outbreaks at certain locales.

The introduction of LPAIVs into poultry likely comes from aquatic and/or migratory birds, which are naturally attracted to dams or watercourses on or near farms. Shedding of the virus into the water and sharing of this contaminated water source with flocks facilitate the initial infection. Lack of proper biosecurity measures (particularly in developing countries) and the movement of people, equipment, and feed in and out of the farms contribute to spread and persistence. Further spread of the disease occurs when an infected bird is taken to a live bird market or housed with uninfected birds on the farm. Transmission of LPAIVs from bird to bird leads to genetic changes that alter its fitness and potential to cause an outbreak or increase its pathogenicity.

In H5 and H7 influenza viruses, several mechanisms and genetic changes have been identified that are important for a shift from LPAIV to HPAIV in poultry. The major genetic change takes place at the cleavage site of the HA protein by insertion or substitution of multiple amino acids. LPAIVs possess a single arginine at the cleavage site, whereas HPAIVs contain multiple basic amino acids (arginine/lysine). This small change in the amino acid motif at the cleavage site can dramatically change the virulence of the virus by allowing the HA protein to be cleaved by a wider variety of proteases found in different cell types. This increases virus tropism and leads to systemic infection. The ability of the virus to replicate in various organs significantly increases the mortality. The change from LPAIV to HPAIV might also occur through recombination between the HA protein and an internal gene (20, 21). Additional genetic changes, such as deletion in the stalk region of the NA protein, might play a role in the adaptation of these viruses to chickens. It has also been shown that a mutation from aspartic acid to asparagine at position 701 of the PB2 protein contributes to the change from LPAIV to HPAIV (Fig. 1) (22).

The conversion from LPAIV to HPAIV in poultry might take several days to several years before causing an outbreak. Although disease signs vary for birds infected with HPAIVs, the major signs are ruffled feathers, edema of the head and face, sinusitis, respiratory signs, excessive lacrimation, decreased egg production, huddling, paralysis, incoordination, torticollis, cyanosis of unfeathered skin (especially of the combs and wattles), diarrhea, and nervous disorders (5, 23). HPAIV infections often progress rapidly and result in high mortality. Once the virus becomes highly pathogenic, it might reenter migratory waterfowl and wild birds around the outbreak area and even transmit to humans in case of direct contact with the infected or dead birds.

Although not HPAIVs, the H9N2 influenza A viruses have become a major concern for outbreaks in chickens, turkeys, ostriches, pheasant, and quail in Asia, Europe,
North America, and the Middle East since the 1990s (24). The virus has become endemic in many countries, and vaccination efforts on poultry farms are not sufficient to control or eradicate the disease.

The outbreaks caused by LPAIVs and HPAIVs in domestic and commercial poultry result in the slaughter of millions of birds and a considerable economic loss. The degree of loss varies depending on the number of domestic and commercial poultry farms in close vicinity to an outbreak and the precautionary biosecurity measures taken. Proper biosecurity measures to avoid spread of the outbreak include monitoring the health of birds on a regular basis for disease symptoms, keeping the shared food and water sources clean, and taking extra precautions while transferring equipment and supplies between farms so as to avoid mechanical transfer of the disease. In addition, active surveillance studies should be conducted at both poultry farms and live bird markets, especially in areas at high risk of influenza A outbreaks.

Swine

Influenza infection in pigs manifests clinically as fever, lethargy, anorexia, and weight loss, and virus is shed by coughing and sneezing. The nasal discharge and aerosol droplets are highly communicable, and intraherd spread is often exacerbated by housing livestock in small, confined areas (25). Although mortality is rare, infected animals may exhibit reproductive disorders and stunting of growth, which increases time to market and contributes to an agricultural economic burden (26).

Pigs play a crucial role in influenza evolution and transmission by serving as a platform to breach spatial, physiological, and molecular aspects of the species barrier. Because pigs are a popular livestock worldwide, their interaction with other influenza hosts such as birds (wild and domestic) and humans is common. Physiological features such as warmer temperatures in the upper respiratory tract (36 to 37°C) than that in humans may facilitate replication of avian viruses (25). Respiratory epithelia of pigs also contain both the human-type [α(2-6)] and avian-type [α(2-3)] influenza receptors. Thus, pigs are susceptible to infection with both human and avian influenza viruses. In the event of a coinfection with both types of viruses, reassortment may occur and resulting variants can be transmitted back to an immunologically naïve human host (27). The concept of pigs as a “mixing vessel” has emerged as a partial explanation for the ability of viruses to breach the influenza species barrier and may contribute to the generation of new epidemic and pandemic viruses. Numerous cases of antigenic mixing and cross-species infections (both human and avian) from pigs have been reported, lending credence to the mixing vessel hypothesis (25–28). A recent example is the 2009 H1N1 pandemic, in which a swine virus introduced into the human population was shown to be a triple reassortant with gene segments from human, avian, and swine influenza viruses (29).

Experimentally, pigs are susceptible to most HA subtypes (H1 to H13). However, only viruses of H1, H3, N1, and N2 subtypes have a sustained presence in pig populations. Much of our understanding of the epizootic nature of swine influenza viruses comes from surveillance data from the North American and European herds during the past century. Clinical signs of influenza in pigs were first observed in North America during the 1918 pandemic. In 1930, Shope and colleagues identified the causative agent as an H1N1 influenza virus that is commonly referred to as “classical swine H1N1” (25, 26). Most reports identify this virus as being genetically similar to the progenitor pandemic 1918 H1N1 virus, and it may have passed from birds into pigs (30). The classical swine H1N1 virus maintained epizootic and relatively genetically stable circulation in North American swine populations for nearly 70 years (26). During this time period, sporadic cases of human infection or seropositivity were reported, but they were usually a result of close contact with swine. An exception was an outbreak of H1N1 at Fort Dix, NJ, in 1976, in which the virus was of swine origin and infected more than 200 trainees, causing 12 hospitalizations and 1 death. A national immunization campaign was initiated, but proved unnecessary as the virus did not spread beyond the facility. Immediate contact with pigs was not reported among the infected trainees, though the possibility of introduction of the virus from an incoming trainee could not be ruled out (28).

In Europe, the first reported swine influenza isolates in 1938 and again in the late 1970s were viruses closely related to H1N1s currently circulating in humans. In both cases, isolated viruses contained only human influenza-like genes, providing further evidence that wholly human influenza viruses could be transferred into pigs. The latter half of the 20th century witnessed the introduction of classical swine H1N1 (possibly from import of North American pigs) into European herds. However, an important divergence from classical swine H1N1 dominance occurred in 1976 when an entirely avian H1N1 was transferred, likely from wild ducks, into Belgian and German herds. This “avian-like swine H1N1” continued to circulate and quickly became the dominant lineage in Europe (25).
The predominance of H1N1 viruses in North American and European swine was overshadowed in the late 20th century when reassortment events gave rise to the novel subtype H3N2. In both North America (1997) and Europe (1973), genetic profiles of early H3N2 viruses suggested transmission from humans into pigs. Later triple-reassortant isolates contained mixtures of influenza genes from swine, human, and avian hosts, and there have been instances of reassortment between the H3N2 viruses and cocirculating swine H1N1 (25–27). In the latter case, such cocirculation gave rise to “second-generation” reassortant viruses of the novel subtype H1N2. Although H1N2 has rarely been isolated outside of pigs, infections in mink (31), humans (32), and turkeys (triple-reassortant H1N2) (33) have occurred in the past decade.

In recent years, viruses outside the commonly circulating swine subtypes (H1N1, H3N2, and H1N2) have caused disease in pigs. In Canada (1999), H4N6 viruses with genes exclusively of avian origin were likely passed to pigs from local ducks, and the resulting virus proved efficient at spreading among pigs (26). In the United States (2006), two H2N3 viruses were isolated from sick pigs on two separate farms. Although the H4N6 and H2N3 isolates did not spread outside the respective isolation zones, each virus displayed genetic signatures (receptor binding and structure) of adaptation to mammals and represented the possible introduction of a new HA subtype into swine populations (26, 34).

Endemic and epizootic infections of pigs with influenza viruses represent a significant risk to global health given the role of this species in generating novel influenza reassortants. Continual surveillance is key to both limiting spread and identifying new virus threats.

**Horses**

As in other mammalian hosts, equine influenza is a localized upper respiratory tract infection causing fever, coughing, and lethargy. It is a significant burden to the equestrian community and thoroughbred industry because outbreaks can delay competitive events and activities (35, 36). Respiratory epithelia of horses are dominated by α(2-3) receptors, suggesting a preferential susceptibility to avian viruses (37). Viruses readily spread to closely housed horses via nasal discharge and respiratory droplets expelled by persistent coughing (35). Equestrian events and competitions often serve as outbreak sites when animals from various geographic locations are brought together. To combat this spread, vaccination strategies are widely used in North America and Europe (36).

Equine influenza was first reported in 1956 with an outbreak of H7N7 virus in Prague, but it has not been isolated in more than 30 years and has likely disappeared from horse populations (38, 39). Susceptibility of horses to the H7 subtype and isolation of H7 LPAIVs and HPAIVs from domestic poultry and birds over the past decade suggest that reemergence of the H7 subtype in horses is a valid risk that needs to be explored.

The H3N8 subtype, initially isolated from horses in Miami, FL, in 1963, is the sole subtype that continually circulates and causes sporadic epizootic outbreaks worldwide. In most cases, a breach in biosecurity involving unvaccinated animals has been implicated in the outbreaks rather than introduction of a virus from another host (36). However, introduction of influenza to horses from a possible avian source was documented in 1989 in northeastern China. More than 13,000 animals were infected, resulting in a mortality rate of 20%. Genetic analysis of these viruses suggested a common avian progenitor, likely a result of transmission from Central Asian ducks into herds (40), yet this virus did not persist or spread outside the region.

In the past decade, outbreaks of equine-like H3N8 in dogs have been documented in several countries, including the United Kingdom, United States, and Australia. There is evidence of cross-species transmission from horses to dogs in at least three instances, which may have occurred due to housing or transport of dogs in close contact with infected horses or by feeding dogs meat from infected horses (37).

The presence of avian-like receptors in the respiratory epithelia and past evidence of cross-species infection by avian viruses demonstrate that horses are potential recipients of other influenza subtypes. Experimental evidence of the ability of equine isolates to infect humans (41) and the recent spread into dogs highlight the role of horses in the transmissibility and evolution of influenza.

**Other Species**

Although it occurs less commonly, aquatic mammals can serve as hosts for multiple subtypes of influenza virus. In 1979 to 1980, a severe outbreak of H7N7 virus was reported in harbor seals in New England. Shortly thereafter, an H4N5 (1982) virus was isolated from a sick seal in the same region. Both viruses were composed of genes entirely from avian influenza sources (42, 43). Over the next decade, H4N6 (1991) and H3N3 (1992) viruses were isolated from seals in Massachusetts and were closely related to circulating North American avian influenza viruses. These isolates caused varying degree of illness, from neurological replication and mortality
(H7N7) to acute lung replication and increased incidence of stranding noted within the time periods of isolation (43). Serological analyses of Arctic ringed seals (1984 to 1998) and South American fur seals demonstrated varying degrees of seropositivity (2 to 27%) to influenza viruses of different subtypes (44, 45). In addition to seals, viruses have also been isolated from striped whales in the South Pacific (H1N2; 1978) and pilot whales in New England (H1N2/9; 1984), and seropositivity of Canadian beluga whales has been reported (1991) (45–47). In each instance, genotypic analyses suggest possible virus introduction from wild birds, likely as a result of the shared habitats of seals and various species of shorebirds. To date, infections in water mammals remain transient, with limited evidence to suggest seal-to-seal spread of these viruses. However, a recent harbor seal H3N8 isolate (New England, 2011) had molecular features and receptor-binding properties that indicated adaptation to mammals, increasing the potential for interspecies transmission (48).

Natural infections in mink have been reported for H10N4 (Sweden, 1984), H5N1 (Sweden, 2006), H3N2 (Canada, 2006), and H1N2 (United States, 2010) (31, 49). The H10N4 virus was likely introduced into mink on a farm setting from wild mallard ducks. The H3N2 virus was a triple reassortant and may have come from uncooked feed containing infected pig tissues (including lung). Additionally, mink have been experimentally infected with influenza viruses of avian, human, swine, and equine origin. Their evolutionary ancestor, the ferret, shares this property and is the gold standard for modeling influenza infections and transmission.

Seroprevalence and natural influenza infection of wild and domestic felids has been reported for subtypes H1N1, H3N2, and H5N1, whereas experimental infection studies show that domestic cats are susceptible to a wide range of influenza viruses of avian and human origin. Infection with highly pathogenic avian H5N1 was thought to have occurred by ingestion of bird meat of previously infected animals and has since been experimentally recapitulated (50). The presence of influenza viruses from various subtypes in aquatic mammals, mink, and felids demonstrates the potential for broad host adaptation and warrants further study into the potential of these hosts to mediate antigenic mixing and interspecies transmission.

**Humans**

Humans are a highly mobile species with an expanding population and living environment. Interaction with wild animals is an increasingly common occurrence that escalates the threat of emerging infectious diseases, particularly novel influenza viruses from the wild bird reservoir. Common agricultural livestock, including chickens and pigs, serves as the intermediate host of influenza viruses and further elevates the risk of transmission of viruses into the human population. Thus, influenza infection in humans is an excellent example of the interplay between human and animal health and its importance to the spread and interspecies transmission of pathogens.

Despite vaccination programs and antiviral therapies, influenza viruses remain endemic in humans. Infecting approximately 5 to 15% of the world population annually, and causing 36,000 deaths every year in the United States alone, influenza remains a significant public health and economic burden (51). Infections are most commonly acute and limited to the upper respiratory tract. The virus receptor predominant in the human upper respiratory tract is the α(2-6) linkage. Thus, humans are less susceptible to avian viruses than are other mammalian hosts such as pigs and horses. However, α(2-3) linkages become more abundant in the lower lung, possibly allowing replication of highly virulent avian viruses such as H5N1 (52). In healthy adults, the virus incubation period is 2 to 5 days, followed by 5 to 8 days of replication with associated clinical signs such as fever, fatigue, general body aches, and nasal congestion. In rare cases, infection may be associated with conjunctivitis, pneumonia, and secondary bacterial infections, or with gastrointestinal symptoms in children. The virus is shed in high titers via respiratory discharge from sneezing and coughing and is highly communicable (51, 53). Influenza viruses circulate in yearly epidemics, often during winter months, in the Northern and Southern Hemispheres. Currently, two subtypes of influenza A are endemic in humans (H1N1 and H3N2), and one subtype (H2N2) has disappeared from the human population (51).

All three subtypes that previously circulated or are currently circulating in humans have caused one or more pandemics. Before the 20th century, evidence of influenza pandemics was recorded largely on the basis of clinical symptoms or outbreaks of respiratory illness (30). Advances in microbiology, medicine, and recordkeeping allowed a more thorough study of the first pandemic of the 20th century. The 1918 Spanish influenza, caused by the H1N1 virus, was the most severe pandemic in terms of morbidity and mortality in recent history. An estimated 20 million to 40 million people were killed worldwide, but the high mortality rate may have been due to the medical care of the time and the
high incidence of bacterial coinfections (30, 54). The exact origin of the virus remains unclear. The surface proteins from human isolates are most similar to those from classical swine isolates, leading to the hypothesis that virus entry into humans occurred via an intermediate pig host. However, this hypothesis is complicated by the facts that surface proteins from the human isolate retained avian-like characteristics and that the disease in pigs was not clinically documented until after the virus had circulated in humans for several months. The latter observation suggests that humans may have transmitted the virus to pigs (30, 55, 56). Regardless of its origin, the pandemic progenitor likely emerged from the avian reservoir and entered pigs and humans, and two lineages (human H1N1 and classical swine H1N1) persisted in their respective populations for several decades subsequently (55, 56).

The human H1N1 virus was supplanted 4 decades later by a new pandemic virus (H2N2) that had acquired avian surface proteins H2 and N2 on the backbone of the previous pandemic virus. The 1957 Asian influenza was replaced a decade later by a new pandemic. The 1968 H3N2 Hong Kong influenza virus had again acquired an avian surface protein (H3) on the backbone of the previous pandemic strain. Both the 1957 and 1968 pandemics were less severe than the 1918 pandemic. This drop in morbidity and mortality was likely because of advances in medicine and antimicrobial agents rather than drastic changes in the virulence and communicability of the viruses themselves (57). In 1977, a pseudopandemic (Russian influenza) and reemergence of the H1N1 subtype in humans may have been the result of accidental release of the virus from freezer storage (56). The H3N2 and H1N1 subtypes have continued to circulate endemically in humans on a yearly basis.

The first influenza pandemic of the 21st century was marked by intensive study of viral genetics and epidemiology, concluding that the pandemic swine influenza (H1N1) was a reassortant virus that was transmitted from pigs into humans. The virus was a so-called triple reassortant and contained genes of classical swine (H1, NP, and NS), Eurasian avian-like swine H1N1 (N1 and M1), human H3N2 (PB1), and avian (PB2 and PA) origin. The virus is believed to have circulated in North American swine for nearly 2 decades before emerging in humans (29). Initial cases of respiratory disease were reported in late March 2009 in Mexico, with confirmed cases in the United States first reported in April 2009. By May the virus had spread worldwide, and on July 11, 2009, the World Health Organization (WHO) officially declared a pandemic. Infection rates with the pandemic H1N1 (pH1N1) virus were elevated in traditional high-risk groups such as the very young and elderly and also in pregnant women, the obese, and those younger than 18 years of age. The latter group comprised more than 60% of pH1N1 infections reported in the United States, recalling the infectivity patterns seen with the 1918 pandemic virus. However, the pH1N1 was clinically milder than previous pandemics. The pandemic was declared to have ended in August 2010, after infection rates of 11 to 21% in 218 countries and more than 18,000 laboratory-confirmed deaths. However, mortality in developing countries may have been significantly underreported and the global deaths from pH1N1 may have been significantly higher (29, 58). The relatively “mild” morbidity and low mortality of the pH1N1 virus may be due to several factors such as rapid vaccine production and implementation, use and efficacy of current antivirals, and advances in public health and modern medical care.

The pH1N1 virus serves as an excellent example of the threat of zoonotic transmission and reassortment capabilities of influenza viruses. The virus itself harbored influenza virus genes from three major hosts: birds, pigs, and humans. The genes donated from the swine viruses were themselves a result of reassortment from classical swine and European avian-like swine viruses (29). Together, and possibly through careful mixing within the pig, these elements created a virus that was robust and fit as well as highly infectious and communicable in humans. That the pH1N1 virus may have circulated for years in swine without detection further highlights the need for constant surveillance of influenza viruses and their potential to emerge in various hosts. The past 15 years has seen the emergence of HPAIVs of subtypes H5 and H7 as well as H9 LPAIVs in human hosts without prior adaptation. The endemic nature of these viruses in poultry in many parts of the world is a troubling prospect for the generation of potential pandemic culprits.

**HIGHLY PATHOGENIC H5N1 INFLUENZA—AN UNPRECEDENTED EVENT**

The emergence of highly pathogenic H5N1 influenza virus in the 1990s in Southeast Asia, its subsequent spread to domestic poultry and mammals in more than 60 Eurasian countries, and its reintroduction into migratory waterfowl is a testament to the need for the One Health concept, which integrates ecology with veterinary and human medicine (59). It is noteworthy that this avian influenza virus was first detected in a child in Hong Kong.
Kong in 1997 (60), most probably acquired from exposure to poultry in a live bird market. Retrospective studies indicate that the eight gene segments in the H5N1 influenza virus were of Eurasian avian origin. The wild bird precursor H5N1 LPAIV was presumably transmitted to domestic waterfowl in southern China and then spread to chickens through the live bird market system. Subsequent epidemiological studies have implicated exposure to live poultry markets as a major risk factor for acquiring H5N1 influenza virus (61). Transition of the H5N1 LPAIV to its HPAIV phenotype likely occurred during circulation in commercial chicken farms. After the H5N1 HPAIV emerged in commercial poultry, it was probably transmitted back to waterfowl through live poultry markets in the coastal region of southern China and has served as the continuing source of H5N1 HPAIV for the region (62). The absence of overt disease signs in chickens and other gallinaceous poultry in live bird markets in Hong Kong during the initial outbreak was a puzzling feature. One possible explanation is that the lethality of H5N1 was being masked by cocirculating H9N2 influenza viruses, as the initial H5N1 virus contained “internal” gene segments closely homologous with the G1 lineage of H9N2 influenza viruses.

Culling of all poultry in Hong Kong resulted in an immediate cessation of poultry and human infections. Six of 18 human infections were fatal. The original H5N1 was eradicated, but multiple genotypes of H5N1 reemerged from aquatic birds in southern China with the following chronology of events.

- 2002: Infection of exotic waterfowl in Hong Kong parks
- 2003: Infection of a family of three in Fujian, China
- 2003 to 2004: Spread to poultry and humans in Japan, South Korea, Vietnam, Thailand, Lao PDR, and Cambodia
- 2005: Infection of bar-headed geese and waterfowl at Qinghai Lake, China, with subsequent spread to the Indian subcontinent, Africa, and Europe

To date, 63 countries have been affected by the H5N1 virus. The H5N1 HPAIV was eradicated in most countries in Europe as well as Japan, Thailand, and South Korea by quarantine, culling of poultry, and compensation. In contrast, in countries that opted for control by vaccination (China, Vietnam, Indonesia, and Egypt), the virus has become endemic, with sporadic outbreaks occurring in poultry in neighboring countries. Although vaccines are not being used in the Indian subcontinent, widespread outbreaks seen in Bangladesh and sporadic outbreaks in bordering countries suggest that the H5N1 virus is endemic in poultry in this region. The H5N1 HPAIV has continued to evolve and has multiple distinguishable clades based on the sequence of the hemagglutinin. The following are continuing concerns about the future evolution of high-pathogenicity H5N1 viruses.

- Acquisition of mutations that will make the virus transmissible in mammals, as recently demonstrated in ferrets (63, 64), with the possibility of a pandemic in humans
- Perpetuation in the aquatic bird reservoir with minimal disease signs
- Establishment of a stable lineage in swine, the intermediate host
- Transmission to the Americas by either migratory waterfowl or smuggled birds

These concerns embrace the fields of ecology, veterinary sciences, and human public health, emphasizing the importance of using an integrated approach for the pandemic preparedness efforts to control H5N1 influenza.

**CONTROL STRATEGIES**

The effort to understand and control interspecies transmission of influenza needs to begin with surveillance within the virus reservoir as well as in each susceptible host. Understanding the patterns and identities of viruses circulating in each host (wild birds, domestic poultry, swine, and humans) is the first step to developing methods to adequately control the viruses. Risk assessment of viruses identified in surveillance efforts can further define virus characteristics and the threats posed to birds and mammals. These steps are essential to develop the most common control strategy for influenza, vaccination. Influenza vaccines for both humans and animals are most commonly egg-propagated, inactivated viruses that produce a protective neutralizing antibody to the HA protein (65). A successful vaccine depends on several key factors: (i) antigenic similarity of vaccine seeds to circulating strains, (ii) immunogenicity of the selected virus, and (iii) strain suitability to high titers in eggs (66). Failure of any of these factors can delay lead production or fail to stop spread within populations.

Influenza viruses remain endemic in humans despite readily available vaccines and antivirals. However, annual vaccination with a trivalent (H3N2, H1N1, and
influenza B) influenza vaccine is an important public health effort to limit influenza morbidity, mortality, and associated economic and productivity losses (65–67).

The degree to which livestock vaccination confers protection varies, depending on the species and geographic location of animals. Poultry vaccination, especially against H5 LPAIVs and HPAIVs, is most common in areas where these viruses are endemic, including South and Central Asia and parts of the Middle East. Development of avian H5 vaccines is particularly challenging because of continual virus evolution, maternal antibody interference, and the fact that vaccination may limit morbidity and mortality but not prevent animals from shedding virus. Thus, vaccination may not be the most effective method of control in domestic poultry. Alternate or secondary measures may be necessary to control an outbreak or address endemicity. Quarantine and/or culling of affected animals may quickly and successfully eradicate infection but cause a significant economic burden to farms. Monetary compensation for culled flocks is one option to address this problem and potentially increase compliance with an eradication campaign (68).

Current swine influenza vaccines provide at least partial protection against H3N2, H1N1, and H1N2 viruses and may limit intraherd spread. However, the susceptibility of swine to many types of influenza viruses, as well as their potential to pass new variants to other hosts, indicates that vaccination does not eliminate all risk. Thus, the surveillance of circulating swine viruses in tandem with traditional vaccine control measures is essential to identify novel viruses emerging from this host (69).

The horse population is highly mobile because of the competitive nature of the equine industry. Routine vaccination in countries where equine influenza is endemic (e.g., United States and United Kingdom) and immediate vaccination of horses imported into countries where the disease is not endemic is an important step in limiting outbreaks and virus dissemination (35, 36). In recent years, transmission of equine influenza viruses into dogs has led to the development and licensing of canine H3N8 vaccines (70).

Two classes of antiviral drugs are currently used to control existing influenza infections: entry blockers (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) (71). Joint statements from the WHO, Food and Agricultural Organization, and World Organisation for Animal Health recommend these antivirals for human use only. The emergence of amantadine-resistant H5N1 HPAIVs, possibly through treatment of poultry with this drug, further justifies the exclusion of livestock from treatment with current antivirals (72). Human H1N1 and H3N2 viruses are highly resistant to entry blockers, and resistance to neuraminidase inhibitors in both human seasonal and pH1N1 viruses is on the rise (71, 73). Thus, continued pursuit of novel influenza antivirals should be given high priority.

The combined approach of surveillance, risk assessment, vaccination, and additional biocontainment measures such as culling and isolation of affected animals is a thorough and effective means to limit interspecies transmission and creation of new influenza viruses.

**RISK ASSESSMENT AND RISK MANAGEMENT FOR INFLUENZA A: MORE THAN MEETS THE EYE**

The reservoir for influenza A viruses in nature is enormous, considering all aquatic ecosystems and the bird populations residing in those habitats. In addition, rapid genetic changes in the viral genome due to replication errors and the segmented nature of the virus make prediction of future epidemics and pandemics of influenza A viruses difficult. The zoonotic potential of influenza A viruses significantly increases their importance in terms of public and veterinary health. With the emergence of each pandemic wave, influenza experts from multidisciplinary fields have been working to develop a risk assessment and management program to evaluate the potential risk of influenza A viruses in nature.

Risk assessment and risk management efforts should be conducted as part of both pandemic preparedness and postpandemic evaluation (74). Continuous active surveillance is an indispensable part of prepandemic action. In addition, it is essential to comprehend the biological and genetic aspects of influenza A viruses, such as the molecular changes required for adapting to a new host and/or for transmission among different host systems, the efficiency of sustainability of the virus in a new host population, and the new set of selective pressures imposed on viruses as a result of inevitable changes in environmental conditions (e.g., temperature and humidity).

Risk assessment is conducted by the WHO, the Centers for Disease Control and Prevention (CDC), and external influenza experts. The current risk assessment strategy is based solely on the antigenicity of the virus, and the main result of these endeavors is the development of annual influenza vaccines. The CDC is currently
developing the Influenza Risk Assessment Tool (IRAT) with the help of external influenza experts, which evaluates “the potential pandemic risk posed by influenza A viruses that currently circulate in animals but not in humans.” According to the evaluation criteria of IRAT, each virus can be categorized as low risk, moderate risk, or high risk. Viruses are assessed on the basis of their properties (e.g., genomic variation, receptor binding, transmission in experimental animals, and antiviral treatment susceptibility/resistance), the attributes of the population (e.g., existing population immunity, disease severity and pathogenesis, and antigenic relationship to vaccine candidates), and ecology and epidemiology (e.g., global distribution of the virus in animals, infection in animal species, and human infections) (75).

Overall, establishment of an efficient risk assessment and management program for contemporary and prospective influenza A viruses will accelerate the understanding of the ecology and evolution of these viruses as well as the infection and transmission dynamics among different host species. Information gleaned from these efforts has direct implications for planning and responses to epidemics and pandemics. Thus, the bridge between scientific agencies and governments should remain strong to facilitate interactive communication and mediate implementation of risk management programs for the sake of national and global health interests.

CONCLUDING REMARKS
In the 21st century, the populations of humans and their domesticated livestock are increasing at an unprecedented rate. Such expansion consumes resources from local environments and encroaches upon natural ecosystems. Concomitantly, the effects of global climate change, including rising temperatures and sea levels, are gradually altering these ecosystems and the wild species that inhabit them. Where these two phenomena collide, the potential for emergence of novel influenza viruses is high. The core components of the One Health concept—human and animal health and environmental awareness—are directly applicable to influenza evolution and zoonotic transmission. From the human perspective, clinicians, epidemiologists, and virologists must examine viruses that have circulated in the past to predict what may emerge in epidemic or pandemic form in the future. The identification of emerging viruses has direct implications for vaccine development and antiviral susceptibility, both of which are key control strategies in the public health arena. A similar endeavor, with the inclusion of veterinarians and agricultural workers, is necessary to address the animal health component. Because of the zoonotic nature of influenza viruses, the data collected by experts in each field are important for understanding the pathogenesis of new viruses and their ability to transmit between various human and animal hosts. The acquisition of these data begins with active influenza surveillance from the environments from which these viruses may emerge. While human surveillance of annual circulating viruses is a recognized process for vaccine generation in many countries, animal surveillance outside of endemic and outbreak zones should be increased in practice and scope. This includes sampling of wild birds within natural flyways and aquatic environments as well as agricultural animals on both small and large farms. While we most commonly recognize and focus on the ability of influenza viruses to become highly pathogenic and virulent, the reverse trend is equally possible. A likely evolutionary trait of an influenza virus is to decrease virulence in a host, thus maintaining and facilitating spread. For these reasons, surveillance of not only diseased but also healthy humans and animals should not be overlooked. For humans, this includes incorporation of seroanalysis in current diagnostic protocols. In agricultural settings, surveillance of healthy poultry and swine is absolutely necessary to identify low-pathogenicity viruses that are circulating and simmering asymptptomatically in livestock.

The genetic instability of influenza viruses, combined with their wide host range and potential to transmit between individual hosts, facilitates the genesis of novel viruses with varying degrees of pathogenicity and virulence. It has become increasingly evident that focus in any one area or on any single host, be it human or animal, is not sufficient to accurately understand the role that influenza plays in the health of each of these groups. This is highlighted by the emergence and transmission of virulent H5 and H7 viruses from birds directly to humans in the past decade, as well as the recent H1N1 pandemic, which contained influenza genes donated from wild birds, swine, and humans.

Thus, the future for controlling influenza viruses and other emerging infectious diseases depends on collaboration between ecologists, virologists, veterinarians, and physicians with the aim of predicting the pathogenicity of benign disease agents in their reservoir and host species.

ACKNOWLEDGMENTS
The authors wish to acknowledge support from the National Institute of Allergy and Infectious Diseases, National Institutes
REFERENCES


Emergence of Influenza Viruses and Crossing the Species Barrier


