

SECTION I

Common aspects of animal influenza

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Influenza A virus

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Introduction

Influenza A viruses (IAVs) are important veterinary and human health pathogens that are present worldwide. The category of viruses has a diverse host range, including a large number of avian and mammalian species. The ecology and epidemiology of influenza A viruses are very complex, involving various free-living, captive-raised, and domestic bird hosts as well as various wild and domesticated mammalian hosts within diverse environments, including humans, pigs, horses, dogs, bats, and sporadic infections in miscellaneous mammalian hosts (Figure 1.1). The other key characteristic of the virus is the genetic and antigenic variation that occurs through the combination of a high mutation rate and a segmented genome that provides an ability to rapidly change and adapt to new hosts. In the right conditions, an IAV can adapt to a new host such that it replicates and transmits efficiently to become endemic in a particular species. In general, this adaptation process produces a viral lineage that has some level of host specificity, so that it becomes more difficult to infect other species. For example, a virus that becomes endemic in horses becomes less able to infect other species such as swine or humans. The species barrier can be less clear in avian species, as a chicken-adapted virus will typically also infect other gallinaceous species, but other classes of birds, such as ducks or pigeons, may be resistant to infection. The IAV can cause a wide range of clinical disease that generally relates to the pathogenesis of the virus, whether it infects just on mucosal surfaces or causes systemic infection. The control of IAVs in animals has used a variety of tools, including vaccines, quarantines, and even culling of infected animals. The goal of eradication of the virus from a host population

can in some situations be achieved, but often at a high cost. In many countries, IAVs are endemic and control efforts are used primarily to mitigate economic losses. Because the primordial reservoir for IAVs is wild birds, the ultimate goal of complete eradication is not feasible, and the potential for introduction of new and unique viruses from the wild bird reservoir is a constant threat.

Etiology

Classification

Type A influenza virus (IAV) belongs to the Orthomyxoviridae family of segmented negative-sense RNA viruses that are divided into six different genera accepted by the International Committee on Viral Taxonomy, including influenza types A, B, C, Isavirus, Thogotovirus, and Quarantivirus [130]. Two additional segmented RNA viruses have been proposed as potential new genera, including a potential type D virus associated with respiratory disease in swine and cattle, and a virus associated with cyclic mortality events in eiders in North America, named the Wellfleet Bay virus [4, 23]. The IAVs are the most widespread and important members of the group, infecting many different avian and mammalian species. Type B and C influenza viruses are human pathogens that rarely infect other species, although infection of swine and seals has been reported [100]. The Isavirus group includes the important fish pathogen infectious anemia virus [61], the Thogotoviruses are tick-borne arboviruses that have been isolated from both humans and livestock [71], and the Quarantiviruses are tick-associated viruses that have been detected in humans and birds [117]. The remainder of this chapter will be focused mostly on IAVs of

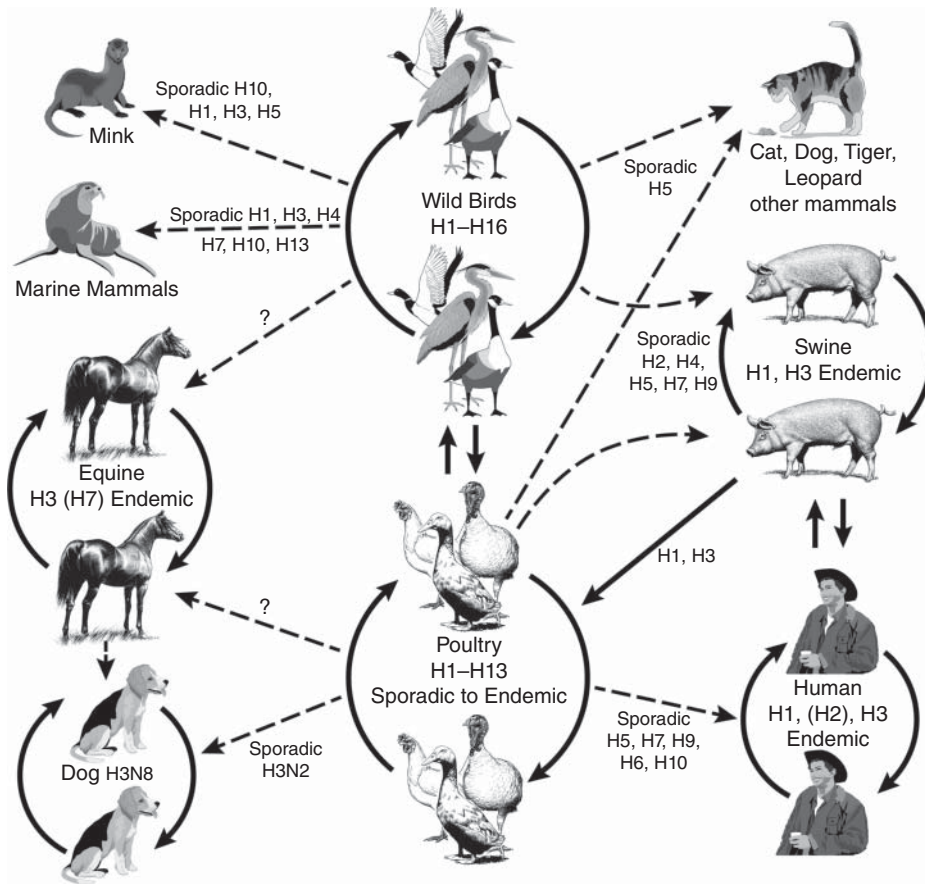


Figure 1.1 Diagrammatic representation of the source and movement of influenza A viruses or their genes within avian and mammalian ecological and epidemiological situations (updated from [160]). H = hemagglutinin subtype, () = subtype previously common but no longer circulating. Source: K. Carter, University of Georgia, and D. Swayne, USDA/ARS.

birds and mammals, but with brief coverage of influenza B viruses contained in human influenza vaccines.

Composition

All IAVs have 8 different gene segments that encode at least 10 different viral proteins. The structural proteins in the mature virion can be divided into the surface proteins that include the hemagglutinin (HA), neuraminidase (NA), and membrane ion channel (M2) proteins. The internal proteins include the nucleoprotein (NP), the matrix protein (M1), and the polymerase complex comprised of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) [103]. Two additional proteins produced

by IAV are the non-structural proteins, namely non-structural protein 1 (NS1) and non-structural protein 2 (NS2), which is also known as the nuclear export protein (NEP) [97]. The NS1 protein is considered to be a true non-structural protein that is not found in the virus particle, but is produced in large amounts in the host cell [14, 172]. The NS2 protein is primarily found in host cells, but some protein can be found in the virion [130]. Several additional accessory proteins have been described that result from transcription from alternative open reading frames, although the function of many of them is poorly understood [177]. The PB1-F2 protein, an 87-amino-acid protein that is transcribed from a different reading frame from the PB1 protein, is a potential virulence factor thought

to be involved in apoptosis in host cells, but it is not found in all IAVs [21]. The PA-X protein, a product of a ribosomal frame shift, has been shown to modulate the mouse immune response [51]. The role and importance of these accessory proteins are still being studied, and their importance to the pathogenesis of the virus is unknown.

The HA protein is categorized into 18 different subtypes, originally based on the hemagglutination inhibition (HI) assay, but now confirmed by gene sequencing and analysis (Table 1.1). The different subtypes are not uniformly distributed among the various bird and mammal species, but the greatest diversity of IAVs occurs in the class Aves, principally in two orders of wild birds, namely the Anseriformes and Charadriiformes. The subtype

distribution is more limited in mammals, with restriction of a few HA subtypes to endemic or sporadic infections of mammals.

Morphology

The IAVs can be morphologically extremely variable, ranging from spherical particles with a diameter of 80–120 nm to filamentous forms that can be several micrometers in length. The filamentous forms seem to predominate in clinical isolates, but after passage in cell culture or embryonating chicken eggs the virus often changes morphology to the spherical forms, at least for human viruses [15, 130]. The morphology appears to be primarily controlled by the matrix 1 protein, and two

Table 1.1 Hemagglutinin subtype distribution^a of influenza A viruses between different birds (class: Aves) and mammals (class: Mammalia).

HA subtype	Host of origin						
	Mammalia				Aves		
	Humans	Swine	Equine	Bats	Anseriformes (e.g. dabbling ducks)	Charadriiformes and Procellariiformes (e.g. shorebirds, gulls, seabirds)	Galliformes (domestic poultry)
H1	++ ^a	++			+	+	++ ^e
H2	(++) ^b	±			+	+	+
H3	++	++	++		++	++	++ ^e
H4		±			++	+	+
H5 ^c	±	±			+	+	++ ^b
H6	±				++	+	+
H7 ^c	±	±	(++) ^b		+	+	++ ^b
H8					±		±
H9	±	±			+	++	++
H10	±				+	+	+
H11					+	++	+
H12					+	+	±
H13					+	++	+
H14 ^d					±		
H15 ^d					±		
H16 ^d						±	
H17				+			
H18				+			

^a± = sporadic, + = multiple reports, ++ = most common.

^b() = Previously common but now not reported.

^cBoth LP and HP viruses.

^dRare subtypes.

^ePrimarily swine influenza virus infections of domestic turkeys.

Modified from Swayne, D. E. and M. Pantin-Jackwood. 2008. Pathobiology of avian influenza virus infections in birds and mammals. In: *Avian Influenza*, D. E. Swayne, ed. Blackwell Publishing: Ames, IA. 87–122.

specific amino acids have been identified as being important [15]. The overall structure of the virus includes a lipid membrane derived from the host cell that has three viral integral membrane proteins, namely the hemagglutinin, neuraminidase, and matrix 2 proteins. The hemagglutinin protein exists as a trimer that appears as spikes on the lipid membrane, and is the most abundant surface protein [25]. The neuraminidase protein exists as tetramers and forms more of a globular structure extending from the lipid membrane. The M2 protein is a small protein that functions as an ion channel that is important for triggering viral uncoating. The M1 protein appears to be the primary bridge between the lipid membrane and the viral core of nucleoprotein, viral RNA, and the polymerase complex.

Propagation

Influenza A viruses are easily propagated in the laboratory, and this has allowed them to be widely studied. Avian, human, swine, and equine IAV were all originally propagated in embryonating chicken eggs, and this method is still commonly used both for diagnostic purposes and for virus propagation, especially for vaccine production. Recently there has been more emphasis, particularly for the mammalian IAV, on growing influenza viruses in cell culture, both in primary and continuous cell lines, for both routine diagnostics and vaccine production [36, 101, 195]. Common cell lines for virus isolation and propagation are chicken embryo fibroblast cells, chicken embryo kidney cells, Madin–Darby canine kidney cells, Vero cells, and others. For avian influenza (AI) viruses (AIVs), the isolation and characterization of viruses is most commonly performed in 9- to 11-day-old embryonating chicken eggs by inoculation of the allantoic cavity. Embryonating chicken eggs provide the added advantage of allowing replication for both low-pathogenicity avian influenza (LPAI) and high-pathogenicity avian influenza (HPAI) viruses [41]. Primary chicken embryo cell cultures are also used, but for LPAI virus (LPAIV), trypsin must be added to the media for efficient virus replication and plaque formation. Alternatively, the use of some cell culture systems, such as primary chicken kidney cells, allows replication and plaque formation of LPAIV without additional trypsin, presumably because it produces a trypsin-like protease as seen with

mammalian kidney cell cultures [62]. Recently, however, the use of chicken eggs has been found to be inadequate for the isolation of some IAVs from humans, swine, and turkeys. As early as 1996, human H3N2 variants were isolated in cell culture that no longer grew well in chicken eggs without adaptation [195]. For these viruses, isolation in mammalian cell culture was more reliable for primary isolation [167], although in one case the use of the egg yolk sac route of inoculation instead of allantoic sac inoculation resulted in a virus isolation [155]. The same viruses that no longer replicate well in chicken eggs also no longer efficiently hemagglutinate chicken red blood cells, which has necessitated the use of alternative red blood cells (RBCs), such as turkey or guinea pig RBCs [90, 155].

Nomenclature

The nomenclature for describing IAVs has been standardized to provide a consistent and informative nomenclature for all IAVs. The features used to name all new IAVs include the following: (1) antigenic type (A, B, C, or D); (2) the host animal from which the virus was isolated, but for human isolates this may be omitted and is simply implied; (3) the geographic origin of the isolate, which can be a city, state, province, or country designation; (4) the unique laboratory or other reference identification number for each isolate; (5) the year of isolation; and (6) the hemagglutinin and neuraminidase subtypes, which are often included in parentheses at the end. For example, an influenza virus isolated from turkeys in Missouri would be A/turkey/Missouri/24093/1999 (H1N2).

Virus life cycle

The initial step in IAV infection is the attachment of the viral hemagglutinin protein to the host cell receptor sialic acid, which initiates endocytosis. Sialic acid is a general term for the terminal sugars found in N- and O-linked glycoproteins that can be made of many derivatives of neuraminic acid. Sialic acid molecules are often classified in terms of how they are linked to the underlying sugars at the α -2 carbon. The most common linkages are the α -2,3 and α -2,6 linkage [158]. These different sialic

acid linkages result in different conformations of the host receptor protein that affects virus binding. The hemagglutinin protein, based on the amino acid structure, will bind different types of sialic acid with different affinity that can determine whether the virus can initiate the infection process. The virus needs to bind strongly enough with the host protein to initiate endocytosis, and typically has strong specificity for either the α -2,3 or α -2,6 linkage. Different animal species will have different patterns and levels of expression of α -2,3 and α -2,6 sialic acid, that may vary between different tissues in the same animal. The α -2,3 sialic acid is predominantly expressed in avian species, and the α -2,6 sialic acid is expressed in humans. The differences in affinity of the hemagglutinin are thought to be one factor that contributes to the species barrier that IAV usually maintains. Although evidence suggests an important role for sialic-acid-binding preferences, some species, including humans, quail, and swine, express both types of sialic acid, although with different tissue distributions and avidities [169, 180]. This receptor distribution can directly affect pathogenesis, as has been proposed for H5N1 infection in humans, where pneumonia is commonly seen and not an upper respiratory tract infection. The pathology appears to correlate with the expression of α -2,3 sialic acid in alveolar type II pneumocytes in the lung [131]. An additional factor is that the specificity of the hemagglutinin for either type of sialic acid is not absolute, and some viruses can bind both α -2,3 and α -2,6 sialic acid [194]. In experimental studies in humans and animals, replication can often occur with many viruses if the subjects are given a large enough challenge dose [11, 46].

The hemagglutinin receptor specificity for sialic acid is not absolute, and can change with as little as two amino acid substitutions at positions 226 and 228 (H3 amino acid numbering) [26, 179]. *In vivo* studies have documented a number of cases of selection of amino acid changes reflecting the host or isolation system in which the virus is being passaged [106, 147].

Pigs have previously been suggested to be a major mixing vessel for human influenza and AIV because they express high levels of both α -2,3 and α -2,6 sialic acid in their respiratory epithelium. The theory was that pigs could be simultaneously infected with human IAV and AIV, and reassortment could

occur between the two viruses, resulting in a new virus that could result in a pandemic strain [125, 183]. The pig as a mixing vessel has some support from field data, and complex reassortant viruses have been isolated from pigs [56, 176]. The 2009 pandemic H1N1 IAV is likely to have been a reassortant virus between two different swine viruses, but the identity of the host and where the reassortment occurred are unknown [138]. However, the outbreaks in humans with AI-like viruses (H5N1, H9N2, H7N7, H7N3, and H7N9), although not resulting in a pandemic virus, show that exposure to infected poultry and not exposure to pigs was the main risk factor for infection [66, 108, 153, 175, 192].

Once viral attachment has occurred the IAV is endocytosed, and when the endosome becomes acidified that triggers the fusion domain of the hemagglutinin protein to become active, and the viral RNA is released into the cytoplasm [146]. The M2 protein plays a key role in the triggering process, as it is an integral membrane protein that allows H⁺ ions to enter in the virion, causing a conformational change of the HA at the lower pH to allow the fusion domain to become active [115]. The adamantane class of antiviral drugs act by blocking the function of the M2 protein, which prevents the fusion of the hemagglutinin within the endosome [43, 157]. The fusion of the viral membrane and the endosomal membrane, mediated by the fusion domain of the hemagglutinin protein, allows the release of the viral RNA–polymerase complex into the cytoplasm, where it is then actively transported to the nucleus because of nuclear localization signals in the nucleoprotein [96].

The negative-sense viral RNA is copied into positive-sense mRNA by the polymerase complex, which includes the three polymerase proteins and the nucleoprotein, in the nucleus. The virus also uses host proteins to initiate mRNA synthesis, including RNA polymerase II. The mRNA requires a 5' capped primer that is stolen from host mRNA by the PB2 protein in a process known as cap snatching [67]. The positive-sense viral mRNA then migrates from the nucleus to begin viral protein translation in the cytoplasm using the host cellular machinery. The positive-sense RNA also serves as a template to produce the negative-sense viral RNA that will be packaged into the virion.

Two viral proteins, the M1 and NEP, are crucial for trafficking of viral proteins to and from the nucleus. The M1 protein also plays a critical role in the assembly and structure of the virion [15]. The viral assembly process includes the three integral membrane proteins, hemagglutinin, neuraminidase, and small amounts of the M2 protein, entering the endoplasmic reticulum, where they are folded and glycosylated before eventually moving to the apical plasma membrane [9]. The M1 protein is believed to be critical in bridging the surface integral membrane proteins and the ribonucleoprotein complex and each of the eight viral gene segments before the virion is complete. All eight viral gene segments have highly conserved regions, 13 and 12 nucleotides long, on the 5' and 3' end of each segment respectively, that are important packaging signals. RNA packaging appears to be an inefficient process, and many viral particles do not package all eight gene segments, creating a high proportion of defective viral particles. It has been estimated that more than 90% of viral particles are non-infectious [29, 31]. The packaging process may also allow multiple gene segments, particularly of the smaller genes, to be included in the virion. This multiple packaging may even affect the phenotype of the virus, since it has been hypothesized that when multiple copies of the NS gene are packaged per virion, an increased resistance to interferon production will occur [127].

The efficient budding of the viral particle from the cellular membrane requires, among other things, the enzymatic activity of the neuraminidase protein to remove sialic acid from the surface glycoproteins, specifically the hemagglutinin protein. This prevents self-binding of the protein and the aggregation of the virus at the cell surface [89, 129]. In experimental studies, viruses that have reduced neuraminidase activity will aggregate on the cell surface because of particles attaching to each other, which can greatly reduce the effective titer of the virus [8]. The loss of neuraminidase activity is not just a theoretical exercise, because one of the markers of AIV adaptation to poultry is the presence of stalk deletions of the neuraminidase protein [88]. These stalk deletions result in a marked decrease in neuraminidase activity. Although the neuraminidase active site is not affected by the stalk deletion, the shorter stalk is thought to reduce flexibility of the protein,

which reduces its ability to attach to the sialic acid substrate. The IAV can at least partially compensate for this reduced neuraminidase activity by making changes in the hemagglutinin protein that reduce the affinity of binding to sialic acid, typically by the addition of extra glycosylation sites near the receptor binding sites [91]. We currently do not understand the selective advantage of neuraminidase stalk deletions in poultry.

For LPAIV, the released viral particles are not infectious until the hemagglutinin protein is cleaved into HA1 and HA2 subunits by trypsin or trypsin-like proteases. The role of HA cleavage will be discussed in more detail in the pathogenesis section.

Virus genetics

Ecology in wild birds

The natural host and reservoir for all type A influenza viruses occur in wild birds, primarily in waterfowl, gulls, and shorebirds [58, 133]. In the natural host the virus appears to be evolving slowly, with most internal genes being highly conserved at the amino acid level [149]. The surface glycoproteins, HA and NA, are much more variable in amino acid sequence, demonstrating the greater diversity of these genes. For both proteins, multiple antigenic subtypes have been characterized, where antibody to one subtype will neutralize, with high specificity, only viruses of that subtype. For the HA protein, 16 subtypes of AIV have been characterized (Figure 1.2), and 9 subtypes have been characterized for the NA protein. At the amino acid level the difference between subtypes is as little 20%, but the most divergent subtypes are up to 63% different. About 25% of the amino acids are conserved among all 16 HA subtypes [95]. Similar comparisons are found for the NA subtypes, with amino acid differences of between 31% and 61%.

In comparing the nucleotide sequence of most of the gene segments from wild bird AIV, including within an HA and NA subtype, a clear separation is found to occur among viruses isolated from Europe, Asia, Africa, and Australia (Eurasian lineage) and those isolated from the Americas (American lineage) [149]. At the amino acid level for the more conserved internal proteins, the distinctions between American and Eurasian lineages

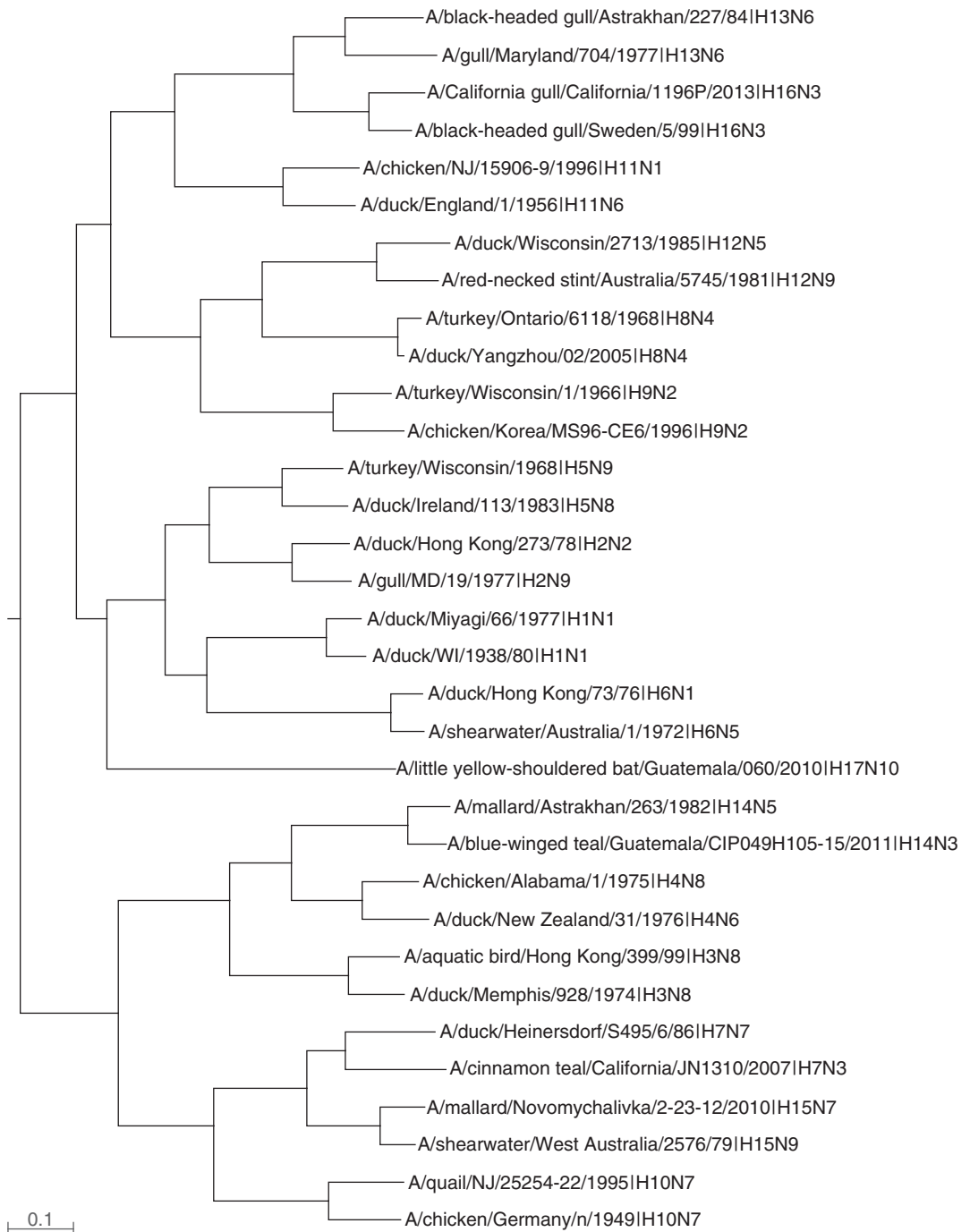


Figure 1.2 Phylogenetic tree of 17 hemagglutinin subtypes. The complete amino acid sequence of representative isolates for all 16 avian HA subtypes and the H17 bat subtype are included, with a representative North American and Eurasian isolate where available. The tree was midpoint rooted using the Influenza Research Database PhyML program, version 3.0 [144].

are lost. The HA and NA genes having greater nucleotide sequence diversity still separate at the amino acid level into clear Eurasian and American lineages for most hemagglutinin subtypes. For the H7 subtype a further division of lineages can be observed between the North American and South American lineages and between the Australian viruses and European and Asian viruses [154]. This distinction of the H7 subtype may reflect the availability of sequences, particularly from South America, where few AIV sequences are available. The differentiation of the wild bird isolates into distinct Old World and New World lineages suggests that infrequent transfer of AIV genes is occurring between these two geographic regions. However, the recent outbreak of Eurasian H5N8 HPAI in North America in 2014 does show that viruses can on occasion move long distances [52].

As more sequence information becomes available from wild bird and poultry isolates, the general rule of American versus Eurasian lineage appears to have more exceptions. For example, the H2 subtype influenza viruses appear to follow the rule of American and Eurasian lineages for poultry and duck isolates, but the North American origin shorebird and gull viruses are more closely related to Eurasian isolates than to other North American H2 isolates [84, 124]. Although the H2 shorebird and gull viruses are more similar to Eurasian viruses, they do cluster as a unique sublineage. A similar Eurasian-like gull and shorebird sublineage also exists for H6 influenza viruses from North America, but the internal genes, including the matrix and non-structural genes, have the anticipated American origin sequence [143]. Therefore these data probably represent a unique subpopulation of the hemagglutinin gene circulating in North America, and not evidence of recent movement of Eurasian-lineage viral genes into the Americas.

The complete host range of AIV in wild birds is not known, but based on sampling studies, two orders of wild birds are most consistently infected, the Anseriformes and the Charadriiformes (Table 1.1). The Anseriformes include ducks, geese, and swans, but the incidence of infection appears to be highest in dabbling ducks, including mallards, pintails, and teal. The incidence of infection appears to be seasonal, with the highest isolation rate being in juvenile birds in the fall of the year [145]. A lower incidence of infection occurs in

the Charadriiformes, which include shorebirds and gulls. Wild bird AIV seems to pass easily between different bird species, and it is not currently possible to predict the species from which the virus was isolated based on the nucleotide sequence. The one possible exception to this rule is that most H13 and H16 viruses are from gulls, and gulls also seem to have a predominant gull lineage for at least some of the internal genes (Figure 1.3) [40, 152]. The ecology of AIV in wild birds is discussed in detail in Chapter 8.

Bat origin influenza

Recently, two unique IAVs have been identified in several species of bats, including yellow-shouldered and flat-faced bats, by molecular detection and sequencing from clinical samples from Central and South America. The bat isolates have not been obtained in eggs or cell culture. The viral sequences show enough similarities to IAV to remain in those genera, but these viruses also have enough unique differences for them to be unlikely to reassort with the traditional type A viruses. The viruses belong to two new subtypes, H17N10 and H18N11 [170, 171]. The internal genes are compatible with human influenza HA and NA genes in a reverse genetics system, but the HA and NA genes have enough structural differences for it to be likely that the HA protein uses a completely different receptor from other type A influenza viruses, and the NA gene has no measurable neuraminidase activity and also probably has a different function [197, 199]. It is not surprising that an influenza-like virus has been detected in bats, as the high density of bats within colonies should favor transmission of the virus, but it is currently not known whether these viruses cause any clinical disease and how widespread the virus may be in bat populations.

Epidemiology in man-made systems

AIVs are unusual in that they can infect and replicate in a wide variety of host species, including chickens, turkeys, swine, horses, humans, and a wide variety of other avian and mammalian species. However, the amount of virus required to infect the host can vary greatly depending on the level of host adaptation, which provides at least some level of species barrier [141, 173]. The virus as it becomes adapted to the new host typically

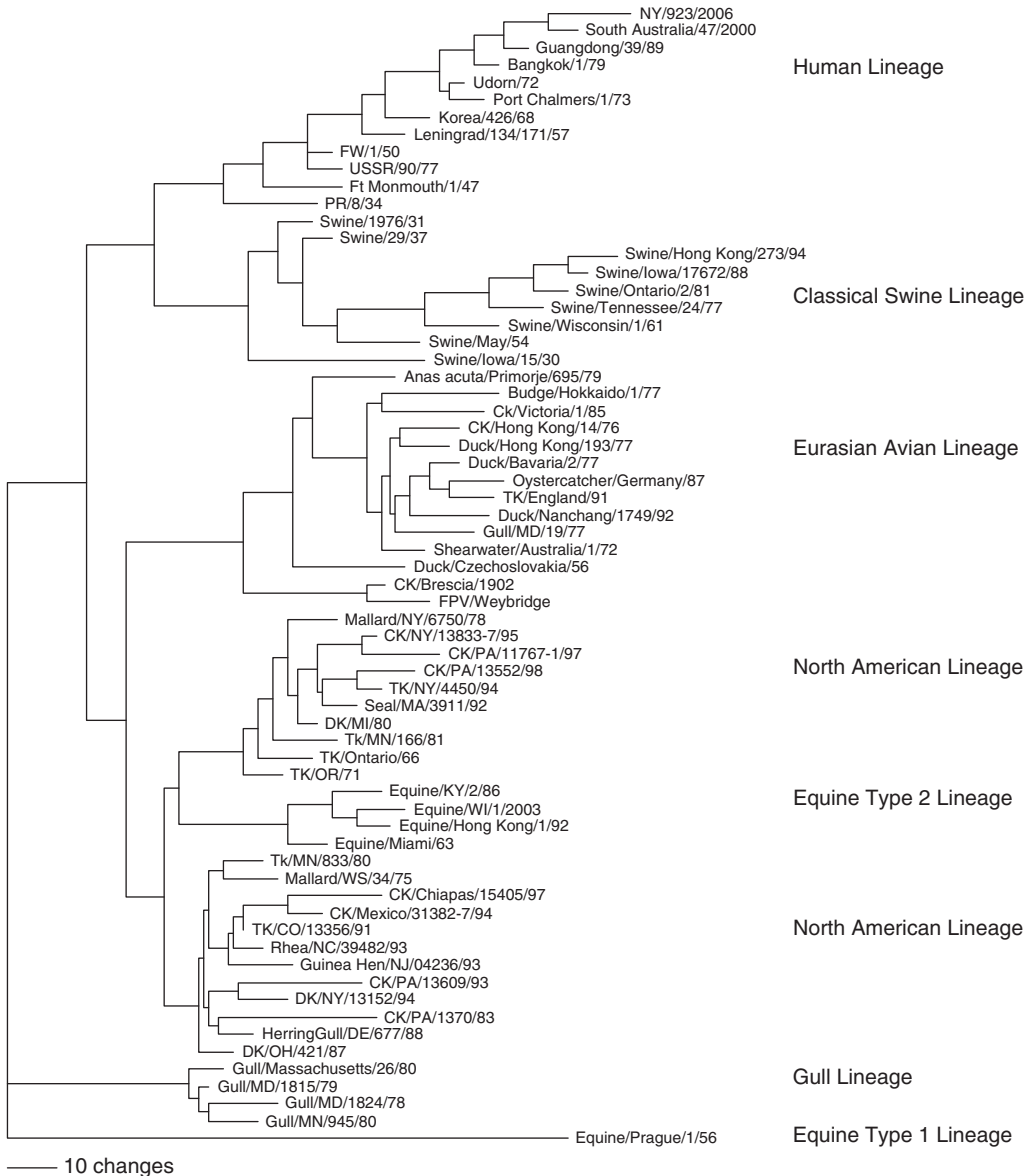


Figure 1.3 Phylogenetic tree of the matrix gene. The tree is based on the complete nucleotide sequence of representative isolates for major groups of type A influenza viruses. The tree is rooted to equine/Prague/1/56, which is the most divergent type A influenza virus. CK = chickens, DK = ducks, TK = turkeys. Standard two-letter abbreviations are used for states from isolates from the USA.

becomes less able to replicate in the original host species, such as wild birds. If the virus is allowed to circulate long enough in the new host, it becomes a human-, chicken-, or swine-adapted virus, and this results in the creation of unique phylogenetic lineages [16, 40]. Influenza viruses in a new host change at a high and predictable level that is the result of the high error rate of the virus and host

selection pressures [17, 40, 126, 150]. For species under immune pressure from natural infection and/or vaccination, the changes in the HA and NA genes can occur at an even faster rate [35, 76]. The changes in both genes are concentrated in specific antigenic sites. For example, the human H3 protein has five antigenic sites that are binding sites for neutralizing antibody [182, 184]. Even with our

current level of understanding, we cannot predict the changes that allow species adaptation or allow the virus to evade the host immune response. However, the number of specific amino acid sites linked to species adaptation continues to grow. Although all eight genes probably play important roles, the HA and PB2 genes are prominent for changes thought to be important for adaptation from avian to mammalian hosts [93].

IAVs have become endemic in a number of species, including humans, swine, horses, and poultry, and once a strain of influenza circulates in a particular species for an extended period of time (months to years), the virus becomes increasingly species specific. Thus human IAVs do not usually infect swine, equine IAVs do not infect turkeys, and poultry IAVs do not infect humans. However, this general rule of host-adapted influenza viruses staying within a single species or related species does have many exceptions. For example, classical swine H1N1 IAV from North America routinely crosses the species barrier from swine to turkeys, causing costly disease outbreaks [45]. The sporadic infection of humans with some AIVs (H5N1, H7N7, H7N3, H7N9, and H9N2) from poultry has been observed, and therefore AIVs do present a public health threat as a zoonotic pathogen, although the risk is considered to be low [66, 108, 156, 175, 192]. Few experimental challenge studies of humans have been performed with AIVs, but in general the viruses replicated poorly and caused little to no clinical disease [11]. It is not understood whether all HA and NA subtypes of AIV have the same ability to infect humans or other species. Currently only a limited number of subtypes have become endemic in humans (H1, H2, H3, N1, and N2) [190].

The movement of AIV from wild birds to domestic bird species is not uncommon, but rarely results in viruses becoming endemic in poultry. Several routes of exposure of wild bird viruses to poultry have been documented or suspected of being the origins of outbreaks. Direct exposure to wild birds is the most likely method, with some of the best documented cases of exposure being in commercial turkeys in Minnesota, where multiple outbreaks of AI were observed yearly in the 1980s and early 1990s [42]. AIVs of many different HA and NA subtypes were isolated from turkeys in different outbreaks, and usually at times when wild ducks were migrating to or from their summer breeding

grounds. During the migratory wild duck season, turkeys were raised outside and the wild birds could fly over or actually land in the turkey pens. During the 1990s the management system was changed so that the turkeys were reared in confinement for their entire lives, and the incidence of AIV was greatly decreased [164]. Limiting exposure of poultry to wild birds through confinement rearing and other biosecurity measures provides an opportunity to reduce the risk of AIV introduction from wild birds.

Another source of introduction of AIV to poultry is the live poultry marketing (LPM) system, which is found in many countries around the world, including the USA. LPMs typically offer a variety of birds that can be slaughtered and used for human food consumption. For many developing countries where refrigeration is not available, LPMs provide a way to maintain freshness until the product is sold. For other countries, such as the USA or Hong Kong, the LPM system caters to consumer preferences at a premium price for specific selection of a food bird compared with the purchase of a chilled or frozen bird from a supermarket. However, this marketing system provides an ideal environment for introducing and maintaining AIV in the poultry population [70, 150]. A common scenario is when domestic waterfowl, primarily ducks, are raised on ponds where exposure to wild ducks and other birds is common [10]. This creates a high risk of infection for domestic ducks, which can be transported to the LPM system where there is close contact with other poultry, including chickens, quail, and other gallinaceous birds. A constant supply of AIV-naive poultry continues to enter the LPM system, and provides the opportunity for viruses to become adapted to chickens and other avian species. Once AIV becomes entrenched in the LPM system, it provides an ongoing source of infection back to commercial poultry. One example is the H7N2 AIV that began circulating in the north-east USA in 1994 and was associated with at least five different outbreaks in industrialized poultry in seven states before it was eradicated [142]. The concern for LPMs in the introduction of AIV has resulted in Hong Kong banning the selling of live ducks and geese in the markets, a comprehensive surveillance program, and stricter sanitary requirements [70]. Quail have also been implicated as a highly susceptible species that may play an important transition role for viruses in the

market [85, 106]. These biosecurity and management changes have been effective in reducing the incidence of infected poultry in the markets.

An additional risk of introduction to farms is through the birds' drinking water. Typically this occurs when surface water sources, such as lakes or rivers, are used for drinking water or other purposes. If the drinking water is not properly purified, AIV from wild birds can be introduced to the poultry flock. The use of raw drinking water was suggested to be the source of AI outbreaks in the USA, Australia, and Chile [47, 132, 154].

At least one other common source of transmission of IAV for turkeys is exposure to pigs infected with swine influenza virus (SIV). Turkeys are susceptible to SIV, and having a turkey farm and swine farm in close proximity is a risk factor for the introduction of SIV. Infections with both classical H1N1 SIV and the more recent reassortant H1N2 and H3N2 SIV, and pH1N1 viruses in turkeys have been reported [45, 105, 155, 191]. Swine influenza has a unique and complex history that has some similarities to the disease in poultry, but also some important differences. SIV genes are also thought to be of wild bird AIV origin, but the detection of AIV genes in swine IAV either *in toto* or as a reassortment with endemic SIV is relatively rare.

The circulating strains of SIV in North America and Europe were quite distinct before the human pandemic H1N1 (pH1N1) virus that emerged in 2009. The pH1N1 virus was able to infect not only humans, but also swine, turkeys, ferrets, and sporadic cases in other species [105, 178]. The origin of swine influenza in North America is associated with the H1N1 Spanish flu pandemic in 1918. The virus diverged from the human isolate and was relatively stable for almost 80 years, and is considered to be "classical swine influenza." In 1998, new SIV emerged in the USA that had a unique internal gene cassette that consisted of swine, human, and avian IAV genes and human influenza-like H3 and N2 genes [181, 198]. The triple reassortment internal gene (TRIG) cassette allowed for multiple reassortment viruses of different HA and NA subtypes. The TRIG cassette included multiple genes that formed the basis of the human pandemic H1N1 virus in 2009 [138]. Since 1999, multiple antigenic variants and multiple reassortment events with human viruses have created an ever changing collection of viruses in North America [5].

Classical SIV circulated in Europe for many years, but it was replaced by avian-origin IAV in 1979 [68]. The avian-like swine virus reassorted with human H3N2 viruses in 1984 to establish a stable lineage. Many additional reassortant viruses of different origins were detected, with H1N2 viruses being commonly observed [72]. The human pH1N1 added to the picture in 2009, and currently avian-like H1N1, human-like H3N2 and H1N2 with different internal gene cassettes, and pH1N1 genes are circulating in the European swine population [178].

Although the surveillance in North American and European swine was far from comprehensive for SIV, enough representative isolates are available to document the major variants of the virus. Surveillance in Asia was sporadic in nature, but it did document a variety of viruses circulating in swine, including classical SIV, European avian-like SIV, human influenza viruses, and additional H1N1, H3N2, and H1N2 viruses not found in Europe and North America. The high density of swine and the importation of pigs to the region provided a unique mixing site for viruses from around the world [178]. Swine surveillance was almost non-existent in Australia, Africa, and South America before 2009, when the human pandemic H1N1 emerged. Studies have documented swine being infected with the pH1N1 virus on all three continents, and for Australia they were the first detections, as the continent had previously been free of SIV [28, 94]. Multiple subtypes of virus were identified in Argentina, including unique human-influenza-origin viruses as well as pH1N1 [110].

The emergence of pH1N1 provided a new impetus to increase surveillance of swine, because the pH1N1 had clear origins in SIV, but exactly when and where this viral lineage emerged is still unknown. The emergence of new viruses in swine indicates that viral genes can come from a variety of sources, including avian and human ones. The restricted movement of swine has allowed unique lineages of virus to develop in Europe and North America, although there is overlap of viruses in Asia. Evidence of infection of swine with avian-origin IAV, either from wild birds or from poultry, continues to be reported, and to pose a threat of introduction of novel viruses with both veterinary and human health implications [44, 55, 83].

Equine and canine influenza

Only two lineages of equine influenza viruses (EIVs) have been reported to be endemic in the horse population. The original subtype detected was H7N7 virus that was first isolated in 1956. The H7N7 EIV lineage based on the sequence divergence from other influenza viruses had been present in the horse population for an extended period of time [189]. The introduction of H3N8 in 1963 resulted in the likely extinction of the H7N7 lineage. The H3N8 lineage infected horses worldwide, probably as the result of frequent international movement of horses for racing and other equestrian sporting events. More similar to human influenza, which also has a worldwide distribution, the H3N8 virus has continued to evolve into unique sublineages, although there are only a limited number of these, presumably because the most fit virus outcompetes the less fit viruses. Currently two clades from the Florida sublineage are the dominant strains [24, 39]. In one of the clearest examples of influenza viruses jumping the species barrier, the H3N8 Florida clade 1 EIV jumped into dogs, probably in Florida, which resulted in the establishment of a unique canine influenza lineage of virus [24]. A recent study has shown that the canine-adapted virus has greatly reduced virulence in horses [119]. A second unique event was also reported, with H3N8 jumping from horses to dogs in Australia during the equine epidemic in that country in 2007 [63].

Clinical disease in poultry

Field presentation

Influenza infections in poultry, primarily chickens and turkeys, can be asymptomatic, but often cause production losses and a range of clinical disease from mild to severe in affected flocks. The virus can be generally divided into viruses that cause mucosal infections in the respiratory and/or enteric tract, and those viruses that also cause systemic infections. The viruses that cause mucosal infections are usually referred to as LPAIV, and typically these viruses do not cause high mortality in affected flocks. The viruses that cause systemic infections usually cause high mortality and are referred to HPAIV (they were historically known as fowl plague viruses) [64].

The LPAIV can cause asymptomatic infections, but typically the most common symptoms are mild to severe respiratory disease. A decrease in feed or water consumption is another common indication of flock infection when careful records of consumption are kept. For layer flocks or breeder flocks, drops in egg production can also be observed. The drops in egg production can be severe, with the flocks never returning to full production, as is commonly seen in turkey breeders infected with swine-like influenza viruses [45, 92]. In large flocks, small increases in daily mortality can be observed as the virus spreads through the flock. The LPAIV infection at least contributes to this increased mortality, because diagnostic testing of the daily mortality is considered to be a sensitive way to identify LPAIV infection [3, 151]. In some situations, infection with LPAIV may result in high mortality, generally in association with concurrent or secondary pathogens and/or poor environmental conditions [7]. On rare occasions, LPAIV may cause specific lesions in internal organs, either through direct infection or by other indirect causes [200].

The disease and lesions caused by AIV infections in domestic ducks will be discussed in more detail in Chapter 14, and in the chapter on pathobiology of avian influenza virus infections in birds and mammals in the previous edition of this book [160]. Elsewhere in the present volume, disease and lesions of IAV infections in humans (Chapter 5), pigs (Chapter 16), horses (Chapter 20), dogs (Chapter 22), miscellaneous mammals (Chapter 23), and laboratory mammalian models (Chapter 24) are presented.

Molecular and biological features of low- and high-pathogenicity avian influenza viruses

The LPAIVs can be of many different hemagglutinin and neuraminidase subtypes. The HPAIVs, for unknown reasons, have been restricted to the H5 and H7 subtypes, but most H5 and H7 influenza viruses are of low pathogenicity. It is only rare that these LPAIVs mutate into the HPAIV. It is generally believed that HPAIVs arise from H5 and H7 LPAIVs that have been allowed to circulate in poultry for extended periods of time. For example, LPAIV circulated for several months to years in poultry flocks in the H5 outbreaks in Pennsylvania in 1983

and Mexico in 1994, and the H7 outbreak in Italy in 1999, before the viruses mutated to become HPAI [49, 59, 196]. The selection pressures for viruses to change from LPAIV to HPAIV are not currently known, but the replication of virus in gallinaceous birds, including chickens, turkeys, and quail, is considered a critical part of the process. HPAIVs are not believed to be normally present in the wild bird host reservoir [121]. However, on four separate occasions HPAI has been detected in wild birds. The first outbreak was in terns in South Africa in 1961, which was not associated with a poultry source [12]. Three widespread outbreaks of H5 HPAI in wild birds have been reported in the last 10 years that were all associated with poultry outbreaks. The initial spillover event in 2005 of a clade 2.2 H5N1 virus resulted in mortality events in multiple wild bird species. The virus moved through wild birds to eventually reach most of Europe and several countries in Africa. This lineage of virus did not persist permanently in wild birds [82]. The second spillover event was a clade 2.3.2.1 H5N1 virus first detected in 2007 [139]. The virus was detected primarily in East Asia, but spread to Eastern Europe and Southern Asian countries in 2010 and 2011, and became established in poultry populations in Bangladesh. Isolates from wild birds were often from dead or sick birds, but were not associated with large mortality events in wild birds that characterized the initial introduction of the clade 2.2 viruses. Experimental testing showed continued high virulence in chickens, but variable mortality in different duck species [22, 33, 53, 99]. It is unclear whether this lineage is persisting in wild birds.

The third wild bird epornitic was detected in late 2013 and has spread from East Asia to Europe and North America. This virus includes multiple reassortants, with N8 being predominant, but N2 and N1 reassortants have also been detected. The hemagglutinin gene is classified as clade 2.3.4.4. This virus has also not been associated with mass mortality events in wild birds, and appears to have less virulence in chickens than previously characterized H5N1 viruses [32, 140].

Cellular pathobiology and hemagglutinin cleavage

The primary virulence characteristic that separates the LPAIVs and the HPAIVs in chickens and other gallinaceous birds is the ability of the hemagglutinin

protein of HPAIVs to be cleaved by the ubiquitous proteases found within most cells in the host. Influenza viruses must have the HA protein, which is produced as a single polypeptide, cleaved into the HA1 and HA2 subunits before it can become infectious. This cleavage is necessary for the fusion domain to be activated during the uncoating step of virus replication. Normally trypsin or trypsin-like proteases (plasmin, blood clotting factor-like proteases, tryptase Clara, bacterial proteases) cleave the hemagglutinin protein by recognizing a single arginine in the extracellular environment [41, 62, 65, 73]. The distribution of LPAIVs in the host is believed to be highly influenced by the local availability of these trypsin-like proteases in the respiratory and enteric tracts [65]. Other proteases can also cleave influenza, and in chick embryos it is believed to be a prothrombin-like enzyme similar to blood clotting factor X [41]. However, when multiple basic amino acids (lysine and arginine) are present at the HA cleavage site, particularly by the insertion of multiple basic amino acids, the cleavage site becomes accessible to furin or other ubiquitous proteases that are found in most cells of the body [148]. The HPAIVs' HA protein is cleaved during the assembly stage of virus replication, and therefore is infectious when it is released from the cell [146, 148]. This allows the HPAIV to greatly expand its ability to replicate in a number of different cell types, including a range of cell types in the brain, heart, skeletal muscle, and pancreas. The damage to critical organs or to endothelial cells lining the blood vessels can cause a variety of disease symptoms that often lead to the death of the bird [111, 159]. Other viral genes are also important in determining the virulence of the virus, but the hemagglutinin cleavage site is by far the most important virulence trait in gallinaceous birds [81, 123].

Impact of host and virus strain on pathogenicity

The HPAIV phenotype by definition causes high mortality in 4- to 6-week-old specific pathogen-free chickens [188], but just because it is HPAI in chickens does not necessarily provide a predictor for disease in other species. Few studies have characterized the pathogenicity of a single isolate in a number of different species after experimental challenge. One of the broadest series of studies examined an H5N1 HPAI 1997 chicken isolate

from Hong Kong that was used as an experimental inoculum for a variety of avian species. The Hong Kong 97 strain caused high mortality in all of the gallinaceous species tested, including chickens, turkeys, quail, and pheasants, although differences in mean death time were observed among species [111]. Most other species tested had less severe or in some cases no clinical disease signs, although most were infected based on the ability to reisolate virus from challenged birds [112–114]. Predictions of virulence, outside of the gallinaceous species, could not be made for different orders of birds. For example, some geese when challenged had neurological signs and lesions that correlated with virus replication sites in the brain [112]. However, ducks tested from the same order of birds, Anseriformes, had limited infection in the respiratory tract but did not show any evidence of disease [112]. It seems clear that the virulence associated with hemagglutinin cleavability is not the only factor that determines virulence in other species. This has been clearly shown in ducks with the recent Asian H5N1 viruses. In a 2-week-old Peking duck model, the early H5N1 viruses from 1997 to 2001 could infect but did not cause morbidity or mortality. However, starting with some isolates in 2002, increased mortality was observed, with 100% mortality being seen with more recent viruses [104, 161]. The Asian H5N1 viruses all have an H5 gene from the same lineage and identical or nearly identical hemagglutinin cleavage site sequence with an insert of multiple basic amino acids, and all remain highly pathogenic for chickens. However, the internal genes for these viruses are variable, and it is believed that these internal gene differences account for the difference in virulence [78].

For mammalian species, including swine and humans, naturally infected with HPAIV, severe clinical disease is associated with severe atypical pneumonia, reflecting replication primarily in the respiratory tract, and systemic replication is not commonly observed. Other mammalian species, including ferrets, cats, and dogs, may have more systemic spread of the virus that contributes to high mortality for some strains of HPAIV [69]. The pathogenesis of HPAIV is difficult to characterize for all species, and as the virus changes, the clinical presentation of disease also often changes.

Hemagglutinin changes associated with high pathogenicity

The hemagglutinin cleavage site remains the best but not a perfect predictor of viral virulence in chickens and other gallinaceous birds. As previously mentioned, the presence of multiple basic amino acids upstream of the HA1 and HA2 cleavage site is correlated with virulence [122]. Only the H5 and H7 subtypes of AI are currently known to have an HPAI phenotype, for reasons that are not readily apparent. Sequence comparisons show the H5 and H7 subtypes to be distinctly different from each other. Although both H5 and H7 proteins maintain the general principle of the cleavage site being between arginine and glycine and multiple basic amino acids at the cleavage site resulting in an HPAIV phenotype, there are distinct differences between the subtypes. The typical cleavage site sequences of wild bird LPAIV of H5 and H7s viruses are different [121]. H5s viruses typically have a QRETR/G sequence with arginine at the -1 and -4 position. H7s typically have an NPKTR/G sequence with a lysine and arginine at the -1 and -3 positions. The change to virulence for H5s can occur by substitution of non-basic to basic amino acids or by an insertion of basic and non-basic amino acids at the cleavage site (Table 1.2). The chicken/Scotland/59 H5N1 virus has four basic amino acids at the cleavage site RKKR/G [27], presumably through site substitution that results in an HPAI phenotype. More commonly, additional basic amino acids are inserted at the cleavage site, with two, three, and four additional amino acids being observed. For example, the chicken/Hong Kong/97 H5N1 virus had a sequence of QRERRRKKR/G [153]. The mechanism of insertion of amino acids is not clear, but a duplication event appears likely for several of the H5 HPAIVs [109]. Other parts of the hemagglutinin protein can also play a role in the phenotype of the virus. The best example is the presence or absence of a glycosylation site at position 10–12 of the HA1 protein. In 1983, an LPAI H5N2 virus, chicken/Pennsylvania/1/1983, was isolated that had four basic amino acids, QRKKR/G, at the cleavage site. Six months later, an HPAIV emerged in Pennsylvania, chicken/Pennsylvania/1370/83, which had the same HA cleavage site, but this virus had lost a glycosylation site at position 10–12 in the HA1 protein. The glycosylation site is structurally extremely close to the HA cleavage

Table 1.2 Examples of genetic mechanisms for LP to HP change based on deduced amino acid sequence of HA proteolytic cleavage sites in H5 and H7 AIV.

Influenza virus	Subtype	Pathotype	Amino acid sequence	Mechanism ^a					References
				1	2	3	4	5	
Typical H5 LPAI	H5	LP	PQ.....RETR*GLF						[128]
A/turkey/England/1991	H5N1	HP	PQ... <u>RR</u> KRTR*GLF	X	X				[128]
A/chicken/PA/1370/1983	H5N2	HP	PQ..... <u>KKKR</u> *GLF	X				X	[128]
A/tern/South Africa/1961	H5N9	HP	PQ <u>RETR</u> RQKR*GLF	X		X			[128]
A/chicken/Puebla/8623-607/1994	H5N2	HP	PQ... <u>RR</u> KRTR*GLF	X	X				[37, 49]
A/chicken/Queretaro/14588-19/1995	H5N2	HP	PQ <u>RRKR</u> KRTR*GLF	X	X				[37]
Typical H7 LPAI	H7	LP	PEIP.....KTR*GLF						[128]
A/chicken/Victoria/1985	H7N7	HP	PEIP..... <u>KKREKR</u> *GLF				X		[128]
A/turkey/Italy/4580/1999	H7N1	HP	PEIPKG... <u>SRVRR</u> *GLF				X		[19]
A/chicken/Chile/176822/2002	H7N3	HP	PEKPKT <u>CSPLSR</u> CRETR*GLF ^b					X	[154]
A/chicken/Canada/AVFV2/2004	H7N3	HP	PENPK... <u>QAYRKR</u> MTR*GLF ^c				X		[107]
A/chicken/Saskatchewan/HR-00011/2007	H7N3	HP	PENPK <u>TKPRPRR</u> *GLF ^d				X		[13]
A/chicken/Jalisco/12383/2012	H7N3	HP	PENPK <u>DRKSRHR</u> TR-GLF ^e				X		[54]

^aMechanisms: (1) substitutions of non-basic with basic amino acids; (2) insertions of multiple basic amino acids from codons duplicated from hemagglutinin cleavage site; (3) short inserts of basic and non-basic amino acids from unknown source; (4) non-homologous recombination with inserts which lengthen the proteolytic cleavage site; (5) loss of the shielding glycosylation site at residue 13.

^b30 nucleotides from nucleoprotein of same virus gene coding 10-amino-acid insert.

^c21 nucleotides from matrix of same virus gene coding 7-amino-acid insert.

^d18 nucleotides from unidentified chicken gene coding 6-amino-acid insert.

^e24 nucleotides from 28S chicken ribosomal RNA coding 8-amino-acid insert.

Modified from Swayne, D. E., D. L. Suarez, and L. D. Sims. 2103. Influenza. In: *Diseases of Poultry*, 13th edition, D. E. Swayne, J. R. Glisson, L. R. McDougald, V. Nair, L. K. Nolan, and D. L. Suarez, eds. Wiley-Blackwell: Ames, IA. 181–218.

site, and it is believed that the loss of the sugars allowed greater access to the cleavage site, making it accessible to the ubiquitous proteases that changed the phenotype of the virus [59]. This and other glycosylation sites have also been shown experimentally to be important in virulence [50].

The change from LPAIV to HPAIV for H7 viruses appears to have several important differences. First, all HPAI H7 viruses have insertions of 2 to 10 additional amino acids at the cleavage site. The mechanism for such insertions also appears to be different in many cases. Although a duplication event appears likely for some viruses, in several recent cases non-homologous recombination is the likely method of insertion. In the Chilean outbreak in 2002, the Canadian outbreak in 2004, and the Mexican H7N3 outbreak in 2012 an insertion of 30 nucleotides from the nucleoprotein gene, 24 nucleotides from the matrix gene, and 24 nucleotides from host chicken 28s ribosomal RNA, respectively, resulted in the increase in virulence [54, 107, 154]. Other cases of non-homologous

recombination have been seen in experimental studies where nucleoprotein and host ribosomal RNA sequence was inserted at the cleavage site [60, 98]. In all five examples, the insertions had some basic amino acids, but they were a minority of the insert. In these examples the increased spacing in the cleavage site loop appears to be the more important factor for increasing virulence, as opposed to just the addition of basic amino acids. Almost all of the H7 HPAI outbreak viruses appear to have become HP by unique events at the cleavage site, which makes the prediction of minimum changes to define HPAI by sequence alone difficult for H7s.

Other variables that affect pathogenicity

The HPAIV is defined by an *in vivo* pathotyping test in chickens, applicable to any influenza virus, and/or by a sequence analysis of the HA cleavage site for H5 and H7 influenza. The best predictor of HPAIV is when a suspect virus has the same cleavage site as another known HPAIV.

In such situations the virus is reportable to the World Organization of Animal Health (OIE) as an HPAIV. However, an outbreak in the USA (Texas) in 2004 was a clear case where the phenotype and the genotype did not match up. In this case the Texas/04 isolate had the same HA cleavage site sequence as the A/chicken/Scotland/59 virus, and was reported to OIE as an HPAIV, but the virus was LP in the standard chicken pathotyping test [79]. Even though the two tests did not correlate, and high virulence was not seen in the field, the virus was still considered to be virulent, and this resulted in major trade sanctions on poultry exports for a limited period of time. Other examples of discordance between phenotype and genotype have previously been described [186], and a similar case was reported in Taiwan of H5 viruses with four basic amino acids where some were pathogenic after IVPI testing and some were not [74]. Currently no completely accurate molecular prediction scheme has been determined for HPAIV.

It is also clear from experimental studies that the age and route of inoculation as well as species can affect the virulence of AI virus in experimental infections. The age effect has been seen both in chickens and in ducks. For example, when 1-day-old SPF chickens were challenged intravenously with the LPAIV A/turkey/Oregon/1971, mortality was seen in seven of eight chicks. When the same virus was administered to 4-week-old chickens at the same dose and by the same route, mortality was seen in only one of eight chicks. In this example, the virus replicated to high titer in the kidney, which resulted in renal failure leading to death in most of the 1-day-old chicks. The same virus given by the intra-choanal cleft (intranasally) at the same dose caused mortality in only one of eight 1-day-old chicks [20]. This example shows that mortality can be greatly affected by the age of the bird and the route of inoculation. The intravenous inoculation route, which is not a natural route of exposure, probably seeded high levels of virus to the kidney, which led to the high mortality. The intravenous route of challenge, the standard for *in vivo* pathotyping in chickens, can result in sporadic deaths with some LPAIVs, typically because of replication in the kidney resulting in kidney failure [134, 135, 163]. Primary chicken kidney cells allow replication of LPAIVs, presumably because they produce trypsin-like enzymes that cleave the

hemagglutinin protein, and this property allows LPAIVs to be plaqued without the addition of trypsin in primary kidney embryo cell lines [20].

In ducks it has also been shown that there is a marked difference in disease based on age, with younger ducks being more susceptible to severe infection. For example, several Asian H5N1 viruses cause high mortality in 2-week-old ducks, but the same viruses in 4-week-old ducks produce much lower or no mortality [104, 161]. Increased virulence in younger animals is commonly seen, although the reasons for the differences are not clearly defined. The immaturity of the immune response, both innate and adaptive, probably contributes to these differences. For example, the interferon response greatly increases in the embryo as it ages, and presumably the peak interferon response also occurs after hatching [87].

In some cases, virulence can be greater in older birds or in birds in egg production. A common example is swine-like influenza in turkeys. For turkey breeders in production, infection can cause severe drops in egg production, but for flocks not in production the birds often seroconvert with no clinical signs of disease [6, 34, 45, 155]. Increases in mortality have also been seen in layers with egg yolk peritonitis after LPAIV infection, which are not seen in immature birds [200].

Antigenic drift and shift

IAVs have two primary mechanisms to provide diversity in the viral population, namely a high mutation rate and the ability to reassort gene segments [86, 174]. Both methods provide an opportunity for the virus to rapidly change and adapt, which contributes to the ability of the viruses to establish infections in new host species. The ability to rapidly mutate and adapt is not unique among the RNA viruses, but some viruses can tolerate higher levels of sequence changes in at least some viral genes. IAVs, as has been previously described, can differ greatly in amino acid sequence, particularly in the surface glycoproteins, hemagglutinin and neuraminidase [95]. These differences in amino acid sequence result in differences in antigenicity, such that antibodies to H1 IAV will neutralize only H1 viruses, and not any other subtype of IAV. These antigenic differences

have major implications for vaccination, since vaccine protection is mediated primarily by specific antibodies being produced to the hemagglutinin protein, and to a lesser extent to the neuraminidase protein [77]. Therefore current vaccines are limited to providing only subtype protection, and to provide complete protection from IAV would require the addition of 16 different antigens representing each HA subtype.

Although neutralizing antibodies to one HA subtype of influenza should neutralize all viruses within the same subtype, differences in the specificity of the antibody greatly affect the level of protection observed. The impact of antigenic drift on vaccination with human influenza is a well-characterized problem that requires the vaccine seed strain to be evaluated every year to try to achieve the best possible match with the circulating strain [136]. Two different subtypes of IAV are endemic around the world in the human population, namely the H1N1 and H3N2. For both subtypes of virus, a single lineage of virus is present that can be traced back to the time when the virus was introduced to the human population [17, 18, 40]. Unlike what we see with animal influenza viruses, which will be described in more detail later, these two subtypes of virus have evolved with little difference in sequence based on geographic origins of the virus. This worldwide distribution is likely to be the result of widespread and rapid movement of humans between regions that efficiently transmits the virus and that allows only relatively minor variants of the virus to circulate at the same time. However, the viruses do change at a rapid and predictable rate, sometimes called a molecular clock [17]. The observed changes in the genome are not random, but are concentrated primarily in the surface glycoproteins [116]. Influenza viruses, like other RNA viruses, lack a proofreading mechanism in the replication of viral RNA, which results in errors in transcription leading to a high mutation rate [103]. The high mutation rate provides the opportunity for change, but many of the changes introduced by this error-prone transcription are deleterious to the virus, because it creates premature stop codons, changes in amino acids so the virus is less fit, or changes in a regulatory signal that affects virus replication [118]. Most of the deleterious mutations are lost during the selection process to achieve the fittest virus in a population.

The mutation rate for all eight gene segments is probably the same, but because of positive selection, more changes in the HA and NA genes are conserved [116].

One of the primary selective factors on the HA protein is thought to be antibody pressure from the host, either from previous exposure to the virus or by vaccination [116]. For the human IAV H3 protein, five antigenic regions have been characterized where antibody to these regions can be neutralizing to the virus and therefore would be protective for the host during infection. These antigenic regions are on the globular head of the HA protein, with many close to the receptor binding site [182, 184, 185]. Antibodies to the antigenic sites can be neutralizing because they directly block access to the receptor binding site and prevent the virus from attaching to and initiating infection in the host. These antigenic regions, however, can tolerate a significant amount of amino acid diversity, and when changes to key amino acids occur, one of the neutralizing epitopes may be changed so that antibodies can no longer bind [182]. These changes in specificity of the antibody can result in a virus being better able to escape the ability of the host's antibodies to control infection, resulting in greater virus replication and transmission of these escape mutants. The accumulation of these amino acid changes at these antigenic sites is the antigenic drift that results in vaccines for IAV being less protective over time. For humans, the influenza vaccine seed strains, both IAV and influenza B virus, are evaluated yearly to determine whether the currently circulating field strains are still neutralized effectively by antibody produced to the vaccine strain. Comparison of virus sequence is used to identify when new viral variants are occurring and at what frequency [136]. From the sequence information, representative strains are used to produce antibodies to do more in-depth cross-hemagglutination inhibition (HI) studies. If the field strains in the cross-HI studies show a fourfold or greater difference in inhibition, this is evidence that the current vaccine seed strain may be ineffective. As the amount of HI data has increased, the use of computer programs to generate maps of antigenic differences, commonly referred to as antigenic cartography, has become common for both human and veterinary medicine [2, 137]. Vaccination for human influenza requires a close match of vaccine

to field strain, or protection from vaccination is adversely affected [48]. Antigenic differences of more than fourfold appear to be the range where the decrease in antibody specificity affects the protection seen from vaccines. The seed strains are typically changed every 3 to 4 years to compensate for this antigenic drift [136].

For poultry, antigenic drift also occurs, but the interpretation and importance of antigenic drift are much more complicated. The principles of changes at antigenic sites affecting the specificity of neutralizing antibody are the same for the immune response in poultry, but the trigger for when antigenic change necessitates a vaccine change is not defined. In part this is a difference in the pathobiology between influenza in humans and HPAI in chickens. With human influenza, viral infection is a mucosal infection of the respiratory tract, and with HPAI, the virus has both systemic and mucosal replication. Killed vaccines, which are commonly used in humans and poultry, provide high levels of serum IgG (or IgY, the avian counterpart to mammalian IgG) antibody, but little if any secretory IgA, which is the most effective antibody for the control of influenza in experimental mouse models [120]. The transudation of IgG (IgY) that crosses the mucosal surface can provide effective control of clinical disease, but it does not provide ideal protection [166]. In chickens with LPAIVs and for replication of HPAIVs on the mucosal surface, a similar immune response probably occurs. However, the severe clinical disease seen with HPAIV infection is primarily from the systemic replication of the virus, and subtype-specific antibody appears to efficiently block viremia and therefore the systemic replication of the virus [77]. The serum antibody protection appears to be affected less by antigenic drift in its ability to block viremia and prevent severe clinical disease, but it has been shown previously that the level of virus shedding is correlated with the relatedness of the vaccine to challenge strain [76, 162].

An additional concern with AIVs is the wide diversity of viruses that can infect poultry. Since most outbreaks of LPAI and HPAI result from independent introductions of viruses from the diverse wild bird reservoir, most epidemiologically unrelated outbreaks are antigenically different from each other even within the same subtype [38, 75]. This antigenic diversity, as described earlier, is

broken down generally into North American and Eurasian lineages, and the selection of a vaccine seed strain should at a minimum consider matching the HA amino acid sequence as closely as possible to try to obtain the best protection and reduction in shedding [162]. However, many different factors are involved in vaccine seed strain selection.

One additional complication with AIVs and other animal influenza infections is that if an outbreak becomes widespread, geographic separation of viral populations can occur because of limits on the movement of animals and animal products that allows separate evolutionary paths to occur. The geographic separation has been observed with several outbreaks, including H5N2 LPAI in Mexico, H9N2 LPAI in the Middle East and Asia, and the H5N1 HPAI outbreak in Asia, Europe, and Africa [76, 187, 193]. The issue of different HA lineages again complicates vaccine selection, since antigenic drift can occur within a clade or lineage. The current A/goose/Guangdong/1/1996 lineage of H5N1 HPAIVs has separated into multiple lineages of virus described in a clade system based primarily on sequence differences, although this does translate into antigenic changes as measured by hemagglutination inhibition tests. Antigenic drift continues such that fifth-order clades are now defined. For example, the 2.3.2.1 viruses that emerged are now further defined based on sequence differences to 2.3.2.1a, 2.3.2.1b, and 2.3.2.1c [1]. Because of the antigenic differences between different lineages of viruses, China has been using surveillance information to target vaccination with updated reverse-genetics-based vaccines [80].

For long-lived animals, an additional concern with influenza infection is antigenic shift. Antigenic shifts are typically considered for human IAV, but have also been seen in animal IAV. Antigenic shift occurs when a large proportion of the host population has previous exposure, by either infection or vaccination, with a particular HA subtype, and then they become exposed to a different HA subtype [30]. Because the host population has little or no protective immunity to the new virus, it can rapidly spread in the new population, causing a widespread and sometimes severe outbreak of influenza called a pandemic. In the human population, four major pandemics occurred in the last century. The most severe was when an H1N1 virus emerged, probably replacing an H2 human influenza, in 1918, and

resulted in a major pandemic that killed over 40 million people [168]. The second pandemic of the century occurred in 1957, when the H1N1 virus was supplanted by an H2N2 virus. The third pandemic started in 1968, when an H3N2 virus supplanted the H2N2 virus [190]. The most recent pandemic was H1N1 influenza, which emerged in 2009. This virus, although the same subtype as the circulating seasonal H1N1 virus, was antigenically different enough to spread rapidly in the human population, and eventually supplanted the old H1N1 virus from circulation in humans [138]. The origins of new pandemic viruses generally are not clearly understood, although it appears that they can be caused by a completely new IAV being introduced into the human population or by a reassortment event between the circulating human strain and another animal IAV [190]. The 1918 H1N1 virus appeared to be a completely new virus, but the H2N2 and H3N2 viruses were reassortant viruses that changed multiple genes, including, most importantly, the HA gene [190]. The 2009 pH1N1 virus was closely related to SIV circulating in North America, but a reassortment event with an unknown virus contributed two other genes that allowed the virus to replicate and transmit well in humans [138].

The best example of antigenic shift in veterinary medicine is that of EIV. Historically, horses had been infected with an H7N7 subtype IAV that appeared to have circulated in horse populations for a long period of time. In 1963 a new subtype emerged, H3N8, which infected horses worldwide, and eventually completely replaced the historic H7N7 IAV, with the last isolate of that subtype being obtained in 1979 [24, 102]. For swine in the USA, H1N1 was primarily the only strain of influenza that circulated from 1918 to the late 1990s. However, starting in 1998, H3N2 viruses began to be isolated in the USA. These viruses were an unusual reassortant that had H1N1 SIV-like genes, human influenza virus-like genes, and AIV-like genes. The H1N1, H3N2, pH1N1, and even other reassortant viruses (H1N2 and H3N1) currently co-circulate in the USA [56, 57]. Because of the antigenic shift, vaccines for horses and swine needed to be updated to include the new viruses in order to achieve adequate vaccine protection. However, vaccine companies have not been very proactive about updating vaccines, in part because of regulatory concerns, and

many equine vaccines include H7N7 as an antigen, although it has not circulated for over 35 years.

For poultry, antigenic shift has not been a major issue because of the short production lives of most commercially produced poultry. Because infection with AIVs had been uncommon, commercial poultry were not naturally exposed, and vaccination is still not widely practiced except against H5N1 HPAIV in China, Egypt, Indonesia, Vietnam, and Bangladesh. Therefore most poultry are completely susceptible to infection with any influenza subtype. Further details about avian influenza vaccines are provided in Chapter 15.

Conclusions

Influenza remains a major health issue for poultry, swine, and equine populations around the world. The biggest concern for poultry has been HPAIV infection, because of severe clinical disease and the negative impact on trade. However, LPAIV infections also remain a concern because they are able to cause disease and production losses, they occur more widely than HPAIVs, and for the H5s and H7s LPAIVs there is the ever present threat of mutation to HPAIV. AIVs are difficult to control because of the wildlife reservoir, the adaptability of the virus, and the lack of good control tools. The SIV issue continues to grow more complex as rampant reassortment of swine and human IAV makes control through vaccination difficult. EIV also continues to change antigenically, although only two major lineages currently circulate. However, current vaccination tools do not provide long-term protection, and in general remain poorly antigenically matched because vaccines are not updated appropriately. Efforts to increase our understanding of the virus and research to develop new methods for control should be a priority for the veterinary community.

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