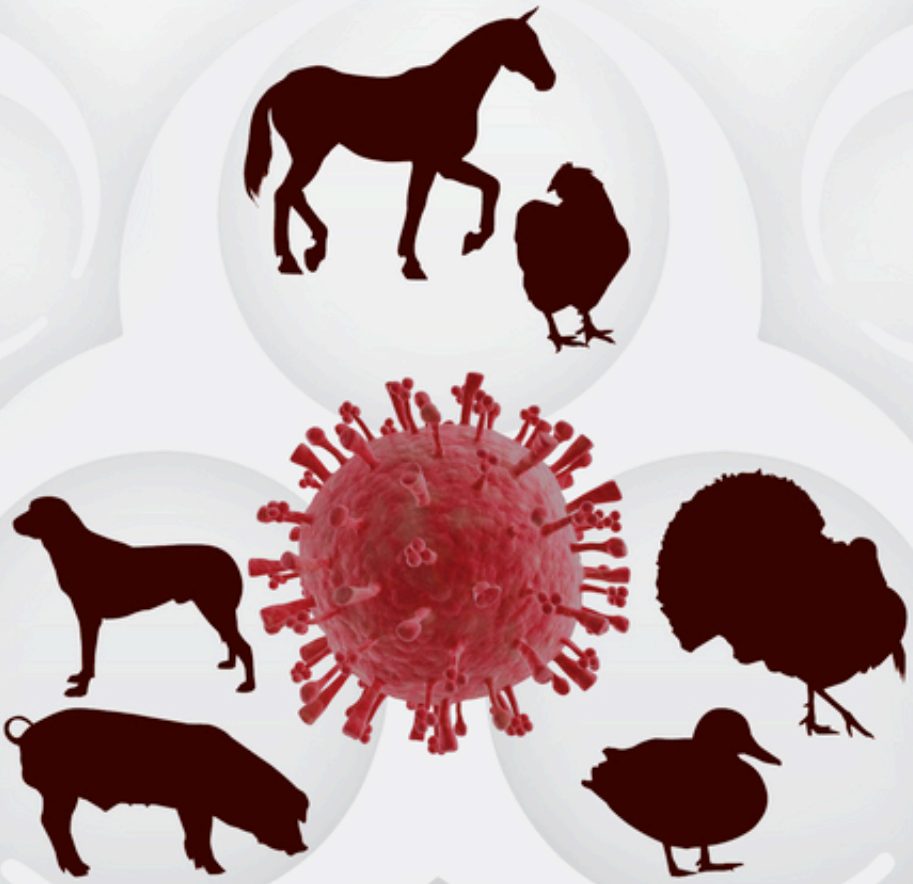
ANIMAL INFLUENZA

SECOND EDITION



Edited by **DAVID E. SWAYNE**

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Animal Influenza

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Second Edition

EDITED BY

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This book is dedicated to the veterinarians and veterinary scientists whose long and distinguished careers focused on studying influenza for the benefit of animal health, and whose discoveries have laid the scientific foundation for all who have followed in the concept of "One World, One Health" for influenza. This book is dedicated to Dennis J. Alexander, Charles W. Beard, Bernard (Barney) C. Easterday, David A. Halvorson, Yoshihiro (Yoshi) Kawaoka, Hans D. Klenk, Rudolph Rott, Werner Schäfer, Richard Slemons, David Stallknecht, and Robert G. Webster.

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Foreword

Over the last two decades, influenza has arguably become the most important disease of poultry, attracting attention from researchers and grant-awarding institutions around the world. Current journals are filled with new findings on influenza. In this respect, this disease resembles Marek's disease (MD) in the 1960s, a challenging time when MD threatened the very existence of the poultry industry. It is fitting, therefore, that a volume dedicated to the study of influenza should be made available to the increasing number of workers in this field. Such a volume will serve to distill current knowledge and present it with an appropriate historical perspective.

As the successor to the earlier text on avian influenza, the present volume has continued its focus on avian species, but with an important expansion to encompass influenza in several important mammalian species. The long history of avian influenza (AI), unlike many other diseases, is reflected in a series of distinct outbreaks or epizootics, each of which is not only unique but also a rich source of information. Each provides lessons by which knowledge is expanded and strategies for control can be improved, justifying a systematic and detailed analysis. The focus is understandably on epizootics caused by high-pathogenicity AI (HPAI) viral strains, and such epizootics have numbered more than 37 since 1959. One of them, involving H5N1 HPAI originating in South-East Asia, has spanned more than a decade and involved multiple animal species and many countries worldwide. It is outbreaks such as this that command the attention of veterinarians, virologists, epidemiologists, and public health specialists, as well as the poultry and animal industries and the general public.

Meanwhile, influenza in swine, horses, and dogs has attracted new interest as certain influenza viruses from both avian and mammalian species have shown a propensity to infect humans. The expansion of this volume to encompass a wider range of host species meshes well with the "One World, One Health" initiative, which recognizes the synergy embodied in a multidisciplinary and multifaceted approach to the study of disease.

This text brings together in a comprehensive manner the knowledge and experience accumulated during more than a century of research and observation of influenza in animal species. The list of authors is impressive and distinctly international. The emphasis on avian influenza is retained and updated, and the nine new chapters on influenza in mammalian species are complemented by the five introductory chapters that deal with both mammalian and avian species. Thus this edition is in some respects totally new, and in other ways represents a logical continuation and updating of the information on avian influenza that was so aptly detailed in the previous edition. Like its predecessor, this book will surely become the major reference source in its field.

The publication of this volume has been sponsored by the American Association of Avian Pathologists, an organization that has long supported the publication of information relevant to poultry medicine. This book continues the tradition of excellent, science-based educational publications produced by this organization.

Like the previous edition, this volume was conceived and edited by Dr. David E. Swayne, who has devoted more than a quarter of a century to the study of avian influenza, and leads the Southeastern Poultry Research Laboratory, Agricultural Research Service, US Department of Agriculture, in Athens, Georgia, which has been a strong contributor to knowledge in this field. Dr. Swayne is a world authority on avian influenza pathobiology and vaccination in poultry, and his work has facilitated the application of vaccines and diagnostic tests used worldwide. He has not only amassed critical knowledge but also translated this into international policy to improve food safety and to protect the USA from HPAI infection. Drawing

on his considerable experience as a pathologist, researcher, international consultant, research leader, and editor, he has personally authored three of the present chapters and contributed to two others as a co-author.

This book will be valued by veterinarians, researchers, and regulatory officials who deal with influenza in avian and mammalian species, and will also assist public health officials in understanding the animal health aspects of this important and complex disease, which will surely pose a continuing threat to animal agriculture and human health.

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Preface

Avian Influenza (2008), the predecessor of the current textbook *Animal Influenza* (2016), was published as a first edition with the intent of periodic updating through successive editions. The impetus for *Avian Influenza* was the emergence of the H5N1 Goose/Guangdong (Gs/GD) lineage of high-pathogenicity avian influenza (HPAI) from the late 1990s to the mid-2000s, which not only caused infections and deaths among poultry in over 60 countries of Asia, Europe, and Africa, but also resulted in infections and deaths among wild birds and numerous mammalian species, including humans. Such an HPAI epizootic involving a large number of animals and dispersion over a wide geographic area had not been seen since the 1920s and 1930s, when fowl plague was endemic or was causing epizootics among poultry in Europe, Asia, Africa, and North and South America. Between 1959 and 2008, 28 epizootics of H5 or H7 HPAI had been reported, with the Gs/GD-lineage H5N1 being larger than all the other 27 epizootics combined, and it was justified in having the designation of a veterinary or agricultural panzootic.

Since the first edition of *Avian Influenza* was published in 2008, H5 or H7 HPAI has caused 13 additional epizootics, and the Gs/GD-lineage H5 HPAI has continued, cumulatively affecting over 70 countries, including outbreaks in North America during 2014–2015, and resulting in deaths or culling of over 500 million poultry. The continuing significance of HPAI has necessitated an update on avian influenza. However, the emergence of a human H1N1 pandemic in 2009, caused by a reassortant influenza A virus with gene segments most closely related to human seasonal influenza, swine influenza, and avian influenza viruses, as well as the identification of cross-transmission of human and swine influenza viruses between humans and pigs in the USA, the emergence of H3N8 influenza in dogs, and the emergence of equine influenza in Australia, has solidified the idea of expanding the *Avian Influenza* text, and its renaming as *Animal Influenza,* for a second edition. Specifically, the first five chapters were broadened from avian influenza alone to more generic animal influenza information. Chapters 6 to 15 were updated with information specific to avian influenza, and Chapters 16 to 24 were added to provide new information about swine influenza, equine influenza, canine influenza, and influenza in other mammalian species.

Both *Avian Influenza* (2008) and *Animal Influenza* (2016) were commissioned by the American Association of Avian Pathologists (AAAP), a non-profit educational foundation whose mission is to promote research and apply such new knowledge to solving avian health problems, which includes providing educational resources to avian veterinarians and health professionals around the world. The authors and editor of this book have received no financial compensation from the sale of this book, but we do acknowledge the valuable professional satisfaction of helping colleagues around the world and advancing the discipline of poultry medicine. All profits have been used to further the educational programs of the AAAP, including donations of educational materials to developing countries.

As editor, I wish to extend special thanks to Anita J. Swayne, my wife, whose patience and encouragement made possible the long journey of this book from idea to reality. I also thank the Board of Directors of the AAAP for commissioning this text, and several colleagues for providing anonymous critiques and reviews of some chapters to ensure accuracy. The highly skilled and professional assistance of John Wiley & Sons, especially of Nancy Turner, Melissa Wahl, Catriona Cooper, and Susan Engelken, over the past three years is much appreciated. Finally, I personally thank Dr. Richard D. Slemons, Dr. Charles W. Beard, and Dr. Max Brugh for introducing me to the exciting world of influenza research, and for their continual career guidance and mentoring, which has made the past

29 years of researching influenza viruses and the diseases that they cause a daily, fun adventure.

Any mention of trade names or commercial products in this book is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the authors. The content of individual chapters is based upon the scientific literature and the knowledge and experience of the individual authors, and is not the official position of the United States Department of Agriculture or other employers of the individual authors.

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SECTION I

Common aspects of animal influenza

1 Influenza A virus

David L. Suarez

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 Quaranfilvirus [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 Quaranfilviruses 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

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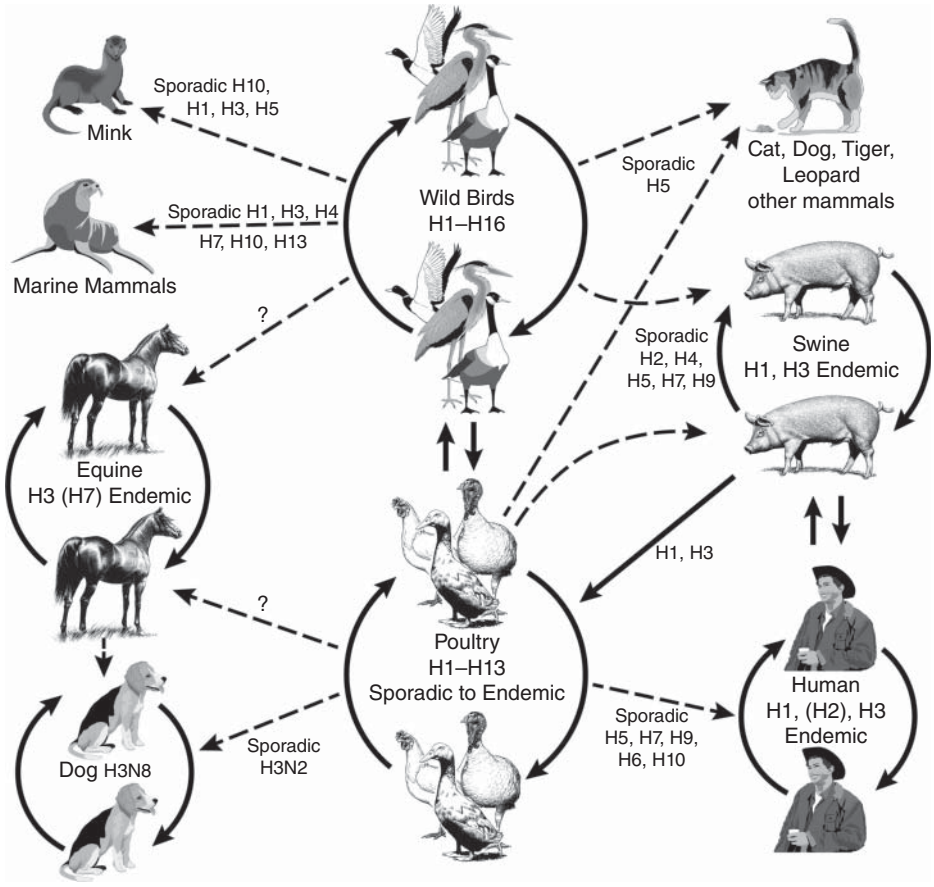

Figure 1.1 Diagrammatic representation of the source and movement of influenza A viruses or their genes within avian and mammalian ecological and epidemiological situations (updated from [160]). H = hemagglutinin subtype, ( ) = subtype previously common but no longer circulating. Source: K. Carter, University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

[c] Both LP and HP viruses.

[d] Rare subtypes.

[e] Primarily 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

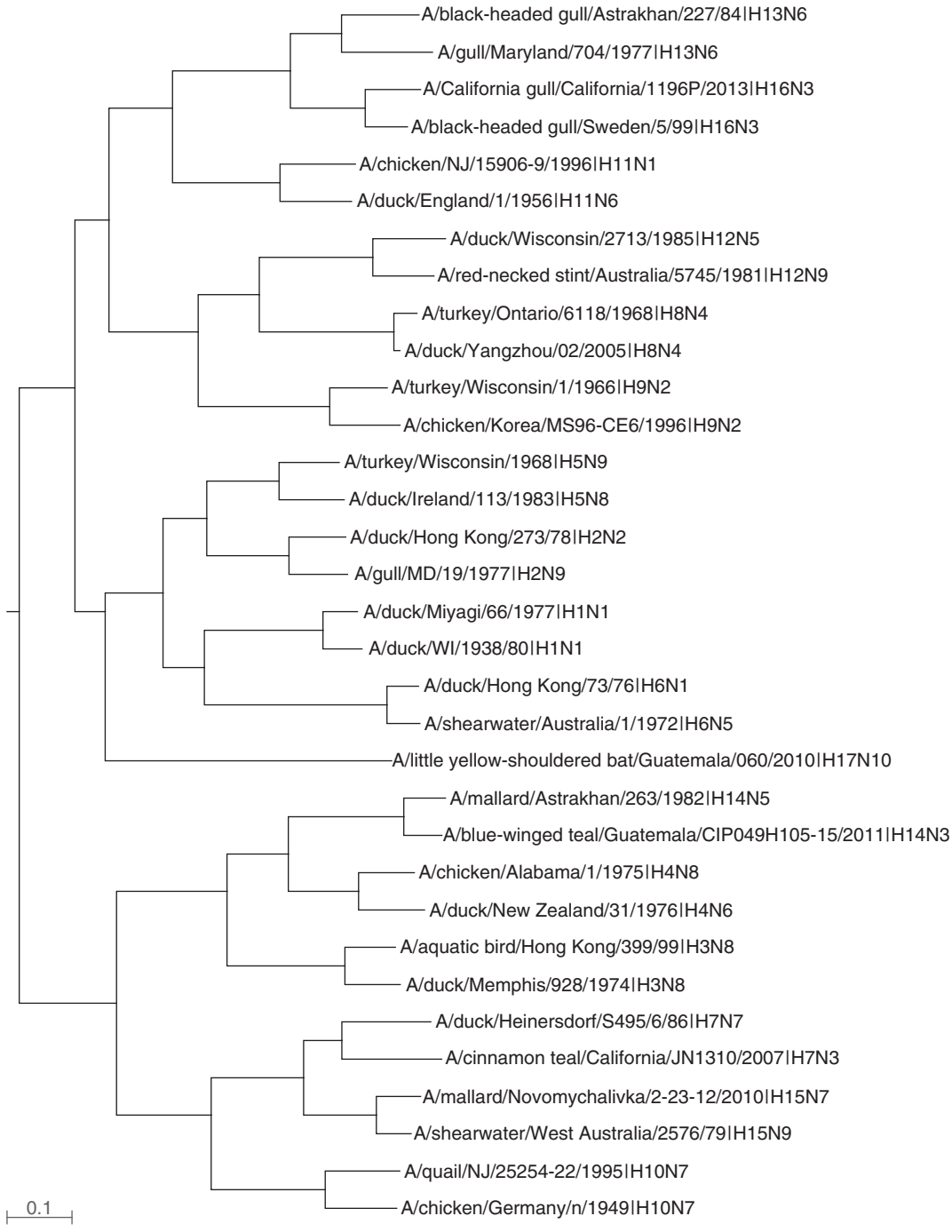

Figure 1.2 Phylogenetic tree of 17 hemagglutinin subtypes. The complete amino acid sequence of representative isolates for all 16 avian HA subtypes and the H17 bat subtype are included, with a representative North American and Eurasian isolate where available. The tree was midpoint rooted using the Influenza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

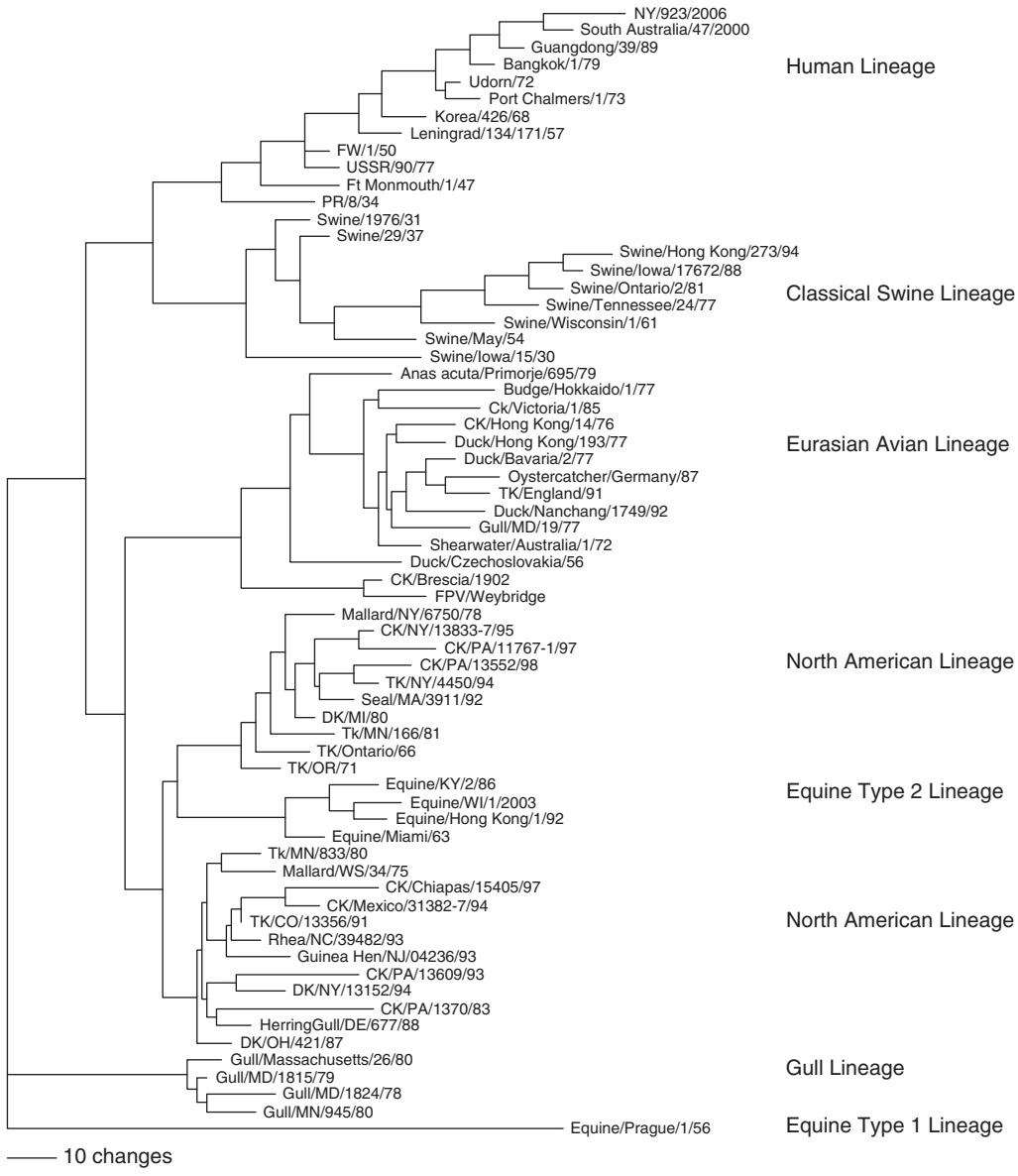

Figure 1.3 Phylogenetic tree of the matrix gene. The tree is based on the complete nucleotide sequence of representative isolates for major groups of type A influenza viruses. The tree is rooted to equine/Prague/1/56, which is the most divergent type A influenza virus. CK = chickens, DK = ducks, TK = 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 . . . .RKRKTR*GLF	X	X				[128]
A/chicken/PA/1370/1983	H5N2	HP	PQ …….. KKKR*GLF	X				X	[128]
A/tern/South Africa/1961	H5N9	HP	PQRETRRQKR*GLF	X		X			[128]
A/chicken/Puebla/8623-607/1994	H5N2	HP	PQ. … RKRKTR*GLF	X	X				[37, 49]
A/chicken/Queretaro/14588-19/1995	H5N2	HP	PQRKRKRKTR*GLF	X	X				[37]
Typical H7 LPAI	H7	LP	PEIP ……. . . .KTR*GLF						[128]
A/chicken/Victoria/1985	H7N7	HP	PEIP . . . ….KKREKR*GLF			X			[128]
A/turkey/Italy/4580/1999	H7N1	HP	PEIPKG . . . .SRVRR*GLF			X			[19]
A/chicken/Chile/176822/2002	H7N3	HP	PEKPKTCSPLSRCRETR*GLF[b]				X		[154]
A/chicken/Canada/AVFV2/2004	H7N3	HP	PENPK … QAYRKRMTR*GLF[c]				X		[107]
A/chicken/Saskatchewan/HR-00011/2007	H7N3	HP	PENPKTTKPRPRR*/GLF[d]				X		[13]
A/chicken/Jalisco/12383/2012	H7N3	HP	PENPKDRKSRHRRTR-GLF[e]				X		[54]

[a]Mechanisms: (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.

[b]30 nucleotides from nucleoprotein of same virus gene coding 10-amino-acid insert.

[c]21 nucleotides from matrix of same virus gene coding 7-amino-acid insert.

[d]18 nucleotides from unidentified chicken gene coding 6-amino-acid insert.

[e]24 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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2 Diagnostics and surveillance methods

Erica Spackman, Giovanni Cattoli and David L. Suarez

Introduction

Detection and diagnosis of influenza A virus (IAV) infection in animals require a laboratory test since disease from IAV presents no pathognomonic signs. Diagnosis and surveillance of animal influenza focus on the detection of virus or type-specific antibodies. Whether one targets the virus or antibodies in testing depends on the goals of the testing. Further characterization of an isolate or antibody specimen may be undertaken in order to define the subtype or other biological features. The specific tests that are employed will vary depending on the species, the goals of testing, and the resources available.

Reflecting the importance of IAVs both for domestic animals and for public health, numerous diagnostic tests have been reported in the literature and are commercially available. In fact IAV is frequently used as the proof-of-concept agent for new diagnostic technology. In addition, because of the importance of IAV, some harmonization of diagnostic and detection methods has been established within certain species and domestic animal groups (e.g. poultry, horses). Standardization of testing methods for poultry is often undertaken at an international level (e.g. World Organization for Animal Health, also known as OIE) or at a regional or national level (e.g. federal government-issued guidelines, National Poultry Improvement Plan in the USA). In contrast, there is sometimes less guidance available for other species. Standard operating procedures and details of the established and most important validated diagnostic methods can be found in a number of references [10, 48, 61, 72] and on the OIE web site (www.oie.int).

Sample types

The type of sample and the processing methods are dependent upon numerous interrelated factors, such as the purpose of testing, the type of tests used, and the target species. It is not uncommon for a single sample to be tested by more than one assay, particularly when the results of a screening test, such as antigen capture immunoassays (ACIAs), must be confirmed by a second, more sensitive test, such as real-time reverse transcription polymerase chain reaction (rRT-PCR) or virus isolation (VI).

Oropharyngeal (or tracheal) swabs and cloacal swabs are the most widely used specimen types for avian species, although tissues are also collected in some cases. Tissues are not optimal for detection of low-pathogenicity (LP) avian influenza virus (AIV), but trachea and lung are recommended if tissue collection is undertaken. Numerous tissues may be collected for high-pathogenicity (HP) AIV, including lung, brain, heart, kidney, and spleen.

Oropharyngeal swabs, which include swabbing of the choanal cleft, are preferred to tracheal swabs for the following reasons: (1) material from the sinuses where the virus replicates is captured from the choanal cleft; (2) these swabs are less invasive and there is not a risk of causing damage to the trachea; (3) less skill is required, as the esophagus is easier to swab and can be confused with the trachea by untrained individuals. A study using rRT-PCR on specimens from experimentally infected animals has shown that oropharyngeal and tracheal swabs are equivalent for detection of influenza from avian species [69].

In most cases the optimal approach is to collect both oropharyngeal and cloacal swabs. Although

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the tropism of AIV for the respiratory or enteric tract is often species specific, there are some strain-dependent exceptions, depending on how a lineage is adapted to a particular species. The general rule is that LPAIV in waterfowl (either domestic or wild) will have a higher tropism for intestinal replication, and therefore more virus will be shed by the cloacal route, resulting in better detection from cloacal swabs [2, 60, 73]. Conversely, in gallinaceous birds, including chickens and turkeys, LPAIV typically has respiratory tract tropism, so it is best to use oropharyngeal or tracheal swabs to collect infectious virus. Importantly, there are insufficient data from many other avian species (pigeons, gulls, shorebirds, etc.) to allow unequivocal recommendation of the use of one swab type or the other; therefore both should be collected. A recent example of an exception to the respiratory–gallinaceous and intestinal–waterfowl tropism rules of thumb is the 2013 lineage of H7N9 viruses from China that replicated well in the upper respiratory tract of both gallinaceous birds and waterfowl. Therefore, when undertaking surveillance for this lineage, oropharyngeal or tracheal swabs are the recommended sample for waterfowl as well as for chickens [49].

More generally, several studies have shown that maximal sensitivity in a population can be achieved by collecting and testing both oropharyngeal (or tracheal) swabs and cloacal swabs, although many investigators do not consider that the increased number of positive samples justifies the greatly increased cost of sampling [33]. However, an approach that has been adopted with wild bird samples involves placing both swab types in the same tube. This approach has been shown to increase the number of positive samples compared with cloacal swabs alone in two independent studies [30, 50].

Since the tissue tropism of IAV tends to be consistent for the respiratory tract in mammals, the optimal samples from mammalian species (swine, horses, and dogs) are nasal swabs. Oral fluids have also been shown to be effective for detecting IAV in swine herds [18, 28, 56]. As is the case for birds, lung tissue may also be used in post-mortem sampling.

Pooling of swab samples by placing numerous swabs in the same tube at the time of collection can help to reduce costs by consolidating samples,

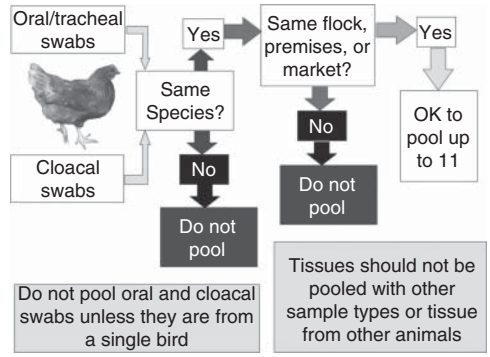

Figure 2.1 Swab pooling for specimens from avian species. Up to 11 oropharyngeal/tracheal swabs or cloacal swabs may be pooled per vial if they are collected from the same species and the birds are housed together as part of the same flock or at the same location or market.

but samples from different species and groups of animals should not be pooled (Figure 2.1). Tissues from different animals should not be pooled because if one animal has developed antibodies they can neutralize the virus in tissues from other animals if the two samples are processed together. In addition, tissues should not be pooled with swab material. Pooling of material later, in the diagnostic laboratory, can dilute positive samples and increase the risk of cross-contamination, and is generally not recommended. Up to 11 oral swabs from experimentally infected chickens have been successfully pooled for both rRT-PCR and VI [35, 64], and up to five swabs with fecal material from mallard ducks have been successfully pooled for rRT-PCR detection in a study using spiked swabs [26].

Sample collection, transport, and storage

The correct procedures for sample collection, transport, and storage are critical for obtaining accurate test results. Swab samples should be collected in a well-buffered, salt-balanced medium containing protein (e.g. brain–heart infusion broth or tryptose phosphate broth) [23, 64]. Influenza is not as stable in salt buffers without protein, such as phosphate-buffered saline, and the sensitivity of virus isolation will be reduced [23, 64]. Samples should never be transported dry, as this has been shown to reduce the sensitivity of rRT-PCR as well as that of VI [55, 64]. Antibiotics may be added to

transport media unless there is a need to test for bacterial agents.

During transport it is important to maintain the cold chain. Ideally, samples should be transported to the diagnostic laboratory within 24 hours of collection, and should be kept at refrigeration temperatures (approximately 4°C). Wet ice may be used to keep the samples cool. Freezing should be avoided, as freeze–thaw cycles will degrade RNA and viable virus. Swab material has been shown to be stable for up to 14 days at 4°C, while freezing for the same period reduced virus detection by rRT-PCR [25, 46].

The metadata associated with a sample are a key part of sample collection. The date of collection, type of sample, location (including global positioning system (GPS) coordinates when possible), clinical condition of the animal(s), age of the animal(s), species, and vaccination status (for domestic animals) should be recorded. This information should be kept with the sample. When collecting samples from wild birds for AIV surveillance, it is important to use the scientific name of the species, as common names can be regional and may not be recognized universally. Historically, countless samples have been labeled "duck", which is insufficient to improving our understanding of IAV biology, as there are numerous species of ducks, with highly variable habitats, migration routes, and genetics.

Since transport of diagnostic samples can be difficult due both to shipping regulations for potentially infectious material, and because the cold chain must be maintained, in situations where only molecular methods (e.g. rRT-PCR, sequencing) will be used, Flinders Technology Associates (FTA) cards (Whatman-GE Healthcare and Bio-sciences, Pittsburgh, PA) may be utilized. Liquid samples may be blotted on this specially manufactured paper card, and once the sample dries the virus will be inactivated and the RNA will be preserved. The card can then be transported with fewer shipping restrictions than samples which may contain live virus. Avian influenza virus RNA has been shown to remain intact for 5 months on FTA cards at ambient temperatures [1], although the sensitivity will be lower than if swab material is used directly [1, 31, 32]. An alternative preservation method for viral RNA that does not require the cold chain involves collecting cloacal swabs from wild birds in 100% ethanol for screening by rRT-PCR [57]. Although this was found to be successful for rRT-PCR, paired swabs needed to be collected in a traditional viral transport medium and maintained at low temperatures to attempt virus isolation [57], so this method is not suitable if virus isolates are needed. Another drawback is that 100% ethanol must be shipped as a flammable chemical. Other commercially available transport media will inactivate samples for ambient-temperature long-term storage, but in the absence of controlled scientific comparisons with avian samples, these products cannot currently be recommended.

Virus detection

Virus detection to identify an active infection can be achieved by attempting VI or by using ACIAs (Table 2.1). Alternatively, viral nucleic acids can be targeted by molecular assays (e.g. rRT-PCR) (Figure 2.2). Typically, rRT-PCR or ACIAs are used to screen samples, and then virus isolation is used to confirm the results.

Virus isolation

The reference standard for the diagnosis of IAV is VI, and although other methods may be used to make a presumptive diagnosis, VI is necessary to confirm the presence of virus in an index case and to undertake further characterization of the virus. The embryonated chicken egg (ECE) from a specific pathogen-free flock (or a flock that is negative for IAV or for IAV antibodies) is considered to be among the most sensitive host systems for the isolation of both avian and mammalian IAVs. Madin–Darby canine kidney (MDCK) cells are also widely used for the isolation of IAV from animal (avian or mammalian) specimens. Although IAV will replicate in other cell lines and in embryonating eggs from other avian species, ECE and MDCK are probably the most widely used systems. The choice of which of these is the optimal laboratory host system is dependent on the strain; some lineages will replicate only in ECE, some only in MDCK cells, and some will replicate well in either system.

One cannot always deduce which system is best based on the sample species of origin. For example, recent swine or swine-like viruses, including H3N2

Table 2.1 Characteristics of selected IAV diagnostic assays.

Assay	Target	Relative sensitivity	Relative specificity	Relative cost per sample	Time to result
Virus isolation	Viable virus	Very high	Moderate	High	1–2 weeks
Antigen detection immunoassays (commercial kits)	IAV protein	Low	High	Moderate	15 minutes
Real-time RT-PCR	IAV RNA	Very high	Very high	Moderate	3 hours
Agar gel immunodiffusion (AGID)	**1** Type A influenza virus nucleoprotein and matrix protein **2** Antibody to type A influenza nucleoprotein and matrix protein	Moderate	High	Moderate	48 hours
ELISA (commercial kits)	Antibody to type A influenza	Moderate	Moderate	Low	2–3 hours
Hemagglutination (HA) inhibition	**1** Identification of HA subtype **2** Antibody to a specific HA subtype	High	Moderate to high	Moderate to high	2 hours
Neuraminidase (NA) inhibition	**1** Identification of NA subtype **2** Antibody to a specific NA subtype	Moderate	Moderate to high	Moderate	3 hours

IAV = influenza A virus, RT-PCR = reverse transcriptase polymerase chain reaction.

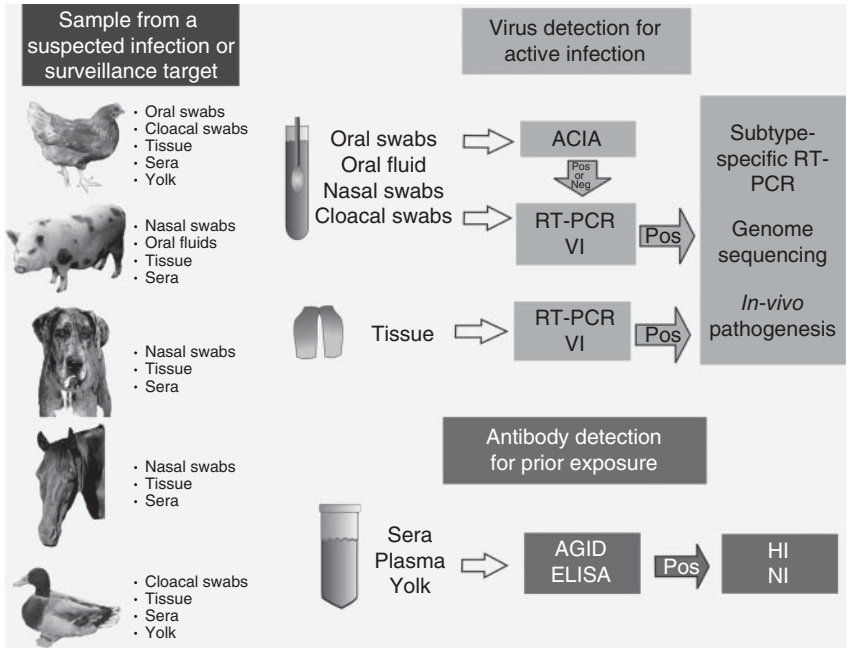

Figure 2.2 Outline of common approaches to influenza A virus (IAV) diagnostic testing. Active virus infection may be detected from swab material, oral fluids, or tissue by antigen capture immunoassay (ACIA), which needs to be confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) or virus isolation (VI), or may be directly tested by RT-PCR or VI. Positive samples and isolates are further characterized by subtype-specific RT-PCR and/or genome sequencing and, in some cases, *in-vivo* pathogenesis studies. Exposure to IAV may be evaluated by antibody detection by agar gel immunodiffusion (AGID) or enzyme-linked immunosorbent assay (ELISA). The subtype specificity of positive samples may then be determined by hemagglutination inhibition (HI) assay or neuraminidase inhibition (NI) assay.

isolates, and pandemic H1N1 lineage viruses may not grow efficiently or at all in ECE, so MDCK cells are preferred. Because turkeys can be infected with swine influenza viruses (SIVs), turkey samples are commonly processed for VI with both ECE and MDCK cells [63]. Numerous other cell lines will support the replication of IAV, but are not so widely utilized.

A limited number of comparisons between ECE and MDCK cells have been reported with samples from different host species. For samples from wild birds, the ECE system is apparently more sensitive, and titers were higher in ECE with samples that replicated in both systems [43, 44], but the data are less clear for samples from domestic poultry and domestic mammals, and MDCK cells are preferred for swine samples [81]. ECE and MDCK cells are considered to be similar in sensitivity for equine and canine samples [12, 20], although some canine isolates may grow preferentially in one system [20]. In some cases, both methods may be used. For example, samples from turkeys which are expected to contain swine influenza are often processed into both ECE and MDCK cells at some reference laboratories.

Other differences between the systems have been noted, such as cost. MDCK cells are less expensive [43], and it has been observed that the equine influenza viruses undergo more selection in MDCK cells than in ECE, which could be important in some situations [29].

Because of the high sensitivity of VI, this method may be used to detect IAV during any stage of an active infection. Depending on numerous host- and virus-related factors, virus may be detected within 24 hours of infection in an individual bird, and for several weeks post exposure in a flock, herd, or population of animals [70]. To achieve optimal sensitivity with either system, it may be necessary to serially passage a specimen (often referred to as a "blind passage") two or three times, but this substantially increases the time it takes to complete the test, and it also increases the risk of false-positive results due to cross-contamination of samples.

Although VI is very sensitive, it is not highly specific or selective, because other agents that may be present in a specimen will readily grow in ECE or cell cultures. For this reason, additional tests on fluids from ECE or MDCK cultures are required to confirm the presence of IAV. Fluids from eggs or cell cultures inoculated with the test material are usually first tested for hemagglutination (HA) by a standard hemagglutination assay [73], or for IAV with an ACIA. With avian samples, an HA-positive sample is often tested by the hemagglutination-inhibition (HI) assay or rRT-PCR to differentiate AIV from other hemagglutinating viruses, most commonly avian paramyxovirus type 1 (i.e. Newcastle disease virus) in poultry specimens.

The presence of IAV in avian or mammalian samples can be confirmed by type-specific tests such as ACIA, agar gel immunodiffusion (AGID) assay for IAV antigen, and rRT-PCR tests on undiluted egg or cell culture fluids. Alternatively, the subtype of the isolate can be identified by HI assay and neuraminidase-inhibition (NI) assays or by gene sequencing. Gene sequencing is the most accurate method of identifying the HA and NA subtypes of IAV, as cross-reactions and false negative results are associated with serological tests and RT-PCR.

Despite the high sensitivity of culture methods for detecting IAV, there are some practical considerations that should be taken into account. First, VI is relatively expensive, and in the case of ECE is not easily scaled up because procurement and incubation of eggs have to be scheduled well in advance. Second, when performing VI the infectious virus can be amplified to a high level, significantly increasing the potential for cross-contamination among samples and exposure of laboratory personnel to infectious virus. For this reason, VI is generally performed in laboratories with enhanced biosecurity (e.g. BSL-3, BSL-3Ag, or P3), especially if it is suspected that the specimen contains HPAI (or any high-consequence pathogen). Virus isolation also requires a high level of technical skill in order to perform the procedure and interpret the results, because culture host systems can support the growth of many different agents, which can complicate diagnosis, as noted above. Furthermore, virus isolation is dependent upon the correct handling of specimens. If the samples are not collected, transported, and stored under the correct conditions, the sample can be degraded and may contain inactivated virions that could lead to false-negative results. Finally, VI has the longest time-to-result of any IAV detection test. The VI procedure may detect virus within 48 hours, but negative samples may take 1–2 weeks to complete, depending upon

the number of passages used and how quickly the virus grows to a high enough titer to be detected by HA or other methods.

Finally, there are some situations where VI is preferred over other methods, such as when it is important to determine whether viable virus is present (e.g. when confirming that cleaning and disinfection have completely inactivated all virus), or when it is necessary to evaluate the antigenic characteristics of an isolate (e.g. to reveal the occurrence of antigenic drift). Virus isolation will also remain in the core IAV diagnostic arsenal because isolates will always be needed for biological characterization.

Antigen capture immunoassays (ACIAs)

Numerous commercial type A influenza ACIA kits in lateral flow device (LFD) and enzyme-linked immunosorbent assay (ELISA) formats are available, but licensing for veterinary use varies among countries. Before any kits were licensed for veterinary use in the USA and elsewhere, kits for human diagnostic testing (Directigen Flu A test, Becton-Dickenson, Franklin Lakes, NJ) had been used successfully in poultry and other species [11, 22, 80]. Within the past few years, several kits have been licensed for use in different countries worldwide.

The LFDs use a monoclonal antibody directed against the highly conserved IAV nucleoprotein to bind viral antigen on a filter strip or membrane. The results can be visualized by the appearance of a band or pattern on the test strip or membrane following a chromatographic immunochemical reaction. Due to the immense interest in the recent Asian H5N1 HPAI virus, commercial H5-specific tests have been developed, and other similar kits may become available, but reports from the field indicate that the sensitivity and specificity are not high. Development of subtype-specific antigen detection tests is challenging because the monoclonal antibodies used in these tests must be directed to the highly variable HA antigen, making the test less reliable than IAV-specific assays.

The primary limitation of antigen detection kits is their low sensitivity. Most kits have an analytical sensitivity of approximately 10^4 to 10^5 mean embryo infectious doses (EID_{50}) [11, 80]. Since birds that present with clinical disease or which die from AIV infection are likely to shed higher levels of virus, they should be targeted for testing. Although clinically healthy birds may in fact be infected and shedding sufficient virus for it to be detected, the chances of obtaining a false-negative result are sufficiently high for the routine testing of clinical healthy birds not to be recommended. Also, it should be recognized that sick and dead birds can shed inadequate virus titers to be detected by ACIA (Spackman, unpublished data).

An effective surveillance approach for AIV, originally used in the 2002 H7N2 LPAIV outbreak in the USA, was to periodically sample 10 birds from the daily mortality on chicken or turkey farms in the surveillance zone in order to identify infected flocks [22]. The targeting of daily mortality has become a standard approach for surveillance of several respiratory diseases of poultry in the USA. Positive results from ACIA tests correlate well with those of other tests, but negative results from ACIA are not reliable and need to be confirmed by further testing.

Advantages of the ACIAs are that they are very rapid, producing results within 15–20 minutes, and highly specific. In addition, commercial antigen detection tests are convenient, self-contained, and easy to use. Therefore they are ideal for use on the farm as a "pen-side" test. Antigen detection tests are also used in diagnostic laboratories as a rapid screening test for IAV in clinical specimens, and for identifying suspect IAV isolates in VI material where titers are likely to be adequate for ACIA detection. The cost per sample of running the commercial antigen detection tests varies according to the manufacturer, but is less expensive than VI and of similar cost to rRT-PCR. It should be noted that the range of sample types that can be used with ACIAs is limited; most of these tests only accommodate tracheal, nasal, or oropharyngeal swab specimens. The tests are species independent, but few data are available to support their reliable use in off-label species, and negative results must be interpreted with caution.

Molecular/nucleic acid-based tests

In recent years, the application of molecular methods for the detection of viral nucleic acid has become an important tool for the detection of IAV and identification of HA and neuraminidase (NA) subtypes. RT-PCR based tests are the

most widely used molecular method, particularly real-time RT-PCR. Alternative amplification methods are also available, including nucleic acid sequence-based amplification (NASBA), an isothermic method for amplifying nucleic acids [14–16, 42], loop-mediated isothermal amplification (LAMP) [6], and insulated isothermal PCR (iiPCR) [5]. Although NASBA and LAMP are similar in sensitivity to rRT-PCR, these methods have not been as widely adopted as rRT-PCR, and iiPCR is too new for predictions to be made as to whether it will attain widespread use. Commercial NASBA, LAMP, and iiPCR kits are available for IAV and selected subtypes (e.g. H5 HPAIV, A(H1N1)pdm09). A report on the LAMP assay is available from the OFFLU website (www.offlu.net); however, full validation data have not yet been published.

Numerous rRT-PCR and conventional RT-PCR tests have been reported for the detection of IAV in poultry, swine, dogs, and horses [27, 37, 39, 40, 53, 54, 66, 68]. RT-PCR tests to identify important HA subtypes, often H1, H1 A(H1N1)pdm09-specific, H3, H5 and H7 [13, 45, 65] and N1 or N2 [74] have been reported. The recent Asian H5N1 HPAIVs have probably been the most targeted, with numerous reports of HA and NA subtype-specific tests [24, 47, 51, 79], although few of the reported tests have been field validated. Test procedures that are maintained by government and regulatory entities or international networks and organizations (e.g. OIE, USDA) are often the most reliable, because they are continually monitored for performance with new IAV lineages (e.g. A(H1N1)pdm09, A(H7N9) 2013 LPAIV lineage from China), and are rapidly updated with validation as needed. USDA-licensed RT-PCR test kits are available for both avian and swine influenza viruses in the USA, with some availability in other countries. Commercial tests from local manufacturers are also available in China and Russia, but it is unclear how sensitive and specific these tests are. The growing availability of commercial tests provides a mechanism for the availability of standardized reagents, internal positive controls, and quality control between reagent lots. The disadvantages of all-in-one kits are that they are more expensive per test, and the primer and probe sequences are proprietary, so *in-silico* specificity analyses cannot be performed by end users.

Molecular methods offer numerous advantages for IAV detection. These include high sensitivity, which is similar to that of VI [3, 11, 52, 65], high specificity, scalability, the ability to accommodate any sample type with proper sample processing, and minimization of contact with infectious materials, as the virus is inactivated at an early stage of sample processing. Real-time RT-PCR, which is more widely used than conventional RT-PCR, offers additional advantages. First, it is among the most rapid molecular tests available, where results can be obtained in less than 3 hours. Second, it is more specific than conventional RT-PCR when used with a hybridization probe. Third, the potential for cross-contamination is reduced because samples are not manipulated after amplification.

The major disadvantage of both conventional and real-time RT-PCR is the high start-up cost for equipment, which has hindered some smaller laboratories from using this technology. Also, the reagents for rRT-PCR are expensive, and although RT-PCR is less expensive than virus isolation, the cost can still be prohibitive for some surveillance efforts. The reagents do require refrigeration, which can be a problem for testing in remote locations. The development of lyophilized reagents for rRT-PCR has been attempted, but compared with conventional RT-PCR the cost of the reagents is higher and the sensitivity is frequently lower [17, 75]. The cost of rRT-PCR is also affected by sample processing (RNA extraction), as this adds to the cost of materials and labor. Another disadvantage of rRT-PCR is that subtype identification has low accuracy due to the high variation of HA and NA sequences. Both false-positive results by cross-reaction with other subtypes and false-negative results have been observed, with an overall accuracy rate of 49.5% being reported [62].

The high sensitivity of RT-PCR does increase the risk of false-positive results by detecting low levels of cross-contaminants. Conversely, decreased sensitivity can occur with RT-PCR due to inhibition with some sample types if these are not processed properly. Both of these risks can be managed with proper controls, including no template RNA extraction controls to test for cross-contamination and internal positive controls [17, 19], and positive extraction controls to verify that inhibitors are not present.

Serological methods

Antibody detection is a common and relatively inexpensive method of surveillance for detecting exposure of animals to IAV. Numerous test formats are used for IAV antibody detection, including AGID assay, HI assay, and ELISA. Of the three assays, HI is the only absolutely quantitative format.

Serology can be performed with sera, plasma, egg yolk from avian species, and sera eluted from blood stored on filter papers (e.g. Nobuto strips) [21]. As with all diagnostic methods, the correct conditions for transport of samples are essential if accurate results are to be obtained. With the exception of blood stored on preservative filter papers, samples should be kept cool, and although antibodies are more robust to freeze–thaw cycles than live virus, freezing and thawing of samples should be minimized.

One of the primary applications of antibody testing for poultry is in the support of trade, to certify flocks or poultry products as free of exposure to AIV. For this reason, antibody tests are performed on millions of samples yearly from US poultry alone. Antibody testing in mammalian species can be used to evaluate exposure to IAV or response to vaccination.

ELISA

ELISAs for influenza A antibody detection are well established, and numerous ELISAs for different species (e.g. avian, swine, equine) are commercially available. Most of the IAV tests are targeted to nucleoprotein antibodies which are produced early after infection, and although these tests are reliable for identifying infected flocks, the ELISA results cannot be used to measure protective antibody levels, because nucleoprotein antibody is not neutralizing. Although both indirect (sandwich) and blocking formats have been used, the utility of the blocking format is broader, since the ELISA is not species specific, and it can therefore be used for surveillance in numerous avian and mammalian species (although performance data are only available for a limited number of species).

ELISA is a high-throughput format which is rapid and easy to use. Although commercial tests are more expensive than in-house-produced tests, the quality control and reagent production are undertaken by the manufacturer. On a per-sample basis, the materials for commercial ELISA cost about twice as much as those for AGID. The amount of specialized equipment required is minimal. An optical microtiter plate reader is needed to evaluate the results. However, many laboratories that run ELISAs routinely also have automated plate washers and even liquid handling stations for diluting samples. For AIV antibody detection, ELISA results need to be confirmed with AGID or preferably HI for H5 or H7, and currently only one AIV antibody ELISA is certified by the OIE.

Commercially available subtype-specific ELISAs are available for H1, H3 swine influenza H5 HA subtype, and N1 and N2 subtypes. However, their specificity is less dependable than that of assays which target type A influenza antibody, due to the variation in the HA protein. When there is a need for a subtype-specific ELISA, whether selecting a commercial test or a procedure that has been reported in the literature, it is essential to ensure that there are adequate verification and validation data for the target species and subtype.

Agar gel immunodiffusion (AGID)

The AGID assay has been used since the 1970s for IAV antibody detection [7]. The principle of AGID is to visualize the immunoprecipitation reaction of AIV antibody and antigen after diffusion in an agar matrix. Although AGID is most widely used in a diagnostic setting to detect antibody using a reference antigen, it can also be used to detect type A influenza antigen (e.g. to confirm the presence of IAV in ECE fluids or cell culture supernatants).

AGID is inexpensive, simple to run, and does not require unusual supplies or expensive equipment. However, preparation of the antigen and control sera with proper quality assurance is expensive and time consuming. For these reasons, many laboratories use antigen and control sera produced by reference laboratories, or that are commercially available. In addition, AGID requires moderate skill and training in interpretation of the test results. The results may be read within 24 hours, but it may take up to 48 hours for weakly positive reactions to become visible.

AGID has moderate sensitivity, and can detect antibody earlier post infection than other antibody detection tests because it reacts with IgM.

Antibody may be detected as early as 5 days post infection, and may be detected for many weeks or months post infection [73], although the response and duration of antibody are affected by both the host and the virus strain. The AGID test is suitable for testing serum, plasma, and egg yolk [8]. Importantly, however, AGID does not produce consistent results with serum from some avian and mammalian species [20, 59, 71].

Hemagglutination inhibition assay

The HI assay can be used as a confirmatory test for the presence of subtype-specific IAV in hemagglutinating egg fluids or cell culture supernatants, to further characterize IAV isolates by identifying the HA subtype, or to identify the subtype-specific antibodies to IAV in serum, plasma, or egg yolk [76].

Suspect isolates are identified by HI with a panel of subtype-specific antisera representing each HA subtype. Because false-positive reactions can be caused by steric inhibition when the reference reagent and test material have a homologous NA subtype (but have different HA subtypes) [58], more than one reference serum per HA subtype is often necessary to assure adequate specificity. The problem of steric inhibition can be overcome by the use of antisera prepared by DNA vaccines containing only the HA gene [38]. In addition, some cross-reaction can occur between HA subtypes, making the results more difficult to interpret. Therefore the specificity of the HI assay is highly dependent upon the quality of the reference reagents.

Specific to the procedure for isolating AIV, HI has historically been used to exclude the presence of avian paramyxovirus type 1 (APMV-1, also known as Newcastle disease virus) in the test material by testing for inhibition with APMV-1-specific antibody. A negative HI assay result with APMV-1 antiserum indicates that APMV-1 is not present and that the specimen is suspect for AIV.

Conversely, the HI assay may be used to identify the HA subtype of IAV antibodies in a specimen by using viruses of known subtype as the antigen in the assay. Again, a panel of all 16 HA subtypes is needed to evaluate all of the different possible subtypes, and the results must be interpreted carefully because of the possibility of cross-reactions, particularly with samples from wild birds where the animal may have been exposed to different subtypes over its lifetime.

Sensitivity is generally not a major concern when the HI assay is used to identify IAV isolate subtypes, because the test is used with amplified virus (as opposed to clinical specimens, where the concentration of virus may be low). However, sensitivity of the HI assay for antibody detection is more of a concern. Reduced sensitivity can occur when significant antigenic drift occurs within a subtype, resulting in low reactivity between the antigens used in the HI assay and antibodies found in test sera. Despite these concerns, the HI assay is still considered to be more sensitive than AGID [41], and it will detect IAV antibody for a longer period post exposure than AGID. Furthermore, the HI assay is not species specific.

From a practical standpoint, the HI assay is relatively expensive and labor intensive when used to identify isolates or when used as a screening test for detecting antibodies, because of the number of antigens or antisera required to test for all 16 HA subtypes. However, the advantages of this assay are that it is rapid (results are available within a couple of hours), simple to perform, and requires only moderate skill to interpret the test results. The HI assay can be useful in some specific applications. For example, for trade purposes some countries require HI testing for the H5 and H7 subtypes, and during an outbreak where the target HA subtype is known, an HI test can be used more efficiently because a specific antigen is targeted. A major advantage of the HI assay is that inactivated antigens can be used, eliminating the need for special biosecurity or biosafety measures in the laboratory.

Neuraminidase inhibition assay

The neuraminidase inhibition (NI) assay can be used to detect NA subtype-specific antibodies or to identify the NA subtype of an isolate. The principle of the NI assay is to inhibit the enzymatic activity of the neuraminidase with subtype-specific antibodies [4]. For characterization of new isolates, a panel of reference antibodies corresponding to all nine NA subtypes is needed to perform the NI assay. The test utilizes a colorimetric reaction which does not occur when the neuraminidase activity is blocked, indicating a match between the antibody and test virus subtype. As with the HI assay, sensitivity is

not a critical characteristic of the NI assay, as virus isolates are used instead of clinical samples. Also, like the HI assay, the specificity is moderate and depends on the quality of the reference sera or antigens used [78].

The current standard NI assay, the thiobarbituric acid (TBA) NI assay, is a more complicated procedure than the HI assay, and although it can be completed within a few hours, it is typically performed in reference laboratories because the substrate used in the test is expensive and the chemicals used are hazardous. The assay can be performed in a 96-well microtiter format or in tubes, but the microtiter assay requires special white-colored plates to make it easier to distinguish color differences. An alternative method for NI antibody detection, the enzyme-linked lectin assay (ELLA) [36], has been increasingly used recently [9, 34, 77]. ELLA is less expensive and uses safer reagents than the TBA assay.

Characterization of influenza isolates

Once an IAV has been isolated it may be genetically and biologically characterized if necessary. The amount of characterization necessary depends on the circumstances. For example, an isolate of an unusual subtype for a species, or from a species not normally associated with influenza infection, will have a higher priority. Isolates from routine diagnostics (e.g. isolation of an H3 from swine) is less likely to be extensively characterized.

Due to the low cost of sequencing, and rapidly improving technology, it has become common to produce the sequence of the HA and NA at a minimum, and often the full genome sequence is produced. Sequencing is the most accurate way to identify both the HA and NA subtype. In addition, partial sequencing of the HA cleavage site is starting to replace the *in-vivo* tests, such as the intravenous pathogenicity index (IVPI), to identify the presence of a multibasic amino acid cleavage site and to classify the AIV pathotype. A list of the multibasic cleavage sites of the HA molecule detected to date for low- and high-pathogenicity H5 and H7 avian influenza viruses is regularly updated and available at the OFFLU website (www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf.). For index-case AIVs, particularly if they are H5 or H7, the *in-vivo* test (i.e. IVPI) should be applied to confirm the pathotype.

For all IAVs, the gene sequence can be used for a basic phylogenetic analysis that provides information about the most closely related isolates for which there are data, and can provide valuable epidemiological information. In addition, as more and more molecular markers for virulence and host range are identified in the literature, likely biological properties can potentially be identified. The Influenza Research Database (www.fludb.org) [67] and the GISAID-EpiFlu database (http://platform.gisaid.org), which contain sequences submitted to public databases, provide annotated lists of possible biological features based on published information for numerous host species. Other characterization may include pathogenesis studies in the host of origin or model species to evaluate potential host range or transmission characteristics. Receptor-binding studies are also becoming more common.

Education and training

The role of the farmer, owner, or animal handler in detecting IAV infections in domestic animals should not be discounted, as their recognition that there is a health problem is necessary for initiation of the diagnostic process. Therefore education of these personnel is of critical importance for early detection, because the signs of influenza can be subtle and non-specific (e.g. LPAI is sometimes first recognized in chickens and turkeys because there is a decrease in food and water consumption). In addition to a description of the clinical signs, education and training should include an explanation of why diagnosing influenza is important, and also describe how and when to increase biosecurity, and the appropriate biosafety measures that should be implemented.

Conclusion

One of the most critical aspects of implementing diagnostic and detection tests for any disease is fitness for purpose. The practical aspects of the test

are as important as its analytical performance. A test such as RRT-PCR may have superior sensitivity and specificity, but the rapid and portable nature of ACIA kits makes them ideal for on-farm testing, whereas RRT-PCR must be performed in a laboratory because of the sample processing required. It is also important to define the goals and outcomes of the testing. For example, active surveillance will have different diagnostic needs to surveillance during an outbreak. Other questions that need to be addressed include what action will be taken if a positive result is obtained, and the consequences of obtaining a false-negative or false-positive result. Finally, regulatory guidelines need to be considered when implementing IAV diagnostics, as these may dictate which tests can be used and how an outbreak or case is handled.

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3 The economics of animal influenza

Anni McLeod and Jan Hinrichs

Introduction

The epidemic of H5N1 highly pathogenic avian influenza (HPAI) that has spread across Asia, Europe, and Africa since 2003 was followed by the emergence and spread of several new influenza subtypes. Although the public has become increasingly well informed about health threats posed by animal influenza, there has been a continuing theme of panic and economic losses even where outbreaks have not occurred, or have been reported and rapidly stamped out, or have been caused by non-zoonotic influenza virus strains. The emergence of H1N1 pandemic virus in Mexico resulted in worldwide human vaccination campaigns in October 2009 [80]. H7N9 low-pathogenicity avian influenza (LPAI) has caused severe disease in humans and abrupt declines in poultry demand since its detection in China in February 2013 [61]. Although equine influenza outbreaks were not harmful to human health, the control program nevertheless caused business disruptions for the Australian horse industry, which led to severe financial losses and financial assistance payments by the Government of Australia in 2007 [69]. Losses caused by animal influenza have been large, and estimates of potential loss are enormous. Equally important, the zoonotic character and pandemic potential of new emerging animal influenza viruses has led to increased collaboration between human and animal health sectors, as well as putting pressure on stakeholders in the livestock sector to increase the safety of livestock production and linked value chains.

Much of the economic impact is driven by the fear that animal influenza viruses may over time lead to a global pandemic with sustained human-to-human transmission. A multitude of animal influenza viruses are known to circulate in different species, but not all of them are zoonotic. Several have low virulence, and only a subset of these are "notifiable" to veterinary health authorities which are then responsible for control interventions. Low-virulent animal influenza viruses decrease the productivity of infected livestock and may reduce the effectiveness of vaccinations against other common diseases of livestock. The newly emerged H7N9 LPAI virus is zoonotic. Table 3.1 gives an overview of the most common avian influenza virus subtypes in poultry. Reducing the prevalence of influenza in livestock reduces the opportunities for genetic reassortments which could potentially cause sustained human-to-human transmission. Control of zoonotic and non-zoonotic influenza viruses in animals therefore has benefits in terms of protecting human health. One recent example is the genesis of zoonotic H7N9 LPAI, which was facilitated by the widespread presence of low-pathogenic and non-zoonotic H9N2 LPAI in poultry production systems [60].

Pandemic prevention has attracted considerable funding from the international donor community, with the aim of reducing the number of infected animals and thus limiting human exposure. Between 2005 and 2009, US$3.9 billion had been committed by bilateral and multilateral donors for the control of pandemic influenza [83]. While strengthening of health services with these funds has probably led to benefits beyond pandemic influenza control, control interventions in livestock value chains have also caused negative economic impacts for value chains actors from movement controls and destruction of livestock. In order to use the animal influenza control funds effectively, it is important to understand the economic and

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Table 3.1 Influenza A virus subtypes that circulated in birds and were found sporadically in people [26, 54, 82].

Subtype	Disease in humans	Impact in animals relevant to economic analysis
H5N1	Fatal human cases reported	High mortality reported
H5N3	No human cases reported	High mortality reported
H5N6	Human cases reported	High mortality reported
H5N8	No human cases reported	High mortality reported
H7N9	Fatal human cases reported	No clinical signs reported
H9N2	Mild symptoms in humans reported	Varying mortality and morbidity reported
H10N8	Fatal human cases reported	?

social factors that affect the success and impact of measures used for control. The absence of apparent losses in livestock production represents a major challenge in engaging stakeholders in the livestock sector in human health-driven control programs for low-virulent influenza viruses. This chapter addresses the economic imperatives faced by decision makers who must deal with different animal influenza virus infections as livestock diseases, while remaining aware of the humanitarian and economic threat of a human pandemic.

Benefits and costs of controlling animal influenza

Three types of benefit justify animal influenza control. Animal influenza covers low-virulent swine and equine influenza viruses, LPAIV, and HPAIV.

Net benefits of avoiding a human pandemic

It is challenging to estimate the potential benefits of preventing a human pandemic. Estimates of the potential number of prevented human fatalities are highly sensitive to assumptions in predictive epidemiological models. Disruption estimates of other economic activities from social distancing and other prevention costs are similarly speculative in nature. The 2009 H1N1 epidemic was considered a mild pandemic, but still caused more than 18 000 laboratory-confirmed deaths during the pandemic phase [79], estimated by one study as equivalent to between 334 000 and 1 973 000 years of life lost (YLL) [76]. Although past pandemics can give rough guidance on potential lives lost, the "valuation" of lost lives presents another challenge. Non-monetary valuations such as YLL or disability adjusted live years (DALY) can be used to prioritize the use of resources among several diseases. Another monetary valuation approach is to use the statistical value of a life saved based on life insurance data. The application of this approach justifies the investment of US$1 billion in influenza risk mitigation if on average 654 people are saved per year [71]. Other economic estimates of potential impact are very large, and this has resulted in considerable international funding for animal and human pandemic control of mainly zoonotic animal influenza viruses, as discussed in the next section under potential impacts of human influenza.

Net benefits of minimizing human disease contracted directly from livestock

Human cases of and deaths from non-pandemic animal influenza viruses, although tragic, have so far been small in number and would not have justified huge international expenditure on disease control. There were 58 781 deaths recorded for malaria in 2013 [81], whereas H5N1 HPAI had caused 402 known deaths and H7N9 LPAI had caused 178 known deaths at the time of writing.

Net benefits from improved livestock productivity through avoiding disease

It is widely agreed that control of the disease at its source in livestock will be the most effective way to prevent the occurrence of a human pandemic of animal origin, and this chapter focuses mainly on the economic impacts of disease and control methods in the livestock sector. Control should be achieved as cost-effectively as possible, and with the minimum disruption to human lives and economies.

Many low-virulent animal influenza viruses, such as the zoonotic H7N9 LPAIV, the pandemic H1N1 influenza virus, and the non-zoonotic H9N2 LPAIV, often cause very mild or barely noticeable disease syndromes. Livestock keepers and traders are therefore often not aware of the invisible productivity losses. Control interventions for low-virulent animal influenza viruses require compliance by livestock keepers. The absence of visible losses does not create compliance incentives, and means that a wider range of livestock production and marketing issues need to be addressed.

A typical pattern of socio-economic effects beginning before an outbreak and progressing towards long-term control measures for notifiable animal influenza viruses is shown in Table 3.2. The length and intensity of each phase are influenced by the virulence of the animal influenza virus, the structure of the livestock sector, and the response capacity of the animal health system.

Market shock is the first economic effect, and may occur even without an outbreak, created by consumer fears. If an outbreak occurs, each element of the disease control process has associated costs and livelihood effects, beginning with reporting of disease, stamping out by culling and movement control, providing compensation for animals culled, and later perhaps the introduction of vaccination. The diverse character of livestock keeping and livestock keepers presents huge challenges in terms of designing control programs that maximize the benefits of containing disease while at the same time balancing the needs of small- and large-scale operators. As disease is brought under control, rehabilitation of the livestock sector begins. This is a straightforward process if an outbreak has been quickly stamped out, but more complicated if it is taking place under conditions of recurring outbreaks. Where there are complex livestock value chains and continuing disease, there is pressure for governments to introduce long-term measures that will restructure the sector in a more biosecure way. However, this carries the risk of excluding smallholders from livestock keeping, with associated loss of livelihoods. It also requires investment to revive animal health systems that have suffered from neglect.

Estimates of net benefits from avoiding disease in livestock need to balance the impact of disease against the impacts of control processes, and assess the differential impact by sector and along value chains. Ideally they will take into account all of the following:

1. Net impact of market shocks. Shocks occur when demand and prices are disrupted by consumer fears of disease or import bans of trading partners. The control process can also cause market disruption by restricting movement and sales or exaggerating consumer fears through ill-judged communication, and may have impacts far beyond the area of infection.
2. Net impact on livestock productivity. Productivity gains from controlling disease must be offset against the losses caused by the control process. These effects are greatest within areas where outbreaks occur, affecting producers and those immediately connected to them through value chains. There may be wider effects if depopulation is extensive.
3. Costs of dealing with diseased livestock. These include treatment (if any) and disposal of carcasses.
4. Direct costs of prevention and control processes. These include all of the human resource,

Table 3.2 Phases of disease and socio-economic issues for notifiable animal influenza outbreaks.

Socio-economic issues	Pre-outbreak	Outbreak(s)	Rehabilitation	Long-term prevention
Market shocks	■	■		
Culling/compensation		■		
Movement control effects		■		
Vaccination costs			■	
Restocking costs			■	
Restructuring investment			■	■
Long-term market access			■	■
Financing animal health			■	■

capital, and consumables needed to carry out surveillance, culling and disposal, movement control, and vaccination.

5 Costs of rehabilitation. Restoring the operation after an outbreak incurs a restocking cost above the normal costs for maintaining production cycles. In addition, it usually requires investment in more biosecure management by farmers, traders, and market managers, as part of the effort to prevent recurrence of disease.

6 Impacts of restructuring. Beyond the immediate impacts of dealing with disease, there may be changes in the structure of the livestock sector resulting from heightened animal health and food safety regulations, or restrictions in the places where production and processing may take place. These measures require investment and will result in improved productivity for some but reduced market access for others. They may also, although this has not yet been evaluated, result in a loss of animal genetic resource.

If the control strategy is well designed and implemented, the losses from control should be considerably less than those that would have occurred from an uncontrolled disease outbreak, but the impacts on different stakeholders may be uneven. Compliance with disease control regulations will depend on the benefit that each stakeholder group perceives from them. For example, providing compensation does not reduce the production loss from culled livestock, but shares the loss between producers and others in society, providing an incentive for producers to cooperate with culling teams.

A complete benefit–cost or cost-effectiveness analysis for global control of zoonotic animal influenza has not yet been attempted. Preliminary estimates have been made for H5N1 HPAI in some countries and regions at different stages of disease. Some of these give a detailed snapshot for a particular country and time, and others talk vaguely of potential costs running into billions, but none of them provide a complete picture.

An example of the scale of losses caused by H5N1 HPAI in the poultry sector was reported for the H5N1 HPAI epidemic in Nepal in 2013, and compared with the costs of short-term response measures as well as long-term investments in animal and human health service infrastructure [41]. Before the onset of the epidemic, the commercial poultry sector had experienced dynamic growth of the commercial poultry population by 50% within 3 years. An annual output of 25.4 million eggs and 1.9 million broilers was achieved, equivalent to US$388 million or 2.04% of Nepal's GDP. More than 1.7 million poultry were culled or died during the HPAI outbreaks in 2013, and control efforts prevented a much wider spread of the disease to more farms. The lost poultry had a domestic market value of about US$9 million.

About 40% of the high-value broiler breeding stock and about 15% of the layer breeding stock were lost. This resulted in supply shortages of replacement progeny for poultry meat and egg production. Nepal has a highly specialized commercial poultry production sector which depends on day-old parent stock imports. Lost parent stock resulted in reduced production for an extended time period after the containment of HPAI, due to the required growth period until birds are productive again and produce eggs for progeny stock. Production took 9 months to recover, and during this period value chain actors had to cope with revenue and income losses.

The value of poultry production declined during this time, resulting in a foregone output value of US$119 million, equivalent to 0.63% of Nepal's annual GDP. This represented a loss to the national economy. Poultry farmers suffered a loss in gross margin totalling US$38.8 million during the 9 months of reduced output. They may also have experienced lost value addition opportunities as the outbreak slowed down the recent dynamic growth in commercial poultry production. It is likely that the reduced domestic poultry production value was to some extent replaced by formal and informal imports from other countries, and by the replacement of poultry meat with other meat substitutes, as Nepal is a net importer of livestock.

Nepal's animal and human health service capacity and infrastructure had been supported with about US$23 million of donor funds earmarked for animal influenza control and prevention between 2006 and 2014. It is highly speculative to attempt to forecast the scale of poultry losses without these additional investments in the animal and human health services. However, the scale of poultry sector losses in 2013 in relation to US$23 million prior to control and prevention investments over a period

of 7 years does indicate the potential benefits in terms of prevented poultry losses if only one epidemic of similar scale is prevented.

One of the difficulties of making a comprehensive global estimate arises from the unreliable and non-specific data on mortality and morbidity losses of livestock from disease. Estimates to date suggest that approximately 232 million poultry had died or been culled in H5N1 HPAI outbreaks between the beginning of 2004 and October 2006 [27]. This figure is probably an underestimate, and does not include mortality from concurrent LPAI viruses. About 40% of all H5N1 HPAI disease events reported to the United Nations Food and Agriculture Organization (FAO) database lack data on mortality or culling quantities. Estimates based on proxy data such as household surveys or agricultural census figures are complicated by the fact that the disease behaves differently in each production system. The effects of LPAI on productivity are often either not noticed, or there is no obligation to report them. Market values of livestock differ substantially even within the same species. Production type and age details of died and culled livestock are not usually reported. Losses in the above-mentioned example from Nepal resulted from dead and culled poultry with market values ranging from less than US$1 for young broiler chicks to more than US$25 for productive broiler parent stocks.

The remainder of this chapter discusses in more detail the main economic effects that have been identified in this section. It covers the potential effects of animal influenza on humans that may be avoided by control of avian influenza in poultry, the contribution of livestock sector diversity to the impact of avian influenza, the effects of market shocks, the effects on food security and livelihoods, the costs and productivity losses associated with outbreak control, the restocking process, and the socio-economic effects of restructuring.

The potential impact of human influenza

It is likely that the next serious discontinuity in world development will originate from either a human influenza pandemic or a transformational world war [68]. Animal influenza has the potential to trigger the next human flu pandemic, and this is a major factor contributing to the concern about animal influenza. In social and humanitarian terms, human pandemics are devastating – witness the impact of the relatively minor global outbreaks of severe acute respiratory syndrome (SARS) in 2003, which killed less than 800 people [11], but seriously disrupted the economies of South-East Asian countries and Canada [14, 20], and the lives of their citizens. The human influenza pandemics in 1918–1919, 1957, and 1968 may have killed 100 million, 2 million, and 1 million people, respectively. In terms of YLL the 2009 H1N1 pandemic is estimated to be comparable to the 1968 pandemic [76].

Pandemic effects depend not only on the numbers of people killed, but also on the demographic distribution of illness and death. A high proportion of infection in economically productive age ranges, as in the case of HIV/AIDS, has the potential to cause long-term damage to economies. Should a human influenza pandemic occur, it is uncertain which age groups would be worst affected. With so many uncertainties, it is impossible to make precise estimates of the economic impact of any new pandemic. The World Bank [8] estimated that the potential economic losses for an influenza pandemic involving 71 million human fatalities would be around US$3 trillion. One of the long-term impacts of a pandemic could be to push large numbers of households below the poverty line [5], and the low level of investment in public health in the poorest countries [59] is a cause for concern.

The economic effects of a pandemic are likely to start with disruptions to businesses and economies, and will place unusually high demands on some services (through stockpiling essential items) and abruptly lower the demand for others (e.g. entertainment, restaurants, hotels). National and corporate plans for operation in times of pandemic aim to allow government and business to continue in the event that employees may be ill, caring for others, or unable to travel to work, and to ensure the availability of the most essential supplies. The 2009 H1N1 pandemic was estimated to have caused Mexico's tourism sector losses of US$2.8 billion within a 5-month period [62]. Considerable resources have been devoted to preparing for a pandemic. It is tenuous to attribute all of this preparation to zoonotic animal influenza virus.

If a human influenza pandemic occurs, it could originate from some other source. Equally, terrorist attacks might create conditions in which travel is impossible and work disrupted. Much of the expenditure on preparedness for disaster, however, would not have been made or planned without the present threat of a human pandemic originating from animal influenza.

Even without a human influenza pandemic, the economic costs of animal influenza have been large, and its control at source is essential. Various contributors to cost are discussed in subsequent sections of this chapter. A number of non-zoonotic animal influenza viruses, which normally would not merit much international attention, are the focus of greater vigilance and stricter control measures than might otherwise be the case, out of concern that they may mutate to zoonotic animal influenza viruses.

The globalized livestock sector

Poultry and pigs are perhaps the most globalized of all livestock. Poultry and pig production and trade have shown steady growth (Table 3.3), and projections suggest that demand will continue to rise. At the same time, both sectors are highly diverse, with production systems ranging from specialized high-intensity units using special-purpose breeds to low-intensity systems using hardy, indigenous breeds. The steady growth in pig and poultry production is a result of efficiency gains from breeding technology, with selection for specific characteristics and a specialization of the required production process for specific breed types and age groups. Selection for high-performance pure-line breeds, hybridization, and artificial insemination, as well as the distribution of production breeds via worldwide market networks, have been a driving force for developing highly productive animals [22].

Poultry production systems

The FAO and the World Organization for Animal Health (OIE) [18, 28] have defined four types of poultry production system, classified as sectors 1 to 4. Sector 1, industrial poultry with high biosecurity, is the system from which the majority of internationally traded poultry is derived. Sector 2 includes large-scale commercial producers with good biosecurity and the farmers under contract to big companies, who raise birds from day-old chicks (DOCs), using feed supplied by the contractors. Contract farming represents an opportunity for new market entrants, requiring technical skill but a lower level of investment than independent farming, because the contractor supplies many of the inputs. During the H5N1 HPAI outbreaks of 2004–2005, contract farmers in Thailand, Vietnam, and Indonesia were buffered from financial loss by their contractors [34, 66]. Sector 3 consists of small- to medium-scale commercial units, in which poultry are confined and fed, but biosecurity investment is low. This is a highly diverse sector. In developed countries, some of the high-value niche-market production, such as organic and free-range products, might be considered to fall within this group, as might specialist producers of rare breeds who keep them in free-range systems. In developing countries, sector 3 consists chiefly of small-scale commercial units with limited investment in facilities, rapid turnover, and a growing market. Their numbers are not high,

Table 3.3 Production and international trade of pig and poultry meat during the period 2002–2011

Year	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Pig production (million tons produced)	178	185	186	190	195	200	206	210	215	215
Pig trade (1 million head)	18 099	19 989	21 846	23 914	26 814	30 628	31 610	32 885	33 317	34 178
Poultry production (million tons produced)	147	151	156	162	166	176	185	190	199	206
Poultry trade (1000 live animals)	868	749	821	917	919	962	1054	1312	1396	1457

Modified from FAOSTAT.