

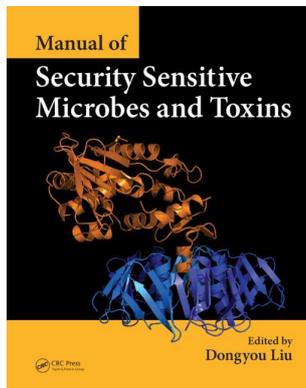
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## Manual of Security Sensitive Microbes and Toxins

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### Avian Influenza Virus (Highly Pathogenic)

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# 3 Avian Influenza Virus (Highly Pathogenic)

Dongyou Liu and Sacha Stelzer-Braid

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## 3.1 INTRODUCTION

The genus *Influenzavirus A* contains a number of segmented, single-stranded, negative sense RNA viruses that are further separated into 17 hemagglutinin (H or HA) subtypes and 9 neuraminidase (N or NA) subtypes. As members of the genus *Influenzavirus A* are adapted in wild aquatic birds (natural reservoir host), they are commonly known as avian influenza viruses (or “avian influenza,” “bird flu”). Among these viruses, several subtypes (e.g., H1N1, H2N2, H3N2, H5N1) are highly pathogenic, with the tendency to induce significant mortality in domestic fowls as well as serious diseases in humans. Hence, they are frequently referred to as highly pathogenic avian influenza viruses (HPAI).

In particular, highly pathogenic avian influenza A (H5N1) virus (HPAI H5N1, sometimes shortened to H5N1) is highly contagious and deadly to domestic poultry. Being capable of direct transmission from poultry to humans, and airborne transmission between mammals, this virus has been responsible for causing sporadic, severe illness in humans and many animal species. After first appearing in Asia in 1997, HPAI H5N1 has created global concern as a potential pandemic threat. Between 2003 and 2011, this virus was responsible for a total of 566 confirmed human cases and 332 deaths. On the other hand, avian influenza subtype H7N9 virus is generally considered as of low pathogenicity affecting birds only and has not been implicated in human outbreaks. However, between February and November 2013, 138 patients have been confirmed in eastern China provinces with avian influenza virus subtype H7N9 infection, resulting in 45 casualties.

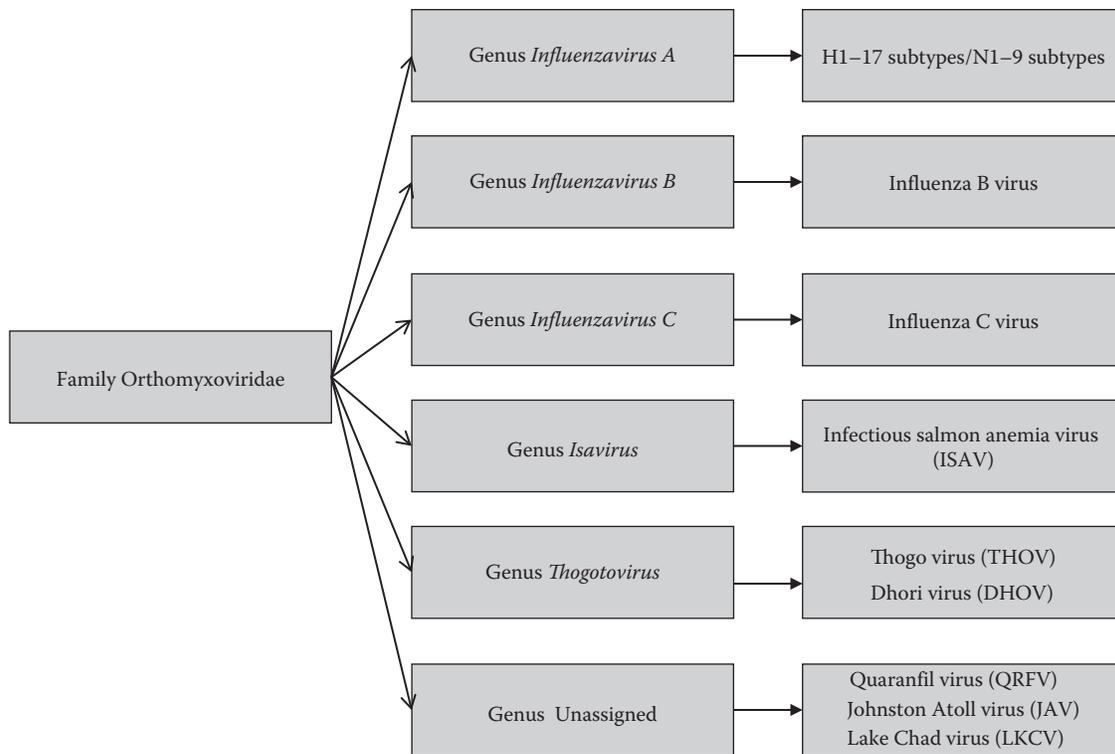
This chapter focuses on HPAI H5N1 as well as the newly emerged H7N9, in relation to its classification, morphology, biology, epidemiology, clinical features, pathogenesis, identification, diagnosis, treatment, and prevention. The other important influenza A virus H1N1, which caused “Spanish flu” in 1918 and “swine flu” in 2009, is discussed in Chapter 11.

## 3.2 CLASSIFICATION, MORPHOLOGY, AND GENOME ORGANIZATION

### 3.2.1 CLASSIFICATION

The family Orthomyxoviridae (orthos means “straight,” myxa means “mucus” in Greek) covers a number of enveloped, segmented, negative-strand RNA viruses that are separated taxonomically into five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Isavirus*, and *Thogotovirus* (Figure 3.1) [1]. A sixth genus has been proposed recently to accommodate three previously unclassified viruses that were originally isolated from ticks and masked weaver bird in the 1950s–1960s [2].

The genus *Influenzavirus A* consists of a single species influenza A virus, which has been subdivided previously into 17 H subtypes (or serotypes) (H1–H17) and 9 N subtypes (N1–N9) based on the antigenicity of viral surface glycoproteins HA or H and NA or N. It is notable that influenza A virus H antigen type 17 (H17) was only recently identified from fruit bats in 2012 [3]. Each influenza A virus has one type of HA and one type of NA antigen; and specific strain/isolate is named by a standard nomenclature specifying virus type,



**FIGURE 3.1** Classification of the family Orthomyxoviridae.

geographical location where it was first isolated, sequential number of isolation, year of isolation, and HA and NA subtype. For example, A/Brisbane/59/2007 (H1N1) denotes that the virus is of Influenza virus A, which was first isolated in Brisbane with a strain number of 59 in the year of 2007, and belongs to subtype H1N1. The genomes of influenza A viruses comprise 8 RNA segments (1–8) encoding at least 11 proteins (HA, NA, PB1, PB2, PB1-F2, PA, NP, M1, M2, NS1, and NS2).

Commonly present in wild aquatic birds, in which they rarely induce overt clinical symptoms, influenza A viruses can be highly pathogenic to domestic fowls and mammals including humans. The influenza A virus serotypes that have caused serious flu pandemics in humans include H1N1 (“Spanish flu” in 1918 and “swine flu” in 2009), H2N2 (“Asian flu” in 1957), H3N2 (“Hong Kong flu” in 1968), H5N1 (pandemic flu or HPAI), H1N2 (endemic in humans and pigs), and more recently H7N9 (bird flu in eastern China in 2013) [4].

Analyses of the HA gene sequences revealed that HPAI H5N1 viruses can be separated into 10 clades, with the main clades consisting of clade 0 (progenitor viruses from Hong Kong and China, 1996–2002), clade 2.1 (avian and human isolates from Indonesia, 2003–2007), clade 2.2 (2005 progenitors from Qinghai Lake and Mongolia and avian and human 2005–2007 isolates from Europe, the Middle East, and Africa), clade 2.3 (avian and human isolates from China, Hong Kong, Vietnam, Thailand, Laos, and Malaysia, 2003–2006), clade 2.4 (avian isolates from Yunnan and Guangxi, China, 2002–2005), and clade 2.5 (avian isolates from Korea, Japan, and China, 2003/2004, and from Shantou, China, 2006) [5].

Furthermore, based on the NA gene sequences, HPAI H5N1 isolates can be divided into two lineages: 1997 human and poultry

isolates and 2005 Vietnamese waterfowl isolates, the latter of which is further divided into two sublineages—GS/GD/1/96-like (containing a full-length NA stalk) or GS/GD/1/96-derived (with a 20-amino acid deletion at amino acids 49–68 in the NA stalk). In addition, phylogenetic comparisons of the PB2, PB1, PA, and NP and M genes of Chinese HPAI H5N1 viruses allow their division into two lineages; and the NS genes of Chinese HPAI H5N1 duck viruses separate them into two alleles, A and B. Combined examination of the HA, N, PB2, PB1, PA, NP, M, and NS gene sequences facilitates the differentiation of HPAI H5N1 viruses into 13 nodes: I [Gs/GD], II [X-series], III [clades 1, 2, 8, 9], IV [clade 1], V [Vietnam, Thailand, Malaysia (VTM)+ precursor], VI [VTM], VII [Indonesia+precursor], VIII [clade 2.1 (Indonesia)], IX [clade 2.2 (Qinghai lineage)], X [clade 2.3], XI [clades 2.3.1, 2.3.2], XII [clades 2.3.3, 2.3.4], and XIII [clade 2.3.4 (Fujian-like)] [6].

The genus *Influenzavirus B* consists of a single species influenza B virus, with only one influenza B serotype. It is mostly a human pathogen, with seal being the only other animal known to be susceptible. Given its reduced rate of antigenic change, and its limited host range (which inhibits cross-species antigenic shift), influenza B virus is less common than influenza A virus and has not been responsible for any pandemics in humans. Similar to that of influenza A viruses, the genome of influenza B virus harbors 8 RNA segments, which encode at least 11 proteins.

The genus *Influenzavirus C* comprises a single species influenza C virus that is infective to humans and pigs. Being less common than the other types, influenza C virus usually causes mild diseases in children. The genome of influenza C virus has seven RNA segments and encodes nine proteins.

The genus *Isavirus* contains a single species infectious salmon anemia virus (ISAV), which is responsible for causing ISAV in Atlantic salmon, Pacific salmon, brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss*). Several distinct strains of the virus have been identified, with a European strain and a North American strain being the most common. The infected fish develop pale gills and tend to swim close to the water surface, gulping for air. Some fish (notably Pacific salmon, brown trout, and rainbow trout) may show no external signs of illness and thus act as carriers of the virus.

The genus *Thogotovirus* consists of two species: Thogotovirus (THOV) and Dhori virus (DHOV). THOV has been isolated from ticks in Africa and southern Europe and is known to infect humans. Phylogenetic analysis of four THOV RNA segments uncovered the existence of a Euro-Asian lineage and an African lineage. DHOV has been isolated from ticks in India, eastern Russia, Egypt, and southern Portugal. The virus is infective to humans, causing febrile illness and encephalitis. DHOV possesses seven RNA segments.

The sixth yet to be named genus of the family Orthomyxovirus currently comprises Quarantivirus (QRFV) (which was isolated from ticks *Argas [Parsicargus] arboreus* collected near Cairo, Egypt, in 1953), Johnston Atoll virus (JAV) (which was isolated from ticks [*Ornithodoros capensis*] in Johnston Atoll, in the central Pacific in 1964), and Lake Chad virus (LKCV) (which was isolated from a masked weaver bird, *Ploceus vitellinus*, at Lake Chad, Nigeria, in 1969) [2]. QRFV is known to cross-react serologically with two other unclassified viruses, JAV and LKCV. While QRFV and JAV PB1 and HA share 80% and 70% amino acid identity to each other, respectively, LKCV PB1 shares 83% amino acid identity with the corresponding region of QRFV PB1. QRFV, JAV, and LKCV are lethal to newborn mice via intracerebral (i.c.) inoculation [2].

### 3.2.2 MORPHOLOGY AND GENOME ORGANIZATION

The virions of influenza A viruses are pleomorphic, mostly spherical or ovoid (50–120 nm in diameter) and filamentous (20 nm in diameter and 200–300 nm in length), with a lipoprotein envelope. The envelope supports about 500 rod-shaped spikelike surface projections (made up of glycoproteins HA and NA), each projecting 10–14 nm from the surface, with the HA protein interposed irregularly by clusters of the NA, or sialidase, protein in a ratio of 4–5 to 1 (HA to NA). Underneath the envelope are matrix (M) proteins (forming capsid) and nucleoproteins (NPs) that enclose the viral genome consisting of eight single-stranded, negative sense RNA segments (ranging from about 900 to 2,500 nt per segment, with the total genome length of about 13,500 nt). The segments all have highly conserved terminal repeats of 9–13 nt at their 5'- and 3'-ends. The longest RNA segment is closely associated with the NP (of 50–130 nm in length and 9–15 nm in diameter) to form helical symmetry.

The eight single-stranded, negative sense RNA segments of the influenza A virus genome encode 11 proteins. Namely, the first segment encodes polymerase basic 2 (PB2);

the second segment encodes polymerase basic 1 (PB1) and PB1-F2 by using alternative reading frames; the third segment encodes polymerase acidic (PA); the fourth segment encodes HA; the fifth segment encodes NP; the sixth segment encodes NA; the seventh segment encodes M proteins M1 and M2 by using alternative reading frames; and the eighth segment encodes nonstructural proteins NS1 and nuclear export protein (NEP) (formally referred to as NS2) due to alternatively splicing events.

Among the proteins encoded by influenza A virus genome, the HA protein is a lectin that mediates the viral binding to the host sialic acid receptors on respiratory epithelia, the fusion of the viral and endosomal membranes, and the subsequent entry to target cells. The HA protein is synthesized as a single polypeptide chain (HA0), which is posttranslationally cleaved by cellular proteases. HA cleavage exposes the hydrophobic N-terminus of HA2, which is critical for the fusion of the viral and endosomal membranes to occur. Interestingly, the HA0 precursor protein of low-pathogenic avian influenza viruses (LPAI) contains a single arginine at the cleavage site and another basic amino acid at position -3 or -4 from the cleavage site, which displays limited cleavage by extracellular host proteases and restricts the viral replication at sites in the host where such enzymes are found, that is, the respiratory and intestinal tracts. On the other hand, the HA0 precursor protein of HPAI possesses multiple basic amino acids (arginine and lysine), which are cleavable by intracellular ubiquitous proteases, enabling the viruses to replicate throughout the bird, causing damages in vital organs and tissues, that lead to severe disease and death. The NA is an enzyme (sialidase) that cleaves sialic acid residues on host cells, resulting in the release of newly produced viral particles from infected cells. NP binds to and encapsidates viral RNA in the infected cell nucleus. M constitutes a proton channel in the virus surface that is targeted by the adamantane class of drugs.

What makes influenza A viruses a constant threat to domestic fowls, humans, and other mammals is their uncanny ability to evolve through genetic alteration and recombination (reassortment) that enhance their capacity to undergo interspecies transmission and/or evade host immune responses. Especially, when two subtypes of influenza A viruses co-occur in a single host, new pandemic viruses may emerge as a result of gene reassortments among RNA segments from different subtypes. In theory, reassortments of influenza A viral RNA segments can lead to at least 256 (2<sup>8</sup>) different genotypes, providing a potentially unlimited supply of new pandemic viruses [7,8].

Indeed, HPAI H5N1 subtype viruses were shown to harbor an HA segment from A/goose/Guangdong/1/96 (H5N1; GS/GD/1/96-like virus), with the remaining seven segments originating from A/teal/Hong Kong/W312/97 (H6N1; W312-like virus). As the HA gene of GS/GD/1/96-lineage has a multi-basic sequence (MBS) (RRKKR) at the cleavage site that is targeted by intracellular endoproteases, including PC6 and furin, the virus containing this gene has an increased ability to infect various cell types and cause systemic infections.

The amino acid changes identified in H5N1 subtype viruses may have also underscored its increased pathogenicity in humans [9,10]. Notable variations in H5N1 subtype viruses were found in the NA protein (H274Y for increased oseltamivir resistance), the NS1 protein (P42S for increased IFN antagonism, D92E for reduced sensitivity to IFN and TNF $\alpha$ , deletion from 85 to 94 for impaired inhibition of IFN production), and the PB2 protein (T271A for increased polymerase activity in mammalian cells, E627K for increased replication in mammalian respiratory tract, D701N for increased ability to replicate in mice) [11–13]. In addition to viral evolution in nature, the introduction of mutations into the H5N1 genome by site-directed mutagenesis and passage in ferrets resulted in a novel, highly contagious strain of H5N1 that has the ability to be transmitted via aerosols among mammals [14].

Similarly, the newly emerged H7N9 viruses from China may be a triple reassortant that has been possibly formed by A/brambling/Beijing/16/2012-like viruses (H9N2) incorporating the gene encoding HA from A/duck/Zhejiang/12/2011 (H7N3, subtype ZJ12) and the gene encoding NA protein from A/wild bird/Korea/A14/2011 (H7N9, subtype KO14) [15–19]. Additionally, a number of amino acid changes have been detected in the H7N9 viruses causing epidemics in China in 2013. These include (1) Q226L substitution in the receptor binding region of the HA protein, (2) 69–73 deletion and R294K substitution (for increased resistance to oseltamivir) in the NA protein, (3) L89V and E627K substitutions (for enhanced polymerase activity and increased virulence in mice) in the PB2 protein, (4) I368V substitution (for increased transmissibility in ferrets) in the PB2 protein, (5) N30D and T215A substitutions (for increased virulence in mice) in the M1 protein, (6) S31N substitution (for increased resistance to amantadine) in the M2 protein, and (7) P42S substitution (for increased virulence in mice) [15–19]. Together, these changes have been responsible for transforming a H7N9 virus of low pathogenicity into a highly virulent one, facilitating its jump from avian to human hosts [20].

### 3.3 BIOLOGY AND EPIDEMIOLOGY

#### 3.3.1 BIOLOGY

The biology of influenza A virus is a complicated process that relies on the involvement of a number of virus- and host-derived molecules. Showing a predilection for respiratory system, influenza A virus employs its HA glycoprotein to bind to sialic acid sugars on the surfaces of epithelial cells in the lung and throat. Assisted by endocytosis, the virus gains entry into the cell and subsequently moves inside the acidic endosome, where with the help of its HA protein, the viral envelope fuses with the vacuole's membrane and the viral contents (viral RNA molecules, accessory proteins, and RNA-dependent RNA polymerase) are released into the cytoplasm. Forming a complex, viral RNA and proteins are transported into the cell nucleus, where the RNA-dependent RNA polymerase transcribes complementary positive-sense RNA (cRNA). The cRNA binds to

newly-synthesized viral proteins to form new viral genome particles, which together with viral core proteins leave the nucleus and enter the membrane protrusion formed by HA and NA molecules. The mature virus buds off from the cell and acquires HA and NA as well as host phospholipid membrane coat. The viruses utilizes its HA to adhere to the cell and its NA to cleave sialic acid residues from the host cell, permitting the mature viruses to detach from the cell. The host cell dies once new influenza virus is released.

Influenza A viruses can remain infectious for 1 week at human body temperature, over 30 days at 0°C, and indefinitely at very low temperatures. Off the host, the viruses can survive in mucus for several hours, in feces on cages for up to 2 weeks, in distilled water for 100 days at room temperature, 200 days at 17°C, and indefinitely when frozen. Influenza viruses are susceptible to disinfectants and detergents (e.g., bleach, 70% ethanol, aldehydes, oxidizing agents, and quaternary ammonium compounds) and are inactivated by heat of 56°C for 60 min or longer, as well as by low pH < 2.

#### 3.3.2 EPIDEMIOLOGY

Influenza A viruses are widespread in nature and commonly occur in wild aquatic birds (of >105 species belonging to 26 families), which, as their natural reservoirs, do not present any noticeable clinical signs [21]. However, when transmitted to domestic fowls such as chicken and ducks, some influenza viruses (so-called HPAI) may cause significant morbidity and mortality, while others (so-called LPAI) only induce a mild respiratory disease, along with a decreased egg production or depression [22].

Transmission of influenza A viruses from aquatic birds to other animals, including domestic poultry and mammals, is often via bird droppings that contaminate the water and food. The virus can be also spread by animal to animal contact, bites and scratches, and the movement of infected live birds, poultry products, or contaminated feed, equipment, and materials. In addition, transmission between infected mammals including humans is possible through aerosols created by coughs or sneezes or through contact with saliva, nasal secretions, feces, and blood [23,24].

Because pigs possess cell surface receptors for both human and avian influenza viruses in their trachea, they provide a milieu conducive to viral replication and genetic reassortment for influenza strains that usually infect three different species, pigs, birds, and humans. Thus, the pigs may act as an “intermediate host” where influenza viruses (e.g., H1N1, H1N2, H2N3, H3N1, and H3N2) might exchange genes, producing new and dangerous strains.

HPAI H5N1 virus, with its first outbreak in 1987 and its first lethal human infection in Hong Kong in 1997, represents the first direct transmission of avian influenza A viruses to humans, without prior reassortment in pigs. There is evidence that the 1918 H1N1 influenza strain may have been the result of an avian virus adapting to humans; however, further sequence analyses are required for confirmation (see Chapter 11). Close contact with sick or dead poultry through

slaughter, food preparation, and defeathering has accounted for a majority of human cases of H5N1 virus infection [25,26]. Since November 2003, >600 human HPAI H5N1 cases have been reported to World Health Organization (WHO) from 15 countries in Asia, Africa, the Pacific, Europe, and the Near East, of which approximately 60% patients have died [27].

H5N1 is a flu virus strain that is capable of infecting more species than any previously known strain and is deadlier than any previously known strain [28]. Besides poultry and humans, HPAI H5N1 virus has been also detected in pigs in China, Indonesia, and Vietnam; domestic cats in Germany, Austria, Thailand, and Iraq; dogs in Thailand; wild stone marten (a weasel-like mammal) in Germany; wild civet cat in Vietnam; and tigers and leopards at zoos in Thailand [29–35].

The recently emerged human pathogenic H7N9 virus is another example of avian influenza A viruses transmitted directly from poultry to humans without apparent involvement of swine. In comparison with H5N1, which is highly pathogenic to both chicken and humans, H7N9 virus appears to possess molecular markers suggestive of “low pathogenicity” in chickens and is able to cause mild or no clinical disease in poultry but severe illness in humans [18,36–40].

### 3.4 CLINICAL FEATURES AND PATHOGENESIS

#### 3.4.1 CLINICAL FEATURES

In humans, infection with HPAI H5N1 virus causes typical flu-like symptoms, with gastrointestinal and respiratory involvements, ranging from (1) cough (dry or productive); (2) diarrhea, vomiting, and abdominal pain; (3) shortness of breath; (4) fever (>38°C); (5) headache; (6) malaise; (7) muscle aches; (8) runny nose; to (9) sore throat. Complications include (1) acute respiratory distress; (2) organ failure; (3) pneumonia; and (4) sepsis. In most cases, fatality (exceeding 60%) results largely from progressive respiratory failure, particularly among children and adults younger than 40 years old [41,42].

Human infections with avian influenza H7N9 subtype show characteristics of influenza, with clinical presentations ranging from fever, cough, shortness of breath, fatigue, muscle aches, hemoptysis, gastrointestinal symptoms, fever, to rapidly progressive pneumonia that does not respond to antibiotics. Encephalopathy and conjunctivitis are uncommon. Laboratory findings consist of normal white cell count, leukocytopenia, lymphocytopenia, thrombocytopenia, and mildly elevated liver enzymes. Most cases are severe, and a few cases deteriorate rapidly within 1–2 days of hospitalization to acute respiratory failure, leading to refractory hypoxemia, disseminated intravascular coagulation with disease progression, impaired liver or renal function, and death. Sputum specimens are more likely to test positive for the H7N9 virus than samples from throat swabs [15,43]. Men and women are equally represented in the youngest age category 20–34 years, but men are two- to threefold more frequent than women in older age groups [44].

In domestic birds, the clinical signs of H5N1 infection include (1) sudden death in several birds; (2) ruffled feathers;

(3) unusual head or neck posture; (4) inability to walk or stand; (5) reluctance to move, eat, or drink; (6) droopy appearance; (7) respiratory distress; (8) diarrhea; (9) swollen head, wattle, or comb; and (10) a drop in egg production. On the other hand, H7N9 infection in poultry is largely asymptomatic.

#### 3.4.2 PATHOGENESIS

The primary target of influenza infection is airway epithelial cells. The infection begins when the HA protein on the viral surface binds to sialic acid residues expressed on the host cell surface. While avian influenza strains (e.g., H5N1) preferentially bind to  $\alpha$ 2,3 sialic linkages (SA $\alpha$ 2,3Gal) (which are found abundantly on the epithelial cells of duck intestine), human influenza strains (e.g., H1N1) bind to  $\alpha$ 2,6 linkages (which are commonly present on airway epithelial cells) [45,46].

The HPAI H5N1 viruses of human origin have avian-type receptor SA $\alpha$  2,3Gal specificity [47]. Given that avian-type receptors exist on the epithelial cells of the lower and, to a lesser extent, upper human respiratory tract as well as on the ciliated cells of *in vitro* differentiated human epithelial cells from tracheal/bronchial tissues, it is no surprise that H5N1 viruses can be directly transmitted from birds to humans and cause serious lower respiratory tract damage in humans. In addition, H5N1 virus is able to infect also nasopharyngeal and oropharyngeal epithelia that apparently do not express SA $\alpha$ 2-3Gal receptor [48–50]. Similarly, the recently reported H7N9 subtype of human origin contains Gln226Leu and Gly186Val substitutions in the HA protein (which are associated with increased affinity for  $\alpha$ -2,6-linked sialic acid receptors) and Asp701Asn mutation in the PB2 protein (which is associated with mammalian adaptation) [15].

Following viral endocytosis and through pattern recognition receptors, influenza virus activates the endosomal Toll-like receptors (TLRs, specifically TLR3 and TLR7) and cytoplasmic retinoic acid-induced gene 1-like receptor (RIG-I), melanoma differentiation-associated gene 5, and NOD2, triggering type I interferon (IFN) secretion (e.g., IFN- $\alpha$  and IFN- $\beta$ ). IFN- $\beta$  stimulates the transcription of anti-viral genes, including RNA-dependent protein kinase (PKR), 2'-5'-oligoadenylate synthetases, Mx protein, and GTPases in neighboring cells through binding of the IFNAR1/IFNAR2 receptor and activation of the IFN-stimulated gene factor 3 transcription factor. Whereas TLR3 stimulation strongly induces NF- $\kappa$ B-dependent inflammatory responses, RIG-I induces antiviral responses, NOD2 activates the inflammasome complex, and TLR7 regulates the induced B-cell response. Apart from the direct damages caused by the virus replication in respiratory and nonrespiratory tissues, an intense inflammatory reaction, possibly enhanced by virus-induced cytokine dysregulation, may add to the severity of pathological changes [51–58].

Among the viral proteins of avian influenza A viruses, the NS-1 protein blocks key pathways of the innate immune response and suppresses the production of type I IFN by both airway epithelial cells and dendritic cells, thus delaying the induction of immune responses that hampers the

viral establishment. NS1 also binds and inhibits the antiviral function of protein kinase R (PKR) by downregulating the translation of the viral mRNA [59–63].

### 3.5 IDENTIFICATION AND DIAGNOSIS

In view of the nonspecific, flu-like symptoms elicited by avian influenza viruses, the diagnosis of human cases on the clinical presentations is difficult. Laboratory tests should be performed on people presenting flu-like signs with a higher risk for developing the avian flu: (1) farmers and others who work with poultry; (2) travelers visiting affected countries; (3) individuals who have touched an infected bird; (4) individuals who eat raw or undercooked poultry meat, eggs, or blood from infected birds; (5) healthcare workers and household contacts of patients with avian influenza; and (6) individuals with an acute respiratory infection and clinical, radiological, or histopathological evidence of pulmonary parenchymal disease (e.g., pneumonia or acute respiratory distress syndrome [ARDS]) and a history of close contact with a laboratory-confirmed case of avian influenza virus infection 2 weeks before illness.

Traditionally, laboratory identification of avian influenza viruses relies on virus isolation in specific pathogen-free (SPF) eggs or in cell cultures. Although virus isolation in fowl's eggs is highly sensitive and remains the gold standard for avian influenza virus detection, it is costly and time-consuming (taking 3–7 days).

The application of enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI), neuraminidase inhibition (NI), and neutralization (VN) tests has enabled differentiation of avian influenza viruses into HA and NA subtypes [64–66]. Microneutralization (MN) assay remains a valuable test for the detection of avian influenza subtype-specific antibodies in humans. However, serological tests are generally unsuitable for identifying newly emerging strains [67].

In recent years, molecular techniques have been increasingly utilized for rapid, sensitive, and specific detection and characterization of avian influenza virus RNA and proteins [68–72]. In particular, reverse transcriptase PCR (RT-PCR) has shown potential for accurate identification, subtyping, and quantitation of avian influenza viral RNA in both cultured and clinical specimens [73–106]. Other molecular procedures used for avian influenza detection include nucleic acid sequencing-based amplification (NASBA), reverse transcription LAMP (RT-LAMP), microarray, and pyrosequencing [107–116].

The common gene targets for avian influenza virus subtyping and specific detection comprise the conserved regions located in the genes encoding for the M proteins (M1&2) or the NP and the variable regions located in the genes encoding for the HA and NA proteins [117].

Considering that the first 12 nucleotides of the 3' terminus (Uni12) and the first 13 nucleotides of 5' terminus (Uni13) are conserved within avian influenza viruses, RT-PCR using Uni12 and Uni13 primers offers a powerful tool for detecting these viruses in human clinical specimens such as nasal swabs [118,119].

Degenerate primers are useful for the detection of unknown or newly emerging subtypes of avian influenza viruses. The use of five sets of degenerated primers covering the HA0 cleavage sites has enabled the detection of 16 HA subtypes of avian influenza viruses.

### 3.6 TREATMENT AND PREVENTION

Currently, two classes of antiviral agents are approved for the treatment and prevention of influenza: the M2 inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir, laninamivir, peramivir and zanamivir). The M2 inhibitors (amantadine and rimantadine) inhibit the M2 proton channel that allows for uncoating of the virus in the endosome, thereby hampering the replication of susceptible influenza A viruses and slowing down the progression to pneumonia. The neuraminidase inhibitors (oseltamivir [GS4104; Tamiflu®], zanamivir [GG167; Relenza®], laninamivir [CS08958; Inavir] and peramivir [BCX-1812 and previously RWJ-270201; Rapiacta and Peramiflu]) inhibit the virus neuraminidase and thereby prevent destruction of sialic acid-bearing receptors that are recognized by influenza virus hemagglutinins, thus stopping the virus from chemically cutting ties with its host cell.

H5N1 influenza viruses are generally susceptible to oseltamivir and zanamivir. Early treatment (within 48 h of symptoms appearing) with the antiviral medication oseltamivir (Tamiflu) or zanamivir (Relenza) may help reduce the disease severity. Oseltamivir may also be used as prophylaxis for persons in close contact with patients diagnosed with avian flu.

H5N1 strains causing human avian flu appear to be resistant to amantadine and rimantadine, which should not be used in case of an H5N1 outbreak. Oseltamivir (Tamiflu)-resistant H5N1 strains (harboring H274Y and N294S mutations in the NA protein) have been detected since 2005, although they remain sensitive to Relenza [120–122].

Most H7N9 viruses appear to be susceptible to the neuraminidase inhibitors (e.g., oseltamivir and zanamivir), but resistant to the adamantanes (amantadine and rimantadine). Therefore, amantadine and rimantadine are not recommended for treatment of H7N9 virus infection.

The Centers for Disease Control and Prevention (CDC) guidance recommends treatment for all hospitalized H7N9 cases, and for confirmed and probable outpatient H7N9 cases. In addition, outpatient cases under investigation who have had recent close contact with a confirmed H7N9 case should receive antiviral treatment, whereas outpatients meeting only the travel exposure criteria for a case under investigation are not recommended to receive antiviral treatment.

For preventive purposes, people working with birds that might be infected should use protective clothing and special breathing masks; avoiding undercooked or uncooked meat reduces the risk of exposure to avian flu and other foodborne diseases; travelers should avoid visits to live-bird markets in areas with an avian flu outbreak; and patients with severe unexplained acute respiratory disease should be tested [123,124].

As a flu vaccine (containing antigens from three or four influenza virus strains—one influenza type A subtype H1N1 virus strain, one influenza type A subtype H3N2 virus strain, and either one or two influenza type B virus strains) is available, its use will help reduce potential infection with avian influenza viruses in humans [125].

### 3.7 CONCLUSION

Members of the genus *Influenzavirus A* are segmented, single-stranded, negative sense RNA viruses that are currently distinguished into 17 H subtypes and 9 N subtypes. Due to their common occurrence in wild aquatic birds (in which no clinical symptoms are observed), influenza A viruses are often referred to as avian influenza viruses (or “avian influenza,” “bird flu”). While some avian influenza virus subtypes (e.g., H1N1, H2N2, H3N2, H5N1) are highly pathogenic and cause significant mortality in domestic fowls and serious diseases in humans, other subtypes are of low virulence. Given their tendency to undergo genetic alterations and reassortments, novel avian influenza virus subtypes that show increased capacity to cross interspecies barriers and induce severe diseases will emerge from time to time and render the control and prevention strategies that have been put in place for the existing virus subtypes ineffective and/or redundant.

The appearance of avian influenza A virus subtype H5N1 in Asia in 1997 represents an excellent example of this organism’s ability to incorporate genetic changes to outsmart host’s immune responses, resulting significant mortalities in domestic poultry and sporadic and severe diseases in humans. Between 2003 and mid-2013, H5N1 has been responsible for causing a total of 630 confirmed human cases and 375 deaths. Another example is the recently emerged avian influenza virus subtype H7N9 that has been responsible for 138 confirmed human cases in eastern China provinces, including 45 casualties between February–November 2013 [126]. Although avian influenza virus subtype H7N9 is generally considered as of low pathogenicity affecting birds only, and has not been implicated in human outbreaks until 2013, the emergence of this novel H7N9 virus reinforces this organism’s capacity to evolve and become a potential threat to human well-being. What is even more remarkable is that this novel H7N9 virus causes silent, asymptomatic infections in domestic poultry and provides no forewarning for human epidemics before the infection is established and much damage has been done [127,128].

To keep an upper hand over the pandemic threats posed by avian influenza viruses such as the highly pathogenic H5N1 and H7N9 subtypes, it is important that rapid, sensitive, and specific molecular techniques are adopted and applied in reference and clinical laboratories for identification and surveillance purposes. Further research to unravel the mechanisms of avian influenza viruses to evade host immune systems will lead to the development of new immunotherapeutics that provide enhanced protection against this organism. In addition, the continued collaboration and knowledge sharing among international and regional frameworks is critical to ensure

any future avian influenza outbreaks that will be dealt with in a timely fashion and appropriate manner.

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