PATHOGENESIS, VIRUS SHEDDING AND SEROLOGIC RESPONSE IN SELECTED DOMESTIC AVIAN SPECIES AGAINST LOW PATHOGENIC AVIAN INFLUENZA (LPAI) WILD BIRD ISOLATES

by

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(Under the Direction of Mark W. Jackwood)

ABSTRACT

This study aims to biologically characterize low pathogenic avian influenza (LPAI) wild bird isolates in selected domestic avian species. Groups of specific pathogen-free chickens and commercial turkeys were intrachoanally infected with 16 LPAI viruses. The pathogenesis, virus shedding and serologic response of these species were determined through clinical signs and histopathological lesions, rRT-PCR and ELISA and HI tests, respectively. All viruses did not cause significant clinical disease except for MN 99-263 in turkeys. Analyses of oropharyngeal and cloacal swabs demonstrated greater virus shedding from both anatomical sites in chickens compared to turkeys. Respiratory and gastro-intestinal tract lesions were predominant in both species, confirming the low pathogenicity of these viruses. More LPAI isolates caused seroconversion in turkeys than in chickens, emphasizing the greater susceptibility of turkeys to AIV infection. This study indicates that most LPAI wild bird isolates can be shed by infected birds without causing overt clinical disease or mortality.

INDEX WORDS: Low pathogenic avian influenza, Wild bird isolates, Specific pathogen-free chickens, Commercial turkeys, Virus shedding, Pathogenesis, Oropharyngeal and cloacal swabs, Quantitative real time RT-PCR, ELISA, HI test

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D.V.M., University of the Philippines, 2002

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

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August 2008

DEDICATION

Mommy, for bringing me out into this world, for her unending love, encouragement, moral support, fervent prayers and personal sacrifices all throughout these years and from whom I draw all my strength and inspiration to pursue all my ambitions.

Daddy, for the discipline, guidance and lessons in life that you have instilled upon me, to you I owe the adamant character I have. Wherever you are, I know you are happy and you are always there to watch over us. We miss you dearly.

My brothers, Alvin, Adie, Allison and Allen, for taking good care of our mother while I was thousands of miles away from home and for all those precious memories we have shared under one roof.

To all of them, this work is humbly dedicated.

ACKNOWLEDGEMENTS

One of the most pleasant and rewarding aspects of writing a thesis is the opportunity to thank those people whose immeasurable efforts greatly contributed to the completion of this endeavor. I would like to express my gratitude to the following individuals to whom I am indebted.

Dr. Mark Walter Jackwood, my academic adviser, for accepting me as a graduate student in his laboratory and for giving me the most challenging (physically and mentally) yet the best research project I have ever laid my hands on. His immeasurable patience, excellent guidance and support in and out of the lab, valuable advice and encouraging comments made me feel that this research is a worthy undertaking. Not to forget his fatherly figure which made me feel at home and at ease while working inside the laboratory. My hat's off to you, sir.

Dr. Maricarmen Garcia and Dr. Guillermo Zavala, members of my advisory committee, for their excellent support and constructive criticisms during the conception, conduct and the defense of this study.

Deborah Ann Hilt, our lab's research coordinator, the "right hand" of the big boss and the most generous chocolate supplier, what else can I say? Thank you very much for the kindness you have shown me throughout my stay in the lab. It is truly an honor to have known you.

Amber McCall and Crystal Polizzi, our lab technicians, for lending their hands in some parts of my experiments. Your presence surely makes laboratory tasks easier and faster.

Fellow graduate students in the lab, Enid McKinley, Sharmi Thor and Jamie Phillips for the camaraderie and for sharing the highs and lows of graduate school life. Thank you for treating me so nicely although I was a "rare specie" in the lab!

Dr. David Swayne (USDA-SEPRL) for endowing us with research funds and Dr. David Stallknecht (SCWDS) for providing us the LPAI wild bird isolates used in this study.

I extend my most sincere appreciation to the faculty, staff and fellow graduate students at the Poultry Diagnostic and Research Center (PDRC). Special mention to those people who had helped me in one way or another: Dr. Egbert Mundt, Dr. Susan Williams, Dr. Pedro Villegas, Dr. Stephan Thayer, Sylva Riblet, Aline Reis, Gwin Kerce, Fred Smith, Donnie Flanagan, Trinton Baird, Ann Duncan and Kim Collins.

To the Fulbright Fellowship Program and the Philippine-American Educational Foundation (PAEF), for giving me this life-changing opportunity to pursue post-graduate studies in the United States of America. My heartfelt thanks to Dr. Esmeralda Cunanan and her efficient staff for their valuable and unending support. Special mention goes out to Ms. Nora Yepes and Ms. Angela Dizon for the excellent assistance and guidance on scholarship-related matters.

Dr. Rosemarie Antegro, for being a good mentor and for taking care of my “business” back home.

Fellow Filipino graduate students at UGA: Marco (and his family), Alvin, Cesar, Joyce and Lotis, thank you very much for all the help, the fun and friendship and the memories here in Athens and for alleviating my chronic case of home-sickness.

To Nikki, for being such a great inspiration in my life.

Finally to God Almighty, the prime mover of everything, for His unconditional love and guidance, for lending me the abilities which I am capable of, for all the life-teaching trials which had molded me into my present character, for all the wonderful blessings and for the courage to pursue my daily activities and long-term goals in life.

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CHAPTER 1

INTRODUCTION AND OBJECTIVE

Avian influenza viruses continue to be a problem worldwide because they are highly infectious, can quickly spread and cause disease in domestic poultry, other animal hosts and even humans. Around the globe, hundreds of thousands of poultry deaths have resulted from direct infection with AIV while millions of poultry flocks at risk have been depopulated as a measure to contain the virus and prevent its further spread (1). Apart from the severe economic losses in commercial poultry, avian influenza viruses can evolve rapidly and cross into other species (2-4). More recently, AI viruses have been isolated from several animal species in which these are not normally found. Some of which, among others, include the incidence of highly pathogenic avian influenza H5N1 infection in wild felids (tigers and leopards) (5), domestic cats (6-9), as well as in dogs (10).

As fears for a potential pandemic rise, international public health agencies take the forefront to address the situation and assist affected countries. The World Health Organization website, as of May 28, 2008, reports 241 deaths out of 383 laboratory confirmed human cases of HPAI H5N1 since 2003 (11). The hardest hit areas are countries of the Southeast Asian region, where backyard farming of different poultry species (e.g. chickens and ducks) and/or unrelated animal species (e.g. chickens and pigs) occurs in a common area (12). In addition, the use of poultry manure as fertilizer for agricultural crops and for fish farming (13) and the use of ducks for pest control in rice paddies in Southeast Asia also contribute to the spread of AI. These, along with the selling, trading and movement (legal or illegal) of poultry and poultry products in live

bird markets (LBM) or along established trade routes (14), enhanced the spread and susceptibility of the poultry population to AIV infection as well as AIV transmission to humans.

Charadriiform (shorebirds and gulls) and anseriform (ducks, geese and swans) birds are known to be the natural hosts of AIV. These wild aquatic birds usually do not show clinical signs of disease following infection with AIV (15). Moreover, HPAI viruses in domestic poultry are thought to have evolved from low pathogenic wild bird influenza viruses through mutations (16, 17). As many of these wild birds fly great distances, they can interact with other species as well as domestic poultry and play a key role in the transmission, evolution, and pathogenesis of the virus (18). However, a long-standing debate still exists as to the exact role of wild birds in the spread of AIV to domestic poultry, with the current focus on HPAI H5N1 type viruses because of their zoonotic potential. For instance, the series of events starting from the death of thousands of wild birds in Qinghai Lake in China in May and June 2005 and the eventual spread of HPAI H5N1 to other parts of Asia, Russia, Middle East, Europe and Africa, provided evidence that wild birds, in some cases, played a role in dissemination of the virus (1). In addition, other subtypes of the virus, particularly H_4, H_6 and H_9 type viruses, are prevalent in wild birds, which may play a role in the replication and interspecies transmission of those viruses (17, 19-21).

Implication of waterfowl and shorebirds in the spread of AIVs, the prevalence of LPAI viruses in wild birds and the potential for some of the virus to mutate into HPAI in chickens warrants the biological characterization of LPAI viruses from wild birds in domestic avian species.

This study aims to biologically characterize LPAI virus wild bird isolates in selected domestic avian species. The pathogenesis, virus shedding and serologic response in specific pathogen-free chickens and commercial turkeys against these virus isolates will be examined.

This research will provide insights towards further understanding of the ecology of avian influenza viruses and will contribute information leading to the development and complementation of strategies for the prevention and control of avian influenza in domestic poultry and ultimately, in humans.

CHAPTER 2

REVIEW OF LITERATURE

Etiology

Avian influenza viruses denote avian strains belonging to the genus Influenzavirus (or Orthomyxovirus) A and are classified under family *Orthomyxoviridae* (18). The virus family derived its name from the Greek words *myxa* (mucus) and *orthos* (straight or standard; correct) due to its ability to bind to sialic acid residues in mucus and are designated as *ortho* to distinguish them from another but related group of enveloped negative strand RNA viruses, the paramyxoviruses (*para* – alternate) (22-24). On the other hand, the term influenza (an Italian word meaning influence) was derived from a medieval Latin term *influentia* and was used to describe disease epidemics that were thought to result from supernatural or occult influences (22).

Influenza viruses are classified as types A, B and C based on antigenic differences in nucleoprotein (NP) and matrix (M) proteins (22, 25). For influenza type A, viruses are further classified into defined antigenic subtypes based on the differences of the primary antigenic structure, hemagglutinin (HA) and the secondary antigenic structure, neuraminidase (NA) (16). Subtyping comprises a combination of one of the known 16 HA glycoproteins with one of the 9 identified NA protein subtypes (24, 26).

Other genera categorized within the same family (but not classified into subtypes) include Influenzavirus B and C (primarily affecting humans) (27, 28), Thogotovirus (tick-borne viruses that have genetic and structural similarities with influenza viruses) and Isavirus (causes infectious anemia in salmonids) (24, 29). While AIVs can cause disease in various bird species

(particularly domestic poultry), other hosts such as swine (30), wild and domestic felids (5, 6), humans (31) and incidental hosts may be infected as well.

Current nomenclature of flu viruses includes virus type (A, B or C), host of origin (animals or if isolated from humans, it is not included by convention), geographic location of first isolation, strain number, year of isolation and antigenic subtype (given parenthetically) (16, 22). For instance, influenza A/PR/8/34 is the 8th human influenza A virus strain isolated from Puerto Rico in 1934 (22).

Virion Structure

Influenza viruses are described as enveloped viruses with a segmented, negative sense (3' to 5'), single-stranded RNA (ssRNA) genome (22). The term negative sense denotes that its genome segments are transcribed to form complementary viral messenger RNAs (mRNA), which are positive stranded by convention (22). Considered as one of its functions, influenza viral genome segments serve as templates for mRNA synthesis, which is important for the production of viral proteins and consequently, viral replication (22). In addition, negative sense genomic RNAs are utilized as templates for the manufacture of anti-genomic positive strands (also known as complementary or template RNA) which are necessary for making additional copies of negative sense RNAs (22).

Varying from spherical or rod-shaped to long and filamentous forms depending on the nature of its host cells, the pleomorphic influenza A (and B) virus contains eight nucleoprotein-encapsidated (5) gene segments which encodes for 10 proteins (22). Influenza C viruses, on the other hand, only contain seven segments since they lack the NA protein (22). Each segment is paired with an RNA-dependent RNA polymerase complex (PB1, PB2 and PA), thus forming the viral ribonucleoproteins (16, 18, 23). These encapsidated particles are surrounded by a host cell-

derived lipid membrane from which two surface glycoproteins (hemagglutinin and neuraminidase) and an integral membrane homotetramer, matrix 2 protein (M2) emanate (Figure 1) (16, 23, 32).

Recently, PB1-F2 protein, a novel 87-amino acid influenza A virus protein, was incidentally discovered by Chen and others (33) while screening for potential antigenic peptides recognized by CD8+ T lymphocytes that are encoded by the PB1 alternative reading frame. Considered as the 11th influenza virus protein, it is variably expressed in infected host cells and localizes in the mitochondria where it induces apoptosis by permeabilization and destabilization of mitochondrial membranes (34).

Giving structural rigidity to the membrane as the most abundant protein in the virion, the matrix protein (M1) lines the inner surface of the lipid bilayer and separates the viral envelope from the ribonucleoprotein complex (16, 18, 22).

The nuclear export protein (NEP) or non-structural protein 2 (NS2) is now established as another protein expressed within the virion and was previously thought to be non-structural, hence its name (22). In contrast, the non-structural protein 1 (NS1) is only expressed in influenza virus-infected cells and has not been found in virions (16, 22).

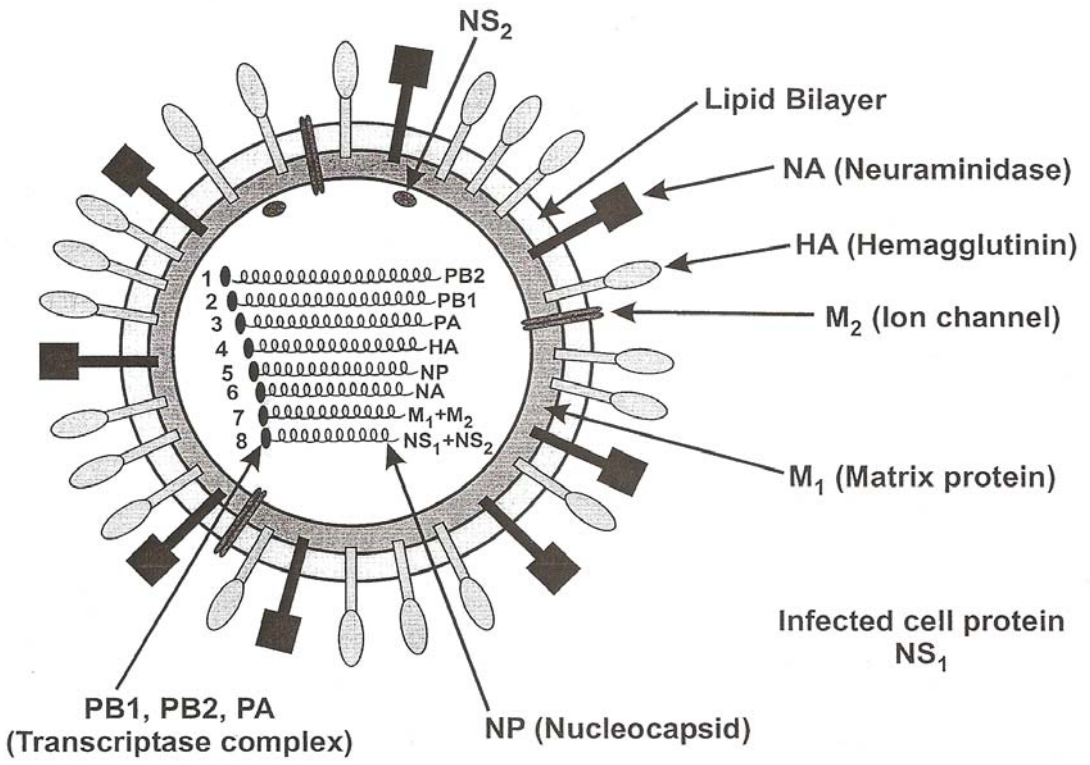

Figure 1: A schematic diagram of the influenza A virus structure [illustration taken from (22)]. Integral proteins hemagglutinin, neuraminidase and matrix 2 project from and are found within the viral envelope, respectively. Underneath the lipid viral membrane lies the matrix protein, which has a close association with the coiled (or helical) ribonucleoproteins (RNPs). Each RNA segment is encapsidated with nucleoprotein and paired with an RNA-dependent RNA polymerase complex (PB1, PB2, PA) to form RNPs. NS1 proteins are found only in virus-infected cells while NS2 (or NEP) plays a role in the export of RNPs out of the nucleus during the late stage of viral infection.

Genome Organization and Encoded Proteins

As mentioned previously, the viral genome contains eight segments. Table 1 below summarizes the protein/s encoded by each gene segment, along with its corresponding segment number (based on its nucleotide length), feature/s and a brief overview of its function/s.

Table 1: Influenza A virus genome RNA segments [taken and modified from (22)]

Segment	Length in nucleotides*	Encoded polypeptide(s)	Feature(s) and Brief Overview of Function(s)
1	2,341	Polymerase basic protein 2 (PB2)	Component of the RNA polymerase complex; cellular messenger RNA (cap) recognition of host cell RNA
2	2,341	Polymerase basic protein 1 (PB1)	Component of the RNA polymerase complex endonuclease activity; catalyzes addition of nucleotides
		Polymerase basic protein 1 F2 (PB1-F2)	Variably expressed in host cells; pro-apoptotic mitochondrial protein
3	2,233	Polymerase acid protein (PA)	Component of the RNA polymerase complex; initiation of transcription
4	1,778	Hemagglutinin (HA)	Major surface glycoprotein and antigenic determinant; receptor (sialic acid) binding activity
5	1,565	Nucleoprotein (NP)	Monomer; binds to RNA to form coiled ribonucleoprotein
6	1,413	Neuraminidase (NA)	Secondary surface protein and antigenic determinant; neuraminidase (sialidase) activity
7	1,027	Matrix 1 protein (M1)	Major protein located underneath the viral lipid bilayer; interacts with RNPs and NS2
		Matrix 2 protein (M2)	Ion channel protein essential for pH regulation and virus uncoating
8	890	Non-structural protein 1 (NS1)	Virion protein expressed only in infected cells; has interferon antagonistic activity
		Non-structural protein 2 (NS2)	Minor virion component; in close association with M1 and involved in nuclear export of RNPs

* based on A/PR/8/34 strain

A. RNA dependent RNA polymerase complex

As seen from Table 1, the three largest influenza genome segments encode PB1, PB2 and PA proteins and form a polymerase complex, collectively referred to as P proteins (35), for transcription and translation of the viral genome in the infected cell. With PB1 acting as the backbone of the complex, the polymerase functions as a trimer which contains a copy of each of its three components (36). Moreover, a recent study that determined its 3D structure has a very compact structure, with no apparent boundaries among subunits (37). Hence, protein to protein interactions among each component are likely to be functionally significant and mutually interdependent, such that factors affecting one component of the polymerase complex may affect the activity of one or more of its components (36, 38). Each protein is discussed below.

Segment 1 – Polymerase Basic Protein 2 (PB2)

The first segment of the influenza A virus encodes one of the three components of the RNA-dependent RNA polymerase, the polymerase basic protein 2 (23). Along with the PB1 and PA, these three proteins are named based on their behavior in isoelectric focusing gels. PB1 and PB2 were found to have a basic charge and PA has an acidic charge (22).

Influenza viruses, unlike other RNA viruses, require the host cell nucleus for viral transcription and translation (39). Therefore, polymerase proteins must be transported to its site of function, whether individually or as a part of a complex. PB2 was found to have bipartite nuclear localization signals necessary for its transport into the nucleus of the infected host cell (40).

In association with the PB1 and PA proteins, PB2 plays a vital role in the recognition and binding to methylated cap structures present in the 5’ end of cellular mRNAs (or pre-mRNAs) (41-43). Its endonuclease activity cleaves 10-13 nucleotides from the bound cap and generates a

short-capped RNA fragment, which acts as a primer for viral mRNA synthesis (44). Thus, PB2 is the main polymerase protein responsible for the initiation of transcription (45). This cap-snatching mechanism of influenza viruses has resemblance to the cap binding process of other well-studied viral proteins (46, 47). Though this mechanism is not completely understood, attempts to determine influenza PB2 regions responsible for cap binding yielded contrasting results. Several amino acids or amino acid sequences have been identified as critical for the cap snatching mechanism through mutagenesis and cross-linking studies (48-50).

Studies have shown that PB2 also plays a role in host range restriction (51, 52) and is suggested to be a major (but not the sole) determinant in the pathogenicity of influenza A virus (53). Several authors demonstrated that a mutation from a glutamic acid (found in avian isolates) to lysine (found in human isolates) at position 627 enhanced viral replication and virulence in non-human primates (squirrel monkeys) (54), humans (55) and mice (53). However, this change has not been associated with increased virulence in other animal models such as ferrets (56) and some severe and fatal cases of human influenza (57).

Segment 2 – Polymerase Basic Protein 1 (PB 1)

Encoded by segment 2 of the viral genome, the PB1 subunit of the RNA polymerase complex is required for the initiation and elongation of newly synthesized viral RNA (vRNA) (58, 59). Possessing independent binding sites for other polymerase components, the N-terminus of PB1 interacts with the C-terminus of PA while the C-terminus of PB1 binds with the N-terminus of PB2 (60). This suggests that PB1 is the core (or backbone) among the three polymerase proteins, hence playing a key role in the assembly of the polymerase complex (61, 62) being responsible for RNA chain elongation (63).

Along with these binding sites, PB1 also possesses four conserved motifs which are present among all viral RNA polymerases. Mutational analyses of these motifs showed that all play important roles in PB1 activity such that changes in these conserved regions abolish transcriptional activity (64) thus, polymerase activity of PB1 is extremely sensitive to structural modifications (38). Furthermore, in experiments using cell free systems (i.e. nuclear extracts from infected cells expressing PB1), it was shown that the PB1 protein was able to catalyze RNA activity (sequential addition of nucleotides to the growing RNA chain), which supports the concept that it is the catalytic subunit of the viral polymerase (65).

In addition to its nucleotide polymerase activity, it was suggested that the endonuclease activity of the viral polymerase resides in its PB1 subunit and not the PB2, as mentioned previously. Through UV light cross-linking and mutagenesis studies, Li and co-workers (49) demonstrated that endonuclease cleavage activity resides in a PB1 sequence that contains 3 essential amino acids, which is similar to the active sites of other polymerases that cut polynucleotides (DNA or RNA) to produce 3'-OH ends, generating capped fragments which serve as primers to initial viral mRNA synthesis. Hence, although the PB2 subunit contains the cap binding motif, the actual cleavage is performed by the PB1 subunit (66). Also, PB1 possesses two discontinuous regions necessary for nuclear localization (67).

Some influenza A virus PB1 genes possess an alternative reading frame containing the PB1-F2 polypeptide, so named because it is encoded by a second open reading frame of the PB1 gene (33). As previously mentioned, this 87 amino acid peptide localizes to the mitochondria (33) where it induces apoptosis. It was later shown that the basic amphiphatic helix in its C-terminal region was responsible for its mitochondrial localization (68). PB1-F2 was found to cause cell death to its host cell through interaction with 2 mitochondrial apoptotic mediators,

adenine nucleotide translocator 3 (ANT3) (at the inner mitochondrial membrane) and the voltage-dependent anion channel 1 (VDAC1) (at the outer mitochondrial membrane) (69). Besides its mitochondrial localization properties, PB1-F2 was also found to have other unique features such as its non-expression in some animal isolates (those of porcine origin), variable expression in individual infected cells and rapid proteosome-dependent degradation (33, 70).

It has been shown recently that PB1-F2 contributes to viral pathogenesis in the mouse model by preventing effective viral clearance in the lungs, thus, delaying the acquired immune response to the infection (69). In another study, McAuley and co-workers (71) showed that it enhances inflammation during primary viral infection of mice and increases both the frequency and severity of secondary bacterial pneumonia. The authors also engineered a PB1-F2 protein expressing influenza virus containing coding changes matching the 1918 pandemic strain. Results showed that the virus was more virulent and induced more pulmonary immunopathology, leading to more severe secondary bacterial pneumonia in mice (71). Hence, the authors suggested that these findings help in explaining the unparalleled virulence of the 1918 strain and the high incidence of fatal pneumonia during that pandemic.

Segment 3 – Polymerase Acid Protein (PA)

Considered as the smallest subunit of the influenza RNA polymerase complex, the PA protein is encoded by the 3rd segment of the viral genome and is the least characterized among the three in terms of function. Its exact role in the influenza virus replication cycle is not known, although early studies of its temperature-sensitive mutants implied a function in viral RNA replication (66). Like the other influenza viral polymerase subunits, it also contains nuclear localization signals required for transport into the nucleus (23). However, mere PA protein expression cannot result to its complete nuclear localization unless it is co-expressed with PB1 or

NS1 (72). This finding was later supported by Fodor and Smith (73) who demonstrated that PB1 requires the co-expression of PA for efficient nuclear accumulation. This implies that PB1 and PA are transported into the nucleus as a complex (73).

This protein has also been recently implicated in the initiation of RNA transcription, possibly by direct participation in the endonuclease cleavage of cellular pre-mRNAs (66). In this study, the authors found that a single PA mutation from histidine to alanine at amino acid position 510 resulted in the inhibition of the transcriptional activity of the polymerase complex in vivo, signifying its important role for transcription initiation (66). A year later, the same authors demonstrated that a single amino acid mutation from arginine to alanine at position 638 promotes the generation of defective interfering RNAs and highlights the role of PA in maintaining appropriate interactions between the RNA polymerase complex and the RNA template. Thus, the authors concluded that the PA subunit could act as an elongation factor (74).

The PA subunit is also known to bind with PB1 but not with PB2 (61), as mentioned previously. It induces a generalized proteolysis and reduces the half-lives of its own accumulated levels and its co-expressed viral proteins (75) but the significance of this proteolytic activity remains unclear (76). Functional analysis of PA deletion mutants identified the amino-terminal one-third (first 247 amino acids) of this protein to be responsible for the protease activity (77). Furthermore, the authors hypothesized an existing correlation between the nuclear localization of the PA protein and the induction of proteolysis, such that the former could be a pre-requisite of the latter (77).

B. Other genome segments and its encoded viral proteins

Segment 4 – Hemagglutinin

Hemagglutinin glycoproteins, along with NA, are embedded within and project from the external viral envelope. A rod-shaped homotrimer encoded by the 4th segment of the influenza A virus, HA is approximately 4 to 5 times the number of the mushroom-shaped NA tetramer and is the major antigen responsible for viral attachment to the sialic acid-containing receptors on the host cell surface, thus initiating virus-cell interaction (22). It also mediates viral entry into the cytoplasm through membrane fusion after endocytosis (78). Moreover, it is considered as a major target for the generation of host neutralizing antibodies and consequently undergoes antigenic variation, which translates to recurrent influenza epidemics (22, 79).

Synthesized as a precursor polypeptide HA_0, it is post-translationally cleaved into 2 disulfide-linked subunits, generating the C terminus of the HA_1 and N terminus of the HA_2. Cleavage is essential for viral infectivity and allows conformational change, leading to release of the fusion peptide, which is important for membrane fusion (22, 80). Generally, the precursor polypeptide is believed to be cleaved by trypsin-like extracellular proteases but the presence of multiple basic amino acids within the site of cleavage renders it susceptible to action of ubiquitous intracellular proteases such as furin (22). Influenza viruses containing HA sequence with insertions of polybasic amino acids adjacent to the cleavage site are regarded as highly pathogenic, allowing infection of a broad range of host cells and cause acute, fatal systemic infection (22). On the contrary, those viruses with two basic amino acid insertions within the HA cleavage site are regarded as low pathogenic, limiting its ability to spread in a host and cause anatomically localized infections with relatively mild symptoms (23, 81, 82). Hence, the

distribution of proteases in the host and the HA cleavage site sequence appear to be the prime determinant of virus pathogenicity (78).

Segment 5 – Nucleoprotein

The nucleoprotein is encoded by segment 5 of the viral genome and is described as a phosphorylated basic protein having a net positive charge at neutral pH. A type-specific antigen, NP encapsidates the viral RNA segments to form RNPs wherein it is the most abundant component (22, 83). It is considered as one of the essential components for transcription and replication (23), having a structural role of maintaining the RNA template in an ordered conformation that is suitable for transcription and/or virion packaging (83).

The potential role in the switch from mRNA transcription to genome replication is a function of NP that has received considerable attention (83). Several lines of evidence indicate NP plays a role in controlling the switching of RNA polymerase from transcription to replication (23). However, the mechanism by which NP might control such switching is not clearly understood (23). A study on a temperature-sensitive NP mutant A/WSN/33 ts56 virus by Shapiro and Krug (84) demonstrated that: 1) nuclear extracts from mutant virus-infected cells failed to synthesize template or complementary RNA (but not mRNA) at a non-permissive (39.5^{o}C) temperature and, 2) *in vitro* synthesis of full-length viral RNA was found to be dependent on a pool of soluble NP. Similarly, several NP temperature sensitive mutants that are defective for replication transcription have been isolated (85, 86). Other authors suggest that the nucleoprotein regulates viral RNA replication through interaction with the viral polymerase subunits (86).

Viral RNA transport across the nuclear membrane is another important function of NP (23). Like the components of the polymerase complex, it possesses nuclear localization signals (87) that regulate its entry into the nucleus during the early stage of viral infection (mediated by

interaction of NP and host cell importin α) (88) and its export into the cytoplasm for viral packaging in the late stage of infection, where progeny vRNAs associate with NP, M1 and NS2 proteins (23, 89). Phosphorylation also plays a role in the regulation of ribonucleoprotein transport (90). Recently, Gabriel and co-workers (91) suggested that the interaction of NP and PB2 with importin α (a component of the nuclear pore complex) plays a role in host range restriction.

Segment 6 – Neuraminidase

Encoded by the 6th segment of the virus and having structural resemblance to a mushroom, neuraminidase is a small homo-tetrameric glycoprotein and is considered as a second major antigenic determinant for neutralizing antibodies (32). It has a receptor-destroying activity that catalyzes the cleavage of a glycosidic linkage between virion surfaces and a terminal sialic acid on the host cell, thereby preventing virion aggregation and promoting release of virus progeny from infected cells (23, 25). The general assumption that NA plays an important role in the release of viral progeny from infected cells in the final stage of influenza infection became the basis for the development of neuraminidase inhibitor drugs which block the NA enzyme active site, thus limiting infection of new host cells and viral spread (92).

The role of NA activity in influenza virus replication, however, is still unclear. Through examination of the binding, entry, protein synthesis and virion assembly of an NA-deficient virus in Madin-Darby canine kidney (MDCK) cells and its replication and infection in a mouse model, Liu and others (93) demonstrated that aggregated NA-deficient viruses are still infectious in cell culture and in mice, suggesting that NA activity is not a requirement for influenza virus entry, replication and assembly, findings of which was later supported by Hughes and co-workers (94). In contrast, Matrosovich and co-workers (95) showed that NA plays a role in early stages of

influenza infection by adding oseltamivir carboxylate (a neuraminidase inhibitor) at different time points after influenza virus infection of human tracheo-bronchial epithelial (HTBE) cells.

Segment 7 – Matrix Proteins (M1 and M2)

The 7th segment of the influenza virus encodes two proteins, M1 and M2. The M1 protein is a colinear transcription product of segment 7 while the M2 protein is encoded by the spliced mRNA of the same segment (23).

1. M1 Protein

As mentioned previously, the M1 protein lies underneath the viral envelope and provides structural rigidity to the virion, being its most abundant component (22). It separates the RNPs from the viral membrane and interacts with both the viral RNA and RNP in the assembly and budding of progeny virions (23).

Several functions have been attributed to the M1 protein. It has an RNA-binding property specific for single-stranded RNA (96) and inhibits viral transcription catalyzed by ribonucleoprotein complexes (97). This protein also contains a nuclear localization signal (98) and modulates the directionality of vRNA transport into and out of the nucleus (99). A study by Bukrinskaya and co-workers (100) found that the dissociation of vRNP with M1 protein is necessary for the entry of RNPs into the nucleus after infection of the host cell. However, at the late stage of infection, M1 protein must enter and associate with the vRNPs inside the nucleus and have the latter transported out for virus assembly, in association with NS2 or nuclear export protein (NEP) (99, 101, 102). Hence, its interaction with vRNP determines whether vRNPs will be transported into or out of the nucleus (101). However, factors such as presence of acidic pH in the cytosol (101) and high temperature (41^{o}C) affect this interaction (102).

M1 protein also affects viral budding and viral morphology. A study by Gomez-Puertas and co-workers (103) demonstrated that the M1 protein is the major virus assembly organizer and the major driving force in the process of virus bud formation. Likewise, it has been suggested that the matrix protein plays an important role in influenza virion morphology (104), although other viral proteins such as hemagglutinin and neuraminidase may contribute (105).

2. M2 Protein

The M2 protein is an integral homotetrameric membrane protein that is abundantly expressed at the surface of virus-infected cells (106). Its gene encodes a 97-amino-acid protein composed of 24 amino acids in the amino terminal domain, 19 trans-membrane amino acids and 54 amino acids in the carboxy-terminal domain (107). M2 is activated at an acidic pH and plays important roles in both early and late stages of virus infection (108). Furthermore, it possesses an ion channel activity (109), wherein among the amino acid residues in its trans-membrane domain, histidine (His37) and tryptophan (Trp41) were found essential for pH-regulated proton conductance (108).

M2 permits the entry of protons into the virion such that acidification of the interior of the incoming viral particle occurs. This process is believed to be important for viral replication since it promotes the dissociation (disruption of protein-to-protein interactions) of the vRNPs from the M1 proteins for nuclear import, as mentioned previously (100, 101). Furthermore, it can affect the status of the conformational form of cleaved HA during intracellular transport (110). M2 modulates the pH of intracellular compartments to prevent acid-induced conformational changes of intracellularly cleaved HAs in the trans-Golgi network, hence stabilizing its form during its transport for viral assembly (110). Studies on M2 protein mutants indicated that different domains of the ion channel are involved in different viral processes (23). The amino

terminal domain of M2 protein, for instance, is important for its incorporation into the virions (107). On the other hand, its carboxyl-terminal residues play an important role in virus replication (111).

Adamantane derivatives, such as amantadine and rimantadine, have been used successfully for the prevention and treatment of influenza A virus infection for more than 30 years (112). These drugs, particularly amantadine, inhibit influenza A virus replication by blocking the M2 protein ion channel via insertion of its active form between amino acids valine (position 27) and serine (position 31) (also known as steric hindrance) (113). This prevents the fusion of viral and host cell membrane and the eventual release of viral RNA into the cytoplasm of infected cells (112). As mentioned previously, the M2 ion channel activity is situated in the trans-membrane region (108) and is also the main site of amino acid changes (seen in residues 27, 30, 31, and 34) found in amantadine-resistant mutants (114). A study on influenza virus mutants without the M2 trans-membrane domain showed that such viruses can still undergo multiple cycles of replication without M2 ion channel activity (115). However, this domain is still essential for efficient viral replication in tissue cultures (116).

Segment 8 – Non-structural proteins (NS1 and NS2)

The 8th RNA segment of the virus encodes NS1 and NS2. The co-linear mRNA transcript encodes NS1 while the spliced mRNA encodes NS2.

1. NS1 protein

As mentioned previously, the NS1 protein is found in large amounts in virus-infected cells but not in virions, hence its designation (22). Existing as an oligomer, it consists of 202-237 amino acids (22), contains two nuclear localization signals (117) and a nuclear export signal (118). A multifunctional protein participating in both protein-to-protein and protein-to-RNA

interactions, it binds non-specifically to double-stranded RNA (dsRNA) and to specific protein targets (119).

Two functional domains have been identified for the NS1 protein. Its N-terminal structural domain (or RNA binding domain) protects the virus against the host anti-viral state induced by interferon α/β (119). On the other hand, its C-terminal domain (or effector domain) inhibits the maturation and export of host cellular anti-viral mRNAs (119). The effector domain is crucial for the function of the RNA binding domain and the dimerization of these domains is essential for the interaction of NS1 with RNA or cellular proteins (119).

NS1 is also known to mediate a generalized cellular pre-mRNA retention in the nucleus and might act as a negative *trans*-regulator of its own mRNA splicing (120). By blocking the nuclear export of cellular mRNAs, NS1 shuts off cellular gene expression, hence making these mRNAs accessible to the viral cap-dependent endonuclease for the production of capped RNA primers needed for viral mRNA synthesis (121, 122). Moreover, it enhances the rate of viral protein expression by stimulating the translation of viral mRNA (23, 121). However, a study done by Garcia-Sastre and co-workers (123) showed the generation of an NS1 deficient-influenza A virus in interferon-deficient cells, suggesting that the NS1 protein is an additional virulence factor in inhibiting the interferon-mediated antiviral responses of the infected host.

The NS1 protein of H5N1 avian flu viruses isolated from Hong Kong in 1997 reportedly induced high levels of pro-inflammatory cytokines in human macrophages, notably the tumor necrosis factor alpha (TNF α) and interferon beta (IFN β) (124). This protein also enables the virus to circumvent the anti-viral effects of these cytokines, thus conferring resistance (125). Although a cytokine storm (dysregulation of cytokines) has widely been hypothesized to be the main cause for the pathology (and ultimately death) in H5N1 infections, a study by Salomon and

co-workers (126) in mice demonstrated that inhibition of the cytokine response does not protect against H5N1 infections and that therapies targeting the virus, and not the cytokines, are suggested.

2. NS2 protein

Encoded by spliced mRNA, the NS2 protein was originally thought to be a non-structural protein (22). However, studies have reported the association of a low amount of this protein in purified virus samples (127, 128). Containing a nuclear export signal (NES) in its amino terminal region, this protein facilitates the nuclear export of viral ribonucleoproteins through its interaction with the cellular nuclear export factor CRM1, which mediates the export of proteins containing classical NES (129, 130). Furthermore, the carboxyl-terminal region of NS2 contains a M1 protein-binding site. Its interaction with M1 is suggested to be involved in the regulation of M1's functions in mRNA, cRNA and vRNA synthesis (128). Based on its role as the viral nuclear export protein, it is now referred to as the nuclear export protein (NEP) (129).

Replication Cycle of Influenza Viruses

A. Virus Adsorption, Entry and Uncoating

Influenza viruses infect cells through the binding of its HA molecules to the sialic acid residues found on the cell surface glycoproteins or glycolipids (22). After binding, the attached virion enters the cells via receptor-mediated endocytosis. The virus enters the cell through a clathrin-coated membrane-bound vesicle (Figure 2), which is removed once it fuses with the endosomes that become increasingly acidic during its late stages (22, 80). Once a virion particle is endocytosed, the low pH in endosomes activates the M2 protein ion channel, permits the flow of ions from the endosome to the virion interior and disrupts the protein-to-protein interaction between the matrix protein and the viral ribonucleoproteins (22). Moreover, the M2 ion channel

activity triggers a conformational change in the HA molecule, rendering it competent to mediate fusion of the viral envelope with the membrane of the endosome and leads to the release of virion contents into the cytoplasm of the host cell (uncoating) (2, 63, 91).

B. Viral mRNA synthesis

Once the virion contents are released to the cytoplasm, the vRNPs migrate into the host cell nucleus using the nuclear localization signal of the nucleoprotein (5) (87). In the nucleus of infected cells, the viral RNAs are transcribed into messenger RNA and copied to produce new vRNAs, as illustrated in Figure 2.

The viral polymerase complex (PB1, PB2 and PA) begins the transcription of vRNAs which requires the initiation by host cell (cap) primers (m^7GpppX^m-containing RNA fragments) derived from host cell RNA polymerase II transcripts (22). PB2 binds to the 5' end of the methylated cap of the actively transcribed cellular mRNAs and a cap-dependent endonuclease activity in PB1 cleaves these capped RNAs 10-13 nucleotides from their 5' end (preferentially after a purine residue) (131). The cleaved products are used as primers for the initiation of viral mRNA synthesis (also known as cap snatching) (22, 44). Transcription (mRNA synthesis) is then initiated by incorporating a G residue complementary to the penultimate C residue on the vRNAs (22) while chain elongation is carried out by the PB1 subunit (64). Transcription continues up to a point where a stretch of uridine residues (located 15-22 nucleotides from the 5' end of the vRNA) is reached. At this stretch, transcription terminates (apparently resulting from the repeated copying of the uridine residues), hence polyadenylate (poly A) residues are added to the 3' ends of the viral mRNAs (22). In summary, it is during transcription where the polymerase complex transcribes the negative-sense viral RNAs into positive-sense mRNAs.

C. Viral RNA replication

Replication of viral RNA involves two steps: 1) synthesis of template or complementary RNAs (positive sense) and 2) viral RNA synthesis (negative sense) (22). The mechanism for the transition from viral mRNA synthesis (primed initiation) to viral RNA (unprimed) replication is not well understood (23). As mentioned previously, there is evidence which indicates that nucleoproteins play a role in controlling this switch mechanism. The production of complementary RNAs was shown to require the presence of free and soluble NPs not associated with nucleocapsids (84). Important for the cessation of cap snatching and anti-termination at poly A sites, NP molecules are hypothesized to bind to the common 5' ends of the growing RNA transcripts (22). Moreover, the addition of more NPs to the elongating chain of RNA prevents the viral mRNA sequence from slipping backward along the vRNA template and blocks the repeated copying of the stretch of U residues in the vRNA template (22).

As mentioned previously, the second step of replication involves progeny vRNA synthesis via the copying of the cRNA template. This stage is primer-independent (does not need PB2 protein for its cap binding activity, hence an unprimed synthesis), requires the presence of PA protein and entails the addition of NP protein to the elongating vRNA (22). This results into producing vRNAs (in nucleocapsid form) which are ready for packaging into progeny virions (22). The transport of viral RNPs is facilitated through its formation of a protein complex with M1 and NS2 (or NEP). Overriding the nuclear localization signals (NLS) found on the polymerase complex and the NPs, the nuclear export signal found on NS2 allows the migration of the vRNPs from the nucleus through its interaction with the chromosome region maintenance 1 protein, a nuclear export factor and the Ran-GTPase protein that shuttles between the nucleus and the cytoplasm (2, 114).

The production of viral proteins is regulated to the time they are required during replication and in relation to the amounts of structural components necessary for virion assembly (132). Based on the quantities of the individual and transcribed mRNA segments, influenza virus infection can be divided into an early and late phase of gene expression (22).

The early phase corresponds to the stage of vRNA replication and amplification of mRNA production wherein the synthesis of specific vRNAs, viral mRNAs and viral proteins are coupled (22). Following primary transcription, equimolar amounts of positive-sense template RNAs are made and from where specific template RNAs are selectively transcribed into vRNAs (22). NS1 and NP vRNAs are preferentially synthesized early due to their involvement in the regulation of transcription and replication of vRNAs whereas the M1 viral mRNA and protein synthesis are both delayed (22).

On the other hand, the late phase of gene expression involves the production of virion structural components (22). Synthesis of all viral mRNAs reaches its peak (but later drops drastically) during the early onset while viral RNA production remains high during this phase (22). M1 proteins are also produced at increased rates along with other structural proteins during this period. It is hypothesized that the synthesis of M1 protein is delayed since it stops the transcription of viral RNA into viral mRNA (97) and is involved in the transport of RNPs from the nucleus to the cytoplasm (22).

Post-transcriptional splicing of NS1 and M1 proteins via the exploited host cell machinery generates functional mRNAs encoding for NS2 and M2, respectively (22). NS splicing is regulated by the *cis*-acting sequences in NS1 mRNAs while both viral and cellular proteins control the splicing of M1 mRNAs (22). Only about 10% of the (NS1 and M1) mRNAs produced are spliced, the amount of which is determined by the rates of splicing versus the rates

of nuclear export of these mRNAs (22). All the spliced and unspliced viral mRNAs are exported to the cytoplasm for translation (22).

During influenza infection, the virus takes over the host cell translation mechanism in two ways (22). First, the NS1 protein suppresses the type I interferon-induced anti-viral state of the host to ensure efficient translation of virus-specific proteins in infected cells (22). Moreover, it prevents the translation of cellular mRNAs by promoting the selective translation of viral mRNAs (22).

D. Virion Assembly and Release

Synthesized on membrane-bound ribosomes, the HA, NA and M2 proteins are translocated across the endoplasmic reticulum (ER) in a signal recognition particle (SRP)-dependent manner (22). The HA's N-terminal signal sequence is cleaved in the ER by signal peptidase while the other two surface proteins do not have such signal sequences (22). After N-linked carbohydrate chains are transferred to HA and NA, these proteins are folded, assembled correctly and transported from the ER to the Golgi apparatus for further processing of its oligosaccharide chains (22). As the last step in intracellular transport, the HA, NA and M2 proteins are expressed at the plasma membrane (Figure 2). The HA is distributed over the surface while the NA and M2 proteins tend to cluster in patches (22).

Critical for both the survival of the virus and its disease-producing ability in the host, the assembly and budding of virus particles are the last but important steps in the virus life cycle (22). Without completing these steps, infected cells will undergo abortive infectious cycles without releasing the complete virus particles (22).

Virion morphogenesis is a complex phenomenon requiring the concerted actions of many viral and host components (133). It is said that the precursor to the envelope of a budding virion

is a patch of cell membrane containing viral envelope proteins (HA, NA and M2), which are believed to determine the site of virus assembly and budding (133). Both HA and NA have been shown to interact with lipid rafts in which vRNA-M1-NS1 complexes exiting the nucleus are transported to (2, 9, 116).

Virus assembly and budding requires the interaction between the viral envelope, vRNP and M1, the latter serving as a bridge between the two (133). M1 is believed to be the key protein in recruiting and assembling viral and host components required for budding at the assembly site of the plasma membrane (133). It interacts with itself, forming dimers and multimers to assist in containing the viral components while excluding host proteins (133). Furthermore, M1 interacts with the inner leaflet of the lipid bilayer and creates asymmetry that causes outward bending for the initiation of bud formation (133). The fusion of the opposing viral and cellular membranes leads to bud formation and closure (22). The virus particle is then completely released into the extracellular environment to infect other cells through the enzymatic activity of neuraminidase (22, 133).

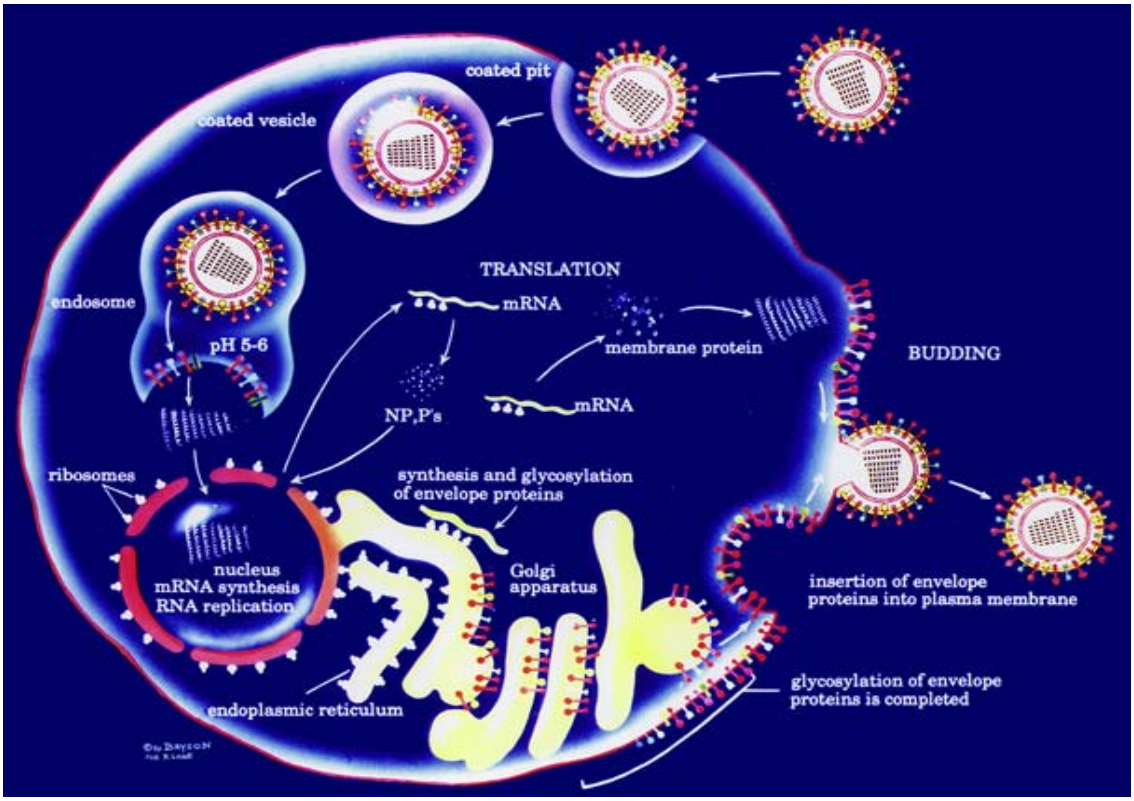

Figure 2: A summarized illustration of the replication cycle of influenza A virus from its attachment to the host cell surface to its eventual release from the plasma membrane [drawing taken from (134)].

Genetics of Influenza Viruses

Masters of disguise, influenza viruses use two main ways to escape host defenses, antigenic shift and antigenic drift (16).

A. Antigenic Drift

Antigenic drift is characterized by slight changes in the antigenic structure resulting from accumulation of amino acid changes occurring within the HA and NA (16, 22). Gradually occurring over a long period of time, antigenic drift results from the inherent lack of proof-reading and error repair mechanisms during virus replication (16), in which substitutions are recognized and selected for by immune pressure (22). Point mutations enable the virus to escape

host defenses since antibodies generated from previous strains (through infection or vaccination) are rendered ineffective (22). Antigenic drift accounts for the nature of seasonal flu epidemics in humans each year (16).

B. Antigenic Shift

Antigenic shift, another mechanism utilized by influenza viruses to evade host defenses, can occur via several means. First, it can result from the simultaneous infection of 2 influenza strains of different subtypes (can be both avian or a combination of an avian and a human flu virus) which combine via genetic reassortment in an infected host (16). This creates a novel and distinct subtype with a combination of HA and NA from both parental strains (22). Antigenic shift is capable of causing unpredictable pandemics wherein the general population is rendered immunologically naïve to the new subtype, as seen with the previous influenza epidemics of 1918-1919 (Spanish), 1957 (Asian) and 1968 (Hong Kong) (16, 22).

History

The earliest written reports of a disease that might have been caused by influenza virus were made by both Hippocrates and Livy in 412 BC, and over the centuries, numerous accounts of epidemics and pandemics have been reported (135). In 1878, Eduardo Perroncito first identified the disease in animals, and described it as “an initially mild disease in domestic birds that after a while turned highly pathogenic, killing virtually all the birds” in Italy (135). In 1901, two other Italian scientists, Centanni and Savonuzzi, identified “Fowl Plague,” so then called, to be a viral disease, but it was not until 1955 that the influenza virus was identified as the causative agent (16).

The first indications that aquatic bird species could be hosts for influenza came in 1972 when antibodies to human influenza NA were identified in Australian pelagic birds. Shortly

after, influenza viruses were isolated from a shearwater and healthy wild ducks (136). Avian influenza viruses are found in waterfowl, shorebirds and gulls, which are its natural reservoirs (136). Swine, horses, humans and sea mammals are aberrant hosts in which increased virulence occurs (137). Although chicken and turkey isolates were grouped with avian isolates, it may be more appropriate to consider these species as aberrant hosts (137).

Between the years 1959 to 1999, 18 outbreaks of avian influenza (caused by highly pathogenic avian influenza) were reported in domestic poultry around the world (138). The impact on the economy for the affected countries was enormous. Millions of raised birds died from the disease or were culled to stop the outbreaks. The first documented HPAI outbreak in the wild bird population was in 1961, where the first avian influenza virus (H5N3) was isolated in Common Terns (*Sterna hirundo*) and killed about 1600 birds in South Africa (137, 139). HPAI viruses are believed to have evolved from low pathogenic wild bird influenza A virus strains that circulate widely in wild birds (1). These highly pathogenic strains were then transmitted back into the wild bird population on several occasions (1). As a result, wild birds not normally seriously affected by influenza virus infection by LPAI virus strains have become ill or died (16).

Low pathogenic avian influenza causes minor sickness or no noticeable signs of disease, and is rarely fatal in wild birds. Evidence of LPAI H5N1 has been found in wild birds in the United States in recent years and is not closely related to the more severe HPAI H5N1 circulating overseas. Examples of historical reports of LPAI H5N1 received by the United States Department of Agriculture are shown in Table 2 (140).

Table 2: Historical reports of LPAI H5N1 received by the USDA [taken from (140)].

YEAR	SPECIES	LOCATION
1975	Wild mallard duck and wild blue goose	Wisconsin
1981 and 1985	Sentinel ducks	Minnesota
1983	Ring-billed gulls	Pennsylvania
1986	Wild mallard duck	Ohio
2002	Turkeys	Michigan
2005	Ducks	Manitoba, Canada
2006	Mute swans, mallard ducks, green-winged teals	Michigan, Maryland, Pennsylvania, Delaware

Influenza A virus was not considered to be a zoonotic disease agent of any importance for some time, even though people suffering from conjunctivitis after being in contact with animals sick with influenza A virus, or when working with highly pathogenic influenza A isolates in the laboratory have been documented (16). But in 1997, a serious outbreak on influenza in chicken farms caused severe respiratory disease in 18 Hong Kong residents positively infected with highly pathogenic H5N1 viruses and six deaths. This outbreak provided the first evidence that avian viruses could be transmitted directly to humans without prior reassortment in a mammalian host or with a human virus, and could cause severe disease (141). Outbreak investigation determined that close contact with live infected poultry was the source of human infection and that the virus had been acquired directly by humans from birds. Rapid destruction of Hong Kong's entire poultry population of 1.5 million birds reduced opportunities for further direct transmission to humans (142). Soon after, a fowl plague outbreak occurred in The Netherlands killing one veterinarian and caused influenza-like illness in 89 persons. HPAI

subtype H7N7, closely related to LPAI, was isolated from infected chickens and detected subsequently in patients (55). More than 30 million birds were killed at the cost of several million euros (55). Unlike HPAI, LPAI strains are not a human health concern including LPAI H5N1.

The worst outbreak of HPAI in modern times is currently plaguing the world. According to the World Health Organization, from the years 2003 to May 2008, the cumulative number of confirmed human cases of H5N1 was 383, of which 241 have died in 14 countries (11). Furthermore, the outbreak of the same H5N1 subtype that caused disease in Hong Kong in 1997 started in Southeast Asia and subsequently spread to most parts of Eurasia and several countries in Africa (16). To date, outbreaks of highly pathogenic form have been limited to subtypes H5 and H7. Highly pathogenic viruses have no natural reservoir and instead, emerge by mutation when a virus carried in its mild form by a wild bird is introduced into poultry (17). The previously stable virus begins to evolve rapidly and can mutate into a highly lethal form of the same initially mild strain (143). The ongoing outbreak has sparked fears of an imminent pandemic in humans. The possibility that humans, if concurrently infected with human and avian influenza strains could serve as a mixing vessel for the emergence of a novel subtype that has the ability to be easily transmitted from person to person increases as more humans become infected (16).

Host Range

Influenza viruses have been shown to infect a great variety of birds including free-living birds, captive-caged birds, domestic ducks, chickens, turkeys and other domestic poultry (144). In North America, domestic turkeys, domestic and wild ducks, domestic and exotic imported

birds and ratites are primarily infected while it is occasionally in chickens, quail and game birds (145).

Avian influenza infection in poultry may have a variety of origins and factors involved. Its incidence and distribution varies considerably with species, age of bird, geographic region and time of the year (82). Moreover, the prevalence of AIV in host-reservoir systems, susceptibility of poultry to circulating subtypes, the number and degree of contacts between the reservoir and poultry and the nature of biosecurity measures are some of the risk factors involved in the transmission of avian influenza (82). Likewise, the alteration of natural bird environments paved way for the creation of man-made ecosystems (e.g. integrated indoor commercial poultry, range-raised commercial poultry, live poultry market, backyard and hobby flocks and bird collection and trading system) that have influenced AI virus ecology (146).

A. Molecular Determinants of Host Range and Pathogenesis

Host range and pathogenesis are polygenic traits (135). Influenza A viruses show host restriction or host adaptation (3), wherein its gene segments may play significant roles (22).

The HA gene plays a central role in the pathogenicity of avian influenza viruses (22) since its cleavability is an important determinant of virulence (132). Based on the virulence in chickens using an intravenous pathogenicity test (IVPI), AI viruses are classified into 2 pathotypes, low and high (3). According to the OIE Terrestrial Animal Health Code (147), HPAI viruses are defined as follows: 1) an infection of poultry caused by any influenza A virus (H5 or H7 subtype) or by any AI virus with an IVPI greater than 1.2 (or as an alternative at least 75% mortality) and 2) any H5 and H7 AI viruses that have amino acid sequences at the HA cleavage site compatible with a HPAI virus. As mentioned previously, the presence of multiple basic amino acids within the cleavage site of an HPAI virus renders it susceptible to the action of

ubiquitous intracellular proteases and allows it to replicate throughout the animal, damaging vital organs and tissues and resulting in disease and death (82). Moreover, these viruses are thought to arise from LPAI strains and several instances of such mutation have been documented (134, 135). As suggested by phylogenetic studies, HPAI viruses do not form separate lineages from viruses of low virulence. This supports the current theory that HPAI viruses arise by mutation from their low pathogenic forms, possibly after the introduction into domestic poultry from their natural reservoirs (148).

On the other hand, LPAI viruses are those isolates that do not meet the above-mentioned criteria for HPAI and are considered the vast majority of AI viruses (3). These possess 2 basic amino acid insertions within the HA cleavage site and are limited by host proteases (trypsin and trypsin-like enzymes), hence replication is limited to sites (respiratory and gastrointestinal tract epithelia) where these enzymes occur (82, 149). LPAI viruses are maintained in the wild bird reservoir and some H5 and H7 LPAI viruses, following transfer and circulation in domestic poultry, have mutated into HPAI viruses and resulted in 24 documented HPAI epizootics since 1959 (3).

The receptor specificity of influenza HAs depends on the host species from which they were isolated (22). In avian influenza, HA specifically binds to sialic acid residues (α2,3 linked to galactose) that are mainly located on the cell surfaces of the gastrointestinal tract of birds and such explains the tissue tropism of the avian flu virus. However, some variation in tissue tropism exists among avian species and AI subtypes, with some species having an abundance of receptors in the respiratory epithelium as well (82, 150). On the other hand, human influenza A virus strains preferentially bind to sialic acid residues by an α2,6 linkage (151). The difference in binding preference appears to be associated with position 226 in the HA receptor-binding site,

with Leu-226 present in human flu viruses instead of Gln-226 that is present in avian strains (22).

The change in host range entails the need to override the selective binding of flu viruses which can be accomplished through an intermediate host carrying both types of above-mentioned receptors thus, creating a new virus through reassortment (132). Pigs are suggested to serve as mixing vessels (intermediate host) for the reassortment of genetic material from human and avian virus strains as swine possess both α2,3 and α2,6 receptors (16). However, the direct transmission of avian influenza virus from poultry to humans in recent years (e.g. H5N1 avian flu in Hong Kong in 1997) demonstrated that reassortment or adaptation in an intermediate host is not necessary for infection of humans by avian flu viruses (136). The ability of the avian H5 virus to replicate in humans may be explained by the finding that human airway epithelium also harbors α2,3-linked sialic acids on ciliated cells (152).

There is evidence for a role of neuraminidase in host range restriction and pathogenicity (22). An investigation by Castrucci and Kawaoka (153) on the biological importance of the NA stalk demonstrated that stalk length affects the host range of influenza A viruses, such that a shortened stalk reduced the ability of the virus to elute from erythrocytes, decreased virus growth in MDCK cells and eggs and decreased the virulence in mice. Furthermore, a study on the HA and NA gene changes prior to the emergence of HPAI H7N1 avian viruses in Italy in 1999 showed that NA stalk deletion followed by the acquisition of additional glycosylation near the receptor binding site of HA may be an adaptation of H7 viruses to a new host species (19). On the other hand, Mitnaul and co-workers (154) continuously passaged a NA mutant in eggs which yielded clones that replicated efficiently through RNA-RNA recombination (exchange of genetic information between RNA segments). Hence, mutations in NA can contribute to the ability of

influenza A viruses to adapt to new environments by undergoing genetic changes, including large insertions and deletions (155). This hypothesis is supported by an early study involving a human-duck reassortant virus that failed to replicate in ducks even though the NA of the human virus has an avian origin (156).

To summarize, a multitude of inter-related determinants (e.g. properties of individual viral components, interaction of such components with one another and with the host cell elements and immunologic and environmental factors) make pathogenicity and host range a very complex phenomena (22).

B. Avian Influenza in Domestic Poultry

1. Chickens

Susceptible to infection with a variety of flu subtypes, chickens are generally not considered as the natural hosts of AI viruses but play a significant role in the ecology of avian influenza (136, 157). Initial virus introduction and circulation in poultry flocks does not cause substantial pathology but may eventually lead to virus adaptation, resulting in the acquisition of mutations in its gene segments which enables further virus spread and lethality, as in the case of the highly pathogenic H5 or H7 subtypes (136).

Isolations of influenza viruses from chickens have been infrequent in comparison to other domestic poultry species such as duck and turkey (158) since most chickens are raised in commercial indoor poultry operations (146). AI has been a rare occurrence in developed countries considering the billions of chickens grown yearly (146) but the probability of virus introduction into these poultry operations increases significantly if it comes into contact with some risk factors such as fomites (159). Once AI infection occurs in the flock, it spreads rapidly

from one farm to another due to poor inter-farm biosecurity practices, resulting in AI epornitics (146, 159).

As mentioned previously, reports of influenza infection in chickens, though having a much higher population worldwide, have been relatively rare in comparison to infections of other domestic poultry. Twelve out of the 18 primary HPAI outbreaks since 1959 were in chickens, despite the low incidence of AI infections. The spread of avian influenza to chicken flocks in Pennsylvania (1983-1984), Mexico (1994-1995), Pakistan (1995), Hong Kong (1997) and Italy (1999-2000) translated to huge economic losses (160).

The Pennsylvania outbreaks of avian influenza that started in late April 1983 resulted in 25 diagnosed cases until early October of the same year (161, 162). Cases were initially thought as infectious laryngotracheitis due to its endemicity in the area and the lesions present in the respiratory tract but it was later confirmed as low pathogenic H5N2 (162). However, a new and dramatically different form of disease was recognized when clinical histories (decreased feed and water consumption, dropping egg production rates and severe mortalities in a few days), clinical signs (depression, tremors or "unusual attitudes of the head") and gross lesions (severe swelling of comb and wattles, periorbital edema, petechial hemorrhage of various serosal and mucosal surfaces) confirmed its mutation from a non-pathogenic agent to its highly pathogenic form (162). This outbreak has resulted in the slaughter of more than 17 million birds, with compensation and other costs in excess of US$ 63 million (163).

The Mexican HPAI episode was in some ways similar to the Pennsylvania outbreaks. Veterinarians and poultry farmers in many of the country's poultry producing areas in late 1993 and early 1994 began reporting respiratory problems with investigations leading to the diagnosis of low pathogenic avian influenza H5N2 via pathogenicity tests in chickens and amino acid

sequencing (164). In early January 1995, poultry farms located in Tehuacán, Puebla and Villa del Marqués, Querétaro had identified an influenza virus characteristic of a highly pathogenic form (164). Strict quarantine actions, serological and virological monitoring and vaccination in poultry production areas were carried out to control the spread of the virus (160, 164).

In Pakistan, an outbreak of H7N3 HPAI initially started in the northern part of the country (wintering area for migratory birds) in December 1994, affecting 3.2 million birds (primarily breeders and broilers) and having 51-100% flock mortality within a 100 km radius (160). Vaccination and biosecurity programs were implemented in the affected regions of the country and since August 1995, no more outbreaks have been recorded (160, 165).

The outbreaks of H5N1 HPAI in Hong Kong during March-May 1997 started (from three separate farms in its northwestern part) with sudden mortalities ranging from 75-100%. H5N1 was first recognized to cause disease in man during this period wherein several cases of human influenza were detected in May and November 1997 (160). Furthermore, the second wave of H5N1 isolations in live bird market stalls and farms in December 1997 prompted the intensive cleaning and disinfection of these establishments and the suspension of bird importation from China, all of which served as a catalyst to depopulate all Hong Kong poultry markets and chicken farms (142, 160).

Italy has been affected by two epidemics of highly pathogenic avian influenza from 1997-1998 and in 1999-2000 (148). The first epidemic, limited to eight premises in backyard and semi-intensive flocks in 4 regions of the country, was caused by a virus of the H5N2 subtype (148). Risk factors identified in the outbreak included marketing of infected birds, presence of mixed species and raising birds in the open (148). On the other hand, the 1999-2000 epidemic that started in northeastern Italy was caused by a HPAI H7N1 virus that originated from a

mutation of its low pathogenic form (148). It eventually spread and affected densely populated poultry areas, causing 413 diagnosed outbreaks and the death or culling of over 13 million birds (148). This resulted to great economic losses to the Italian poultry industry and distress in the social community (148).

Australia had five recorded outbreaks of highly pathogenic avian influenza in 1976, 1985, 1992, 1994 and 1997 (166). All were caused by H7 strains and the origin in each instance was blamed on wild birds but findings were inconclusive (166). Occurring on a combined broiler and egg layer farm located in the outer suburbs of Melbourne, the 1976 outbreak was recognized as being possibly HPAI with the poultry on the affected farms exhibiting "textbook" clinical signs and lesions of the disease (e.g. swollen cyanotic combs and wattles) (167). Similar to the previous outbreak, the 1985 epornitic occurred at Lockwood near the provincial city of Bendigo in Victoria on a medium-sized, multi-age chicken farm, where the virus was isolated from chickens exhibiting clinical signs and lesions consistent with HPAI (168). The third outbreak (1992) occurred again in Bendigo area but in a different location involving broiler breeders with clinical history similar to the previous outbreaks (167). A farm housing chickens of different ages in Brisbane, Queensland was the fourth site of an AI outbreak in 1994. Similar clinical signs were presented by the affected birds indicative of HPAI (167). The fifth outbreak (1997) occurred near the town of Tamworth, New South Wales where a rise in mortalities was observed in a multi-age commercial layer farm (166). Clinical signs observed include diarrhea, "gasping breathing" and cyanotic combs. Three-month old emus from an adjacent farm were also found to be infected with the same virus that had affected the chickens (166). In all outbreaks, all birds on the farms were slaughtered and the farms were disinfected. Neighboring farms were placed into quarantine and monitored by serological and pathological testing (167).

2. Turkeys

Since avian influenza was identified in turkeys in 1963, most of the major turkey-producing countries had problems associated with this virus (160). Since 1964, influenza outbreaks in turkeys have been reported in 19 states in the US, with the majority of the outbreaks being sporadic except for the states of California and Minnesota, the main US turkey producers that are situated in migratory waterfowl flyways (169). Other areas involved in turkey production (also located under waterfowl flyways) in the US include Utah, Wisconsin, Iowa, Missouri, Arkansas, Pennsylvania, West Virginia, and North Carolina, states where turkeys are either raised on the range or in confinement (169).

Two major outbreaks of low pathogenic avian influenza occurred in Sanpete Valley, Utah (H7N3) and central Minnesota (H9N2) in 1995. The Utah outbreak caused about 40% mortality in 0 to 4 week old poults and most mortality cases were associated with secondary *Escherichia coli* or *Pasteurella multocida* infections (169). The Minnesota incident, on the other hand, affected 178 turkey farms and resulted to the worst economic loss to influenza infections recorded in 1 year in the state. In both outbreaks, inactivated vaccines were used to control the infection (169).

During the spring and summer of 2002, an outbreak of low pathogenic AI H7N2 occurred in Shenandoah Valley, Virginia, primarily affecting turkey farms and resulting in the slaughter of over 4.7 million birds (170). Molecular sequencing of the strain isolated in the outbreak revealed striking similarities to the H7N2 strain circulating in the live bird markets of northeastern US. Depopulation and vaccination were used to control the outbreak (170).

In 2007, outbreaks of LPAI were detected in 3 states (West Virginia, Nebraska and Virginia). The West Virginia (H5N2) incident occurred in April 2007, involving a single flock of

25,600 turkeys while the Nebraska (H7N9) outbreak occurred in June 2007, affecting a multi-age turkey operation of 145,000 birds. The third outbreak (H5N1) that happened in Virginia occurred in a flock of 54,000 turkeys in July 2007. All outbreaks were controlled by depopulation (171).

After the absence of fowl plague in the Netherlands for more than 75 years, a suspected outbreak of a highly pathogenic avian influenza virus was reported in late February 2003 on several poultry farms (chicken and turkey) located in the most poultry-dense area in the country (172). The pathogen was identified having an H7N7 subtype and was related to viruses detected in 2000 during the routine AI surveillance in ducks in the Netherlands (173). Post-mortem lesions did not comply with descriptions in the literature, having a lack of hemorrhagic changes in tissues and edema and cyanosis in comb and wattles (172). Lasting for 2 months, the outbreak infected a total of 255 flocks, with 1255 commercial flocks and 17,421 hobby flocks preventively culled, accounting for a total of 30 million birds culled. Moreover, the virus was transmitted to 89 people who were in close contact with infected poultry and one veterinarian died (174).

Avian influenza virus infection in turkeys was first reported in Great Britain in 1963 when it was isolated in Norfolk (a stop-over area for migratory waterfowl) showing severe signs of severe disease (HPAI). However, low pathogenic AI was isolated in turkeys from 1964-1978 wherein the isolated viruses were associated with mild respiratory disease and egg laying problems in the field outbreaks (21).

On the other hand, the months between March and May 1979 marked the isolation of 8 influenza viruses from 16 turkey farms. Subtypes isolated from these outbreaks were H7N2, H7N3, H7N7 (HPAI), H1N1 and H10N4 and 14 out of the 16 outbreaks were situated again in

Norfolk, with the remaining located in Suffolk and Hertfordshire (21). Another HPAI (H5N1) outbreak occurred in a single turkey farm in Norfolk in 1991 (175).

An outbreak of an Asian lineage H5N1 virus in turkeys occurred on a large commercial meat turkey farm in Holton, Suffolk in January 2007 and was reported to authorities following an exceptional increase in morbidity and an unexplained high and escalating mortality (176). However, post-mortem examination revealed no specific changes suggestive of AI infection. Carcasses, blood and cloacal swab samples tested positive for the presence of an AI virus via egg inoculation, rRT-PCR (H5 subtype) and was shown to have a HA_0 cleavage site typical of the Asian lineage of H5N1 HPAI viruses seen in Europe in 2006. The source of the virus was unknown (176).

Another HPAI H5N1 outbreak in Suffolk affected a flock comprised of 5,000 turkeys, 1,118 ducks and 410 geese maintained under a free-range system on the early part of November 2007. This particular isolate was reported to have the closest genetic identity to an isolate from wild birds in the Czech Republic in June and July 2007 while being phylogenetically distinct from the H5N1 strain isolated from the Holton, Suffolk incident (177).

D. Avian Influenza in Wild Birds

The search for the source of influenza viruses that emerged and caused pandemics in humans pointed out to the presence of influenza viruses in wild birds (178). It is said that wild aquatic birds are the natural reservoirs of all influenza viruses (178) and the evidence for such claim is strong (16). Except for Antarctica where there is only serological evidence, influenza viruses have been isolated from birds of all continents (16). However, most of the studies were conducted in developed countries such that Africa, South America and Asia are much less explored (16). All 16 HA and 9 NA subtypes have been isolated from wild birds and most of the

strains isolated were defined as low pathogenic. Almost all of the LPAI strains isolated from more than 105 species from 26 different bird families were from anseriform birds (e.g. teal, ducks, geese and swans) and to a lesser extent charadriiforms (e.g. gulls, terns, red knot. ring-billed gull and ruddy turnstones) (16, 179). On the other hand, the isolation of low pathogenic strains from land-dwelling wild birds is rare (179).

Studies on viral evolution have shown that influenza A virus strains in wild ducks show limited evolution over time (178, 179). Hence, it has been suggested that the virus exists in a state of evolutionary stasis wherein it is nearly perfectly adapted in its natural host and that the continuing mutations do not provide selective advantage and are not favored (178). Analysis of flu strains recovered from the early 20^{th} century museum-preserved wild ducks showed little antigenic drift in avian sequences in 80 years, suggesting the virus has reached its optimum adaptation in its natural host (180). However, this scenario is in contrast to the situation where the virus crosses over to another host (particularly mammals). Introducing avian flu strains into a new host usually results in changes in gene segments which includes the rapid alteration of surface glycoproteins (HA and NA) (181) due to selective pressure on the virus as a measure for host adaptation (182).

As mentioned previously, the first reported isolation of an influenza virus from wild birds was in common terns in South Africa in 1961, where a HPAI H5N3 virus killed about 1,600 birds (139). However, it was not until the mid-1970s that systematic investigations for the presence of the flu virus in wild birds were undertaken (160).

During the first two weeks of December 2002, HPAI H5N1 was detected and caused outbreaks in two separate parks (Penfold and Kowloon), affecting waterfowl (ducks, geese, swan) and other wild birds (little egret, grey heron, black- and white-headed gull) in Hong Kong

(138). Outbreak investigation in Penfold Park noted the presence of new migrating ducks flying into and swimming in the park's man-made ponds, which was then populated with a variety of free-ranging resident waterfowl (138). This outbreak was controlled by strict quarantine and depopulation one week before the second outbreak occurred. Meanwhile, investigation on the Kowloon outbreak observed the presence of a live poultry market close to the park entrance nearest the open ponds. Strict isolation, culling, increased sanitation and vaccination of affected birds contained the second outbreak (138).

An outbreak of HPAI H5N1 occurred among wild birds in Qinghai Lake (a protected natural reserve for wild birds) in western China during the latter part of April through June 2005 (183). The virus was first identified affecting bar-headed geese but soon had spread to other avian species residing in the lake. A total of 6,184 dead birds (bar-headed geese, brown- and black-headed gulls, ruddy shelducks, great cormorants, whooper swans, black-headed cranes and pochards) were found during the span of the outbreak (183). Four different H5N1 genotypes of the virus were found via sequence analysis of 15 viruses that represented 6 avian species affected during the outbreak (183).

More recently, HPAI H5N1 was confirmed in wild mute swans found on the Dorset coast in Great Britain on January 2008 as part of the country's wild bird surveillance program (184). Analysis of the virus indicated its close relationship to a cluster of isolates recovered in the middle to the later part of 2007 from wild and domestic birds in the Czech Republic, Romania and Poland (184). No further evidence of AI infection was found in the wild bird population and domestic poultry within the area (184).

1. Anseriforms

All the 16 hemagglutinin (except for H13) and 9 neuraminidase subtypes have been detected in almost all combinations in waterfowl (82, 185). H3, H4 and H6 are the most dominant HA subtypes detected in waterfowl while the most common NA subtypes are N2, N6 and N8 (65, 175, 176). In addition, the most common combination of HA and NA subtypes isolated from waterfowl are H3N8, H4N6, H4N8, H6N2, H6N8 and H9N2 (186, 187). The H5, H7 and H9 subtypes that can potentially cause disease in poultry and humans were considered rare in North American waterfowl previously but was recently reported having a relatively higher prevalence (20%) in a three-year study conducted by Hanson and co-workers (187) in Minnesota. On the other hand, H8 is an extremely rare subtype in ducks, having been isolated only less than 10 times during the last 20 years in North America (186, 188).

Prevalence of avian flu infection in waterfowl varies with space, time and age of the host population. The pre-migration staging during the late summer (July and August) coincides with the peak of AI transmission, with the highest prevalence of infection found in a large number of immunologically naïve juvenile (less than 1 year old) birds that gather during this period. Such conditions promote the efficient transmission of the virus. In contrast, AI infection declines during the winter season and also on wintering grounds (82, 185).

2. Charadriiforms

Although it is recognized that anseriform species are regarded as important avian flu reservoirs, early studies of AIV infection in wild birds were directed at pelagic species under the Order *Charadriiformes*, a diverse group of birds including those which are not associated with aquatic habitats (185). Distributed worldwide, many of these birds have long range migration patterns and utilize different aquatic habitats (185). Shorebirds and gulls in the Americas were

reported to be the more frequent source of potential precursors of the highly pathogenic H5 and H7 subtypes while it is usually isolated from ducks in Europe and Asia (189).

The prevalence of AIV is highly variable and its epidemiology is not well understood in this group. In contrast with waterfowl, the prevalence of AIV in charadriiforms peaks during spring rather than late summer and increases during fall migration (185). Similar to anseriforms, isolation rates and subtypes vary considerably over time, region and between species (190). A study by Kawaoka and others (191) showed that shorebirds and gulls have variable prevalence of AIV, having isolation rates up to 20% in May 1985, 8% in September 1985 and 14% in May 1987. On the other hand, a research on the prevalence of influenza viruses in North American migrating birds by Krauss and co-workers (186) noted H3 and H11 as the most dominant HA subtypes detected in shorebirds while N2, N4, N8 and N9 were the most common NA subtypes. This is in contrast to an earlier study by Stallknecht (185) which stated that H9 and H13 are the most common HA subtypes in shorebirds and gull isolates. The H9 subtype, isolated from humans during the late 1990s, has also caused AI outbreaks in various parts of the world and has established permanent lineages in domestic poultry (192).

In a European study on the prevalence of influenza A viruses in wild migratory birds, HA subtypes H13, H16 and NA subtypes N3, N6 and N8 were isolated only from charadriiforms. Moreover, the most frequently detected HA and NA subtype combination was H4N6, N7N7 and H6N2 (193).

3. Other wild birds

Although avian influenza has been isolated in a wide range of avian species, little attention has been given to other groups of birds (15). Some species from which AIV have been isolated include pigeons (138, 194), cormorants (183), greater flamingo and tree sparrows (138),

starlings and rock partridge (168, 195). Although there are some exceptions, most of these birds are associated with terrestrial habitats. In general, isolations of AIV in land-based wild birds are not that successful and hence, are not considered as major reservoirs of avian influenza viruses (185). However, their role in the ecology and epidemiology of AIV cannot be discounted since the isolation of avian flu viruses in some of these species were associated with poultry outbreaks, as in the case of starlings in the 1985 epornitic in Australia (168). Furthermore, some of these birds were experimentally infected and found to be susceptible to AIV infection (e.g. pigeon) (186, 187).

Role of Wild Birds in the Spread of Avian Influenza

Wild birds predominantly, anseriforms and charadriiforms, are the known natural hosts of AIV, which generally exist in low pathogenic form and does not usually cause clinical signs of disease in these birds (82). Contact of these birds with other feral birds, domestic poultry or other animals, directly or indirectly through environmental contamination, occasionally transmits the virus in its low pathogenic form (82). Through time, the virus may adapt into its new host via mutations and can transform into its highly pathogenic form, spreading through the flock and causing avian flu outbreaks that can possibly evolve into a human influenza pandemic (16, 17).

The debate on the exact role of wild birds in the spread of HPAI H5N1 to domestic poultry is still long-standing primarily due to the risks it poses to wildlife, human health (zoonotic potential) and agriculture (trade impact) (82). Rarely detected from wild birds, HPAI viruses usually have been geographically or chronologically isolated within or close to the vicinity of an HPAI outbreak in domestic poultry. This scenario is in consonance with the theory that highly pathogenic AI only emerges after the virus has crossed from wild birds to poultry (160). However, HPAI H5N1 is currently “breaking the rules”, indicating that the virus is

continuously evolving (178). Some of these observed changes mentioned by Webster and others (178) in their recent paper include: 1) direct transmission of H5N1 viruses from wild birds to humans (e.g. collection of feathers from dead wild swans in Azerbaijan), 2) transmission of influenza virus genes from domestic poultry to migratory waterfowl, 3) increased tracheal virus shedding in aquatic birds, 4) extensive diversity in pathogenicity for waterfowl (ranging from non-pathogenic to highly lethal) and 5) appearance of at least three distinguishable H5N1 clades in the past 3 years.

Greater attention has been given to the possible role of wild birds in the spread of AIV after the Qinghai Lake outbreak (183). The progressive westward spread of HPAI H5N1 to poultry and feral birds in other parts of Asia, Russia, Middle East, Europe and Africa provided evidence that wild birds, in some cases, played a role in the spread of the virus (1). For instance, the spread of AIV from the southeastern to the northwestern part of Europe suggested a "leap-frog" manner of virus dissemination across the European continent, with infected birds flying for a short distance and transmitting the virus to other susceptible birds before it dies (1). Many of these outbreaks were also associated with local H5N1 poultry epornitics. Hence, it is unclear whether wild birds harbor the virus and asymptomatically transmitted it to local poultry or the other way around, whether locally infected poultry transmit the virus to wild birds, subsequently spilling the virus over to migratory bird populations (1, 82).

Several speculations have risen regarding the true nature of these outbreaks. In the Qinghai Lake incident, it was observed that there were no death trails along wild bird migratory routes and no reported poultry outbreaks within the vicinity of the lake in 2005-2006 although an outbreak was confirmed in Lanzhou (about 300 km away) in 2004 (1). Furthermore, an internet blogger revealed that one of the key migratory species affected (bar-headed geese) during the

outbreak has been raised near the lake as part of an experimental program to domesticate and repopulate these species of birds in the wild (196). Thus, farm-reared bar-headed geese were possibly the source of the outbreak, as domestication increases the chance of contact with infected domestic poultry (196). Despite all these findings, little is still known about the eco-dynamics of HPAI infection in wild birds thus, the importance of migratory birds in the spread of highly pathogenic avian influenza remains a puzzle to be solved, providing us a unique opportunity to further understand its role (182, 189).

Transmission and Spread

The mechanisms by which influenza viruses cause infection from one bird to another are not clearly understood. Attempts to experimentally characterize the transmissibility of influenza viruses in domestic poultry revealed that bird-to-bird transmission is multi-faceted, depending on several factors such as virus strain, sufficient contact of the host to the pathogen, bird species and the environment (16, 17).

Based on natural and experimental infections, virulent viruses are poorly transmitted (in comparison to low pathogenic viruses) from infected to susceptible chickens and turkeys since the extent of viral spread depends on the amount of virus released either through the respiratory or intestinal route (17). Because highly pathogenic AI viruses cause sudden deaths in affected birds, a relatively little amount of virus is excreted during the duration of such infection (17).

During the pre-wintering migration, the perpetuation of influenza viruses in waterfowl takes place through the passage of the virus from adult to juvenile birds on bodies of water where birds congregate (17). This offers an increased opportunity for the transmission of infection via exposure to a contaminated environment (82). Infected birds shed virus-laden feces into the water and susceptible birds get infected by drinking contaminated water or through “cloacal

drinking" (feco-oral route) (17, 82). Therefore, domestic poultry are at risk anytime it shares a common water source with wild aquatic birds (82).

As viruses cannot replicate outside the host, they need to persist in water for sometime (17). Under normal aquatic environmental conditions, AI viruses are relatively stable but are able to persist longer in cooler temperatures (82). In experiments conducted by Stallknecht and co-workers (197), it was determined that avian influenza viruses (10^6 $TCID_{50}$/ml) stored in distilled water at 28^{o}C could remain infective for up to 102 days, at 17^{o}C for up to 207 days and possibly as long as 1000 days at 4^{o}C. In another study, it was also found that the duration of virus infectivity decreases as salinity and pH increases (198). Moreover, avian flu viruses can still remain viable in water after freezing over winter and can still serve as a source of re-infection in waterfowl (199). Under natural conditions, however, the effects of pH, salinity, UV radiation and presence of biologically active material (e.g. bacteria, degrading enzymes) influence the persistence of the virus (16). The above-mentioned information therefore suggests that feco-oral transmission via water is the most likely and efficient mode of transmission and perpetuation of low pathogenic strains in wild birds (16).

Wild bird activity (usually waterfowl) results to the introduction of LPAI viruses into a poultry population where direct contact may not be necessary, as viruses introduced by waterfowl may be spread into domestic poultry farms by humans or other animals not susceptible to the virus thru mechanical transfer (146). Movements of farm personnel, feed and bird delivery trucks entering in and going out of the farm, contaminated food and water supply, density of the infected poultry population, as well as farm supplies and equipments, have all been strongly implicated as a source of spread of AIV (127, 129, 158, 193-195). Moreover, the legal and illegal trade of poultry and poultry products in live bird markets or along established trade routes and

the illegal trade and transport of live birds (e.g. fighting cocks and doves in Malaysia, eagles in Belgium and ducks in Taiwan) had also been found to contribute to the unwanted spread of the virus (14).

Evidence abounds in the implication of waterfowl in most of the LPAI outbreaks since it was found that a higher prevalence of AIV infection exists in poultry situated migratory waterfowl routes (e.g. Minnesota) and poultry kept in exposed conditions (e.g. range turkeys) (144). A seasonal occurrence in high-risk areas is also seen in influenza outbreaks coinciding with migratory activity wherein evidence of probable initial contact with waterfowl was found in most documented specific outbreaks (144). In some instances, virus introduction into poultry resulted from an area where the virus is endemic (e.g. live bird markets) (163).

Clinical Signs and Pathology

Although there are only two pathotypes of avian influenza viruses demonstrable in the laboratory (low and highly pathogenic), natural infection by AIV results in a wide range of clinical outcomes, depending on a variety of factors such as virus strain, host species, sex, age, acquired immunity, concurrent infections and environmental factors (146). Manifestation of AIV infection ranges from no obvious clinical signs to severe mortality, with birds of all ages and most (if not all) avian species being susceptible (145).

A. Low Pathogenic Avian Influenza Viruses

The low pathotype of AIV usually produces a mild or no clinical signs of disease in wild birds (146) but there have been some exceptions wherein high mortality and clinical manifestations were reported (121, 122, 172). Although the range of clinical signs of LPAI may overlap with those of HPAI, as seen with the field mortalities caused by LPAI viruses, it is usually associated with secondary infections (200).

In domestic poultry such as chickens and turkeys, the clinical signs of AI infection ranges from asymptomatic to the manifestation of abnormalities in the respiratory, digestive, urinary and reproductive organs (146). Among domestic poultry, turkeys are considered to be the most susceptible birds and may suffer from serious respiratory disease problems after infection with LPAI viruses (4). Coughing, sneezing, rales, rattles and excessive lacrimation are the most frequent signs representing infection of the respiratory tract (146). Increased broodiness and decreased egg production may be seen in layer and breeder hens (146). General clinical signs including huddling, ruffled feathers, depression, decreased activity, decreased feed and water consumption and an occasional diarrhea may also be observed (146). Moreover, seroconversion without signs of clinical disease may be the only evidence of flock infection for some subtypes of AI (82). On the other hand, experiments involving the inoculation of LPAI virus isolates in SPF birds usually showed much lower mortality than HPAI viruses (200).

Gross lesions caused by LPAI viruses are mostly confined in the respiratory tract (especially the sinuses) and are characterized according to the severity of inflammation (e.g. catarrhal, fibrinous, serofibrinous, mucopurulent, fibrinopurulent) (146). Some of the respiratory tract lesions which may be present in LPAI infections are as follows: 1) congested or hemorrhagic tracheal mucosa, 2) presence of serous or caseous tracheal exudates which can result to asphyxiation due to airway blockage, 3) fibrinous to fibrinopurulent air sacs which may be accompanied by secondary bacterial infections and 4) swollen infra-orbital sinuses with mucus or mucopurulent discharge (146). On the other hand, gross lesions which can be seen in the digestive and reproductive tract are: 1) catarrhal to fibrinous inflammation of the peritoneal cavity (“egg yolk peritonitis” may be observed), 2) catarrhal to fibrinous enteritis in the ceca and/or intestine (turkeys), 3) inflammatory exudates in oviducts of laying birds, 4) regression of

ovaries and involution of the oviduct, 5) swollen kidneys with urate deposition and 6) presence of firm pancreas with pale mottling and hemorrhage (turkeys) (146).

Some of the microscopic lesions which can be found in LPAI-infected poultry include: 1) ventromedial fibrinocellular to peribronchiolar lymphocytic pneumonia, 2) heterophilic to lymphocytic tracheitis and bronchitis, 3) nephrosis and nephritis (caused by IV-inoculated strain-specific viruses), 4) pancreatitis with acinar necrosis (in natural and experimental infection in turkeys) and 5) lymphocyte depletion, necrosis or apoptosis of lymphocytes in the bursa of Fabricius, thymus and spleen in birds that die from LPAI infection (146).

B. Highly Pathogenic Avian Influenza Viruses

In domestic chickens, turkeys and related galliform birds, replication of the virus and the extent of damage it causes on host body organs and systems (circulatory and nervous) reflects the nature of the clinical signs of AI (146). HPAI viruses do not induce clinical signs in some resistant birds but may manifest a different clinical picture in other species (201).

Highly pathogenic AI viruses are capable of causing severe outbreaks in chickens or turkeys and are currently restricted to the H5 and H7 subtypes (145). Turkeys and chickens are the most susceptible birds to HPAI viruses (4). In most cases, the disease is so sudden that some birds die even before any clinical sign can be seen (146). Nervous disorders (e.g. head and neck tremors) are noted in affected birds if the disease is less severe and if birds survive for 3-7 days (146). Decreased activity, noise reduction and decline in feed and water consumption inside poultry houses are also observed due to depression of infected birds (146). Rales, sneezing and coughing of birds can also be seen but are less prominent than LPAI-infected birds (146). In breeders and layers, sudden drops in egg production, including total cessation of production, ensues within 6 days (146). Similar clinical signs can be seen with other galliform birds and

those that survive infection have neurological signs and behavior modifications (146). Mortality rates are very high and can reach up to 100% in some flocks (146).

A variety of edematous, hemorrhagic and necrotic lesions in visceral organs and the skin are produced by HPAI viruses in poultry (146). Gross lesions observed in HPAI-infected birds are as follows: 1) swelling of the head, face, upper neck and feet which may be accompanied by petechial to ecchymotic hemorrhages, 2) periorbital edema, 3) cyanosis of non-feathered skin (e.g. wattle and combs), 4) congested or hemorrhagic lungs, 5) necrotic foci within parenchyma of visceral organs (e.g. pancreas, spleen, heart, liver and kidney), 6) urate deposits in kidney, 7) hemorrhages on serosal and mucosal (e.g. proventriculus and gizzard) surfaces and 8) atrophic cloacal bursa and thymus (146).

Histologic lesions which can be found in HPAI-infected birds consist of multi-organ necrosis and/or inflammation, with the: 1) brain (e.g. lymphocytic meningoencephalitis, neuronal necrosis, edema and hemorrhage), 2) heart (e.g. focal degeneration to multi-focal diffuse coagulative necrosis of cardiac myocytes), 3) lung and respiratory tract (variable lesions from mild to severe), 4) pancreas (e.g. necrosis of pancreatic acinar cells) and the 5) primary and secondary lymphoid organs severely affected (e.g. necrosis, apoptosis and depletion in cloacal bursa, thymus and spleen) (146). Necrosis in skeletal myofibers, kidney tubules, vascular endothelial cells and corticotrophic cells of the adrenal gland were found as lesions commonly associated with virus replication (146). Moreover, underneath the non-feathered skin are numerous microthrombi within dermal and hypodermal capillaries and small blood vessels. Likewise, vasculitis, perivascular to generalized edema, subcutaneous edema and necrosis of the capillary endothelium may also be observed in this area (146).

Diagnosis of Avian Influenza

Since clinical signs of disease and lesions can vary dramatically in AI, diagnosis based on these can only be considered presumptive (145). Therefore, laboratory confirmation is required to establish the etiology of the clinical disease. Direct detection of AI viral proteins in specimens (e.g. tissues, swabs, cell cultures, embryonating eggs), viral isolation and identification and the detection of antibodies to the virus are some of the laboratory methods utilized in the diagnosis of avian influenza (146).

A. Sample Selection and Storage

Most low and highly pathogenic AI viruses replicate in the respiratory and intestinal tracts and are commonly isolated from tracheal or cloacal swabs obtained from either dead or live birds (146). Tracheal and cloacal swabs are best for virus isolation in domestic poultry while the latter works best for wild birds (145). Dry sterile swabs used for getting tracheal and cloacal swab samples should be placed in a sterile transport medium containing antibiotics to control bacterial and fungal growth (145). On the other hand, collected tissues should be placed in sterile tubes or plastic bags with the respiratory organs, intestinal tract and other internal organs separated as virus isolation from internal organs may be an indication of systemic spread. In the case of HPAI infections, the virus can be isolated virtually from every organ because of high viremia levels or replication in parenchymal cells (146). Samples can either be stored at 4^{o}C for up to 48 hours or frozen at -70°C if samples must be kept for extended periods if virus isolation cannot be done immediately (145).

B. Direct Detection of AI Viral Proteins or Nucleic Acids

Influenza viral RNA or viral proteins in animal samples can be detected directly using a variety of diagnostic tools currently available. Virus isolation, antigen capture immunoassays

and molecular diagnostic tests are the three common direct diagnostic tests for avian influenza (202).

1. Virus Isolation

As the gold standard for AI diagnosis, virus isolation is indispensable for biological characterization as well as the full sequence analysis of the isolate (25, 202). Isolation of the virus is usually done in 9-11 day old embryonating SPF chicken eggs through the inoculation (allantoic sac route) of supernatants obtained from tracheal and cloacal swabs or ground tissue suspension processed from infected tissue samples (145). For 3-7 days, eggs are incubated at 35-37°C and if the virus is present, most embryos will die following inoculation but some will not depending on the virulence of the isolate (145). Embryo deaths within 24 hours after inoculation should be discarded and regarded as non-specific (e.g. death due to bacterial contamination or inoculation injury) (146). Eggs should be removed from the incubator after 3 days or death, chilled and allantoic fluids collected for HA test (145). Hemagglutinating activity of the allantoic fluid in chicken erythrocytes indicates the presence of the virus but should be confirmed if it is due to influenza virus or other hemagglutinating viruses (146). Virus isolation, however, has some important drawbacks: 1) time (several days to weeks) spent in diagnosis, 2) availability of eggs, 3) need of appropriate facilities to work with the virus especially in HPAI cases (202).

2. Antigen Capture Immunoassay Tests

Another commonly used diagnostic tool in AIV is antigen capture immunoassay tests. Mostly targeting the nucleoprotein to detect any type A influenza virus, these tests are widely used in humans but are utilized for veterinary purposes (e.g. Beckton-Dickinson - Directigen Flu A Test and Synbiotics - Flu Detect) (202). With these tests, results can be rapidly obtained within 15-30 minutes with minimal laboratory equipment (202). Sensitivity, however, has been an issue

with these tests since it is reported to be much lower than virus isolation or some of the molecular diagnostic tests available (202).

3. Molecular Diagnostic Tests

Amplification of nucleic acids to high levels for the identification of an unknown virus is the basic goal of all molecular-based tests. Rapid technological evolution paved way for a wide selection of molecular diagnostic tests currently available, of which the 1) traditional reverse transcription-polymerase chain reaction, 2) RT-PCR with detection by enzyme-linked immunosorbent assay, 3) real-time reverse transcription-polymerase chain reaction (rRT-PCR) and 4) nucleic acid sequence-based amplification (NASBA) are common examples (202).

The method of detection of the PCR product differentiates the traditional RT-PCR, RT-PCR-ELISA, and rRT-PCR from each other but these tests are essentially similar, providing fast and sensitive diagnostic results (202). In traditional PCR, the product is detected and separated according to size via electrophoresis in an agarose gel. To confirm the specificity of the PCR product, a nuclear acid hybridization ELISA test is also used (RT-PCR-ELISA) (202). The use of fluorescently labeled probes to detect the amplification of the PCR product while running the test describes rRT-PCR wherein results are reported on a real-time basis (202). On the other hand, NASBA is a test which directly amplifies and detects RNA (202).

The RNA extraction step, the RT-PCR amplification step and the primer and probe sequences are crucial elements in achieving a sensitive and specific test (202). The amplification efficiency of RNA extraction is affected by the following: 1) extraction technology used (e.g. organic, silica column or magnetic bead extraction), 2) type of sample processed (e.g. tracheal or oropharyngeal swabs in chickens and turkeys vs. cloacal swabs in ducks) and 3) presence of PCR inhibitors (202). Test performance and results can be critically affected by the choice of RT-PCR

amplification kits and reagents. In general, the RT-PCR procedure can be run either by a: 1) one-step procedure (simpler than the 2-step protocol and offers reduced opportunity for contamination) or 2) two-step procedure (more sensitive than 1-step but may be prone to contamination) (202). Lastly, the sequence of primers and probes is an important factor to consider since these provide the basis for sensitivity and specificity of the test (202).

Although molecular tests are sensitive, have potential for high throughput and can be performed rapidly, some of its disadvantages are: 1) high cost of equipment, 2) proneness for contamination (false-positive results), 3) inability to differentiate live from inactivated virus (not good for environmental testing) and, 4) false negatives (e.g. presence of PCR inhibitors, inefficiency of extraction, genetic diversity of isolates) (25, 202).

4. Serology

The detection of subtype specific antibodies via serologic tests is important for epidemiological investigations during outbreaks of avian influenza (25). There are several procedures used in serologic surveillance and diagnosis, some which are: 1) agar gel immunodiffusion test, 2) hemagglutination-inhibition test and 3) ELISA assays (145).

a. Agar Gel Immunodiffusion Test

Agar gel immunodiffusion (AGID) tests detect the presence of antibodies against the nucleocapsid and matrix proteins, wherein virus preparations containing both or either of these antigens are used (203). Because a single test detects serological response in all bird species and against infection by all type A influenza viruses, AGID is the preferred serologic surveillance test in the United States (145). This test utilizes unknown test sera, test antigen and influenza A positive control sera (145). Depending on the concentrations of the antibody and antigen, precipitin lines can be detected after approximately 24-48 hours and are best viewed against a

dark background that is illuminated from behind (203). When the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well, a positive result is recorded (203).

b. Hemagglutination inhibition test

An essential follow-up for AGID-positive samples, HI is a rapid gold standard test that allows the detection of antibodies produced against the hemagglutinin surface protein of the virus hence, it is subtype-specific (145). Using a suspension of 0.5% chicken erythrocytes as an indicator, four HA units of the virus can be tested against the sera of known H subtypes (145).

However, it has a limited value in the initial screening of suspected flocks or birds unless the infecting virus has already been previously identified (145). Control sera and test antigens for all the known HA subtypes are also necessary when detecting infection from an unknown subtype (145). Since sera of many species contain non-specific inhibitors that may interfere with the specificity of the test, treatment of sera with receptor-destroying enzyme and potassium periodate may be necessary to eliminate non-specific HI reactions during subtyping (146). Moreover, steric hindrance may occur if the antibody used for H subtyping has a homologous N antibody to the unknown isolate, leading to non-specific inhibition (145).

c. Enzyme-Linked Immunosorbent Assay

ELISA is an effective biochemical assay for the detection of antigen-specific antibody in a sample. In the case of avian influenza, antibodies detected in the test are primarily against the nucleoprotein and matrix proteins of the influenza virus (available in commercial kits), resulting in a type specific test (204). The three formats of ELISA that exists are: 1) direct ELISA (for direct detection of antigen or proteins, histopathological specimens, smears), 2) indirect ELISA (uses a single species antibody directed against serum proteins to detect multiple antigens or

antibodies) and 3) competitive ELISA (can be used to detect antigen or antibody; involves competitive binding of the unknown antigen or antibody with a known Ag or Ab for available binding sites) (205).

Useful in surveillance programs, ELISA can be a semi-automated test and is suitable for the rapid screening of large number of samples (145). Furthermore, it can detect antibody titers earlier than virus neutralization (39) or hemagglutination inhibition test and can detect antigens in very low concentrations (205). However, ELISA has certain drawbacks: 1) test is group antigen specific and not type specific (does not differentiate between serotypes), 2) earlier detection of titers is of no real advantage in most practical application of serological data and 3) requires expensive equipment (205).

CHAPTER 3

MATERIALS AND METHODS

Viruses and Virus Titrations

Twenty-four LPAI wild bird virus isolates, used in this study (Table 3) were obtained from the Southeastern Cooperative Wildlife Disease Study (SCWDS), College of Veterinary Medicine, The University of Georgia, Athens, GA. Viruses were inoculated in 9- to 11-day old SPF embryonating chicken eggs to determine infectious titer using the Reed and Muench method (206). Moreover, lesions found in embryos on the last day of a week-long titration were recorded and correlated with the results of a modified rapid hemagglutination test done on the allantoic fluid of all surviving embryos. Out of the 24 LPAI viruses initially titered in embryonating eggs, sixteen viruses with titers above 1 x 10^6 EID_{50}/ml were selected for pathogenicity studies in chickens and turkeys.

Birds and Facilities

Four-week old specific pathogen-free White Leghorn chickens (Merial Select, Gainesville, GA) and three-week old commercial turkeys (Sleepy Creek Hatchery, Goldsboro, NC) were used in this study. A minimum of eight birds per group/virus/isolator were utilized. All experiments were conducted under biosafety level (BSL) 2 Ag+ facilities at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, The University of Georgia, (Athens, GA) in accordance with USDA-APHIS Permit Number 103372. The facility consists of a stand-alone block building with controlled access. Moreover, the building has an ante-room that leads to the main room where the isolators are maintained. Stainless steel and

polycarbonate, negative pressure, HEPA filtered, isolator units with internal dimensions of 42"L X 24"W X 31" H were used to house individual groups of birds. Each isolator unit has its own air handling equipment, a 2 door pass-through access port, radiant heater, height adjustable feeders, nipple drinkers, and a sealed collection canister for manure.

Experimental Design

Viruses were diluted with sterile phosphate buffered saline (PBS, pH 7.4) to adjust the amount of inoculum to 1 x 10^6 EID_{50} per 0.1 ml per bird. For viruses below 1 x 10^6 EID_{50} per 0.1 ml concentration, 0.1ml of undiluted virus per bird was given. The viruses were administered via the oropharyngeal (intrachoanal) route. For each experiment, one group having the same number of birds was not inoculated and served as negative controls. After inoculation, all birds were observed for clinical signs of disease and mortality twice daily for 21 days. Clinical signs of disease were scored and recorded as follows: 0 = no signs, 1 = mild to moderate respiratory signs, 2 = moderate respiratory signs (depressed, not eating), 3 = moderate to severe respiratory signs (not eating, neurological signs) and 4 = moribund birds (were removed and necropsied immediately).

Oropharyngeal and cloacal swabs were collected (in sterile PBS) from each bird at 2, 4 and 7 days post-inoculation (PI). Moreover, fecal samples from all isolators (including the control group) were obtained at the time of swab collection. All swab and fecal samples were stored at -80^{o}C and thawed only once for RNA extraction. Serum was also collected from each bird at 7, 14 and 21 days PI. Samples were initially stored at -20^{o}C and sent to the PDRC Diagnostic Laboratory for ELISA testing. The HI test was also conducted on all serum samples from each bird. Tissue samples for histopathology were collected from 3 birds per isolator/group at 3 days PI.

RNA Extraction and Quantitative real-time RT-PCR

Viral RNA template was extracted from swabs and fecal material using the MagMax-96 Total RNA Isolation Kit (Ambion Incorporated, Austin, TX) and KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA), according to the manufacturer's recommendations.

The Ambion Ag Path ID One Step RT-PCR kit (Ambion Incorporated, Austin, TX) was used for nucleic acid amplification with a 25 μL reaction mixture containing the following reagents: 12.5 μL of kit-supplied 2X RT-PCR buffer, 1 μL of kit-supplied 25x RT-PCR enzyme mix and 10 μL of extracted viral RNA template. Each reaction mixture utilized 10 picomoles of matrix gene primers (forward and reverse) and probe sequences (all three using 0.5 μL each), following the protocol of the real time RT-PCR assay developed for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes (207). Real-time RT-PCR was carried out in a Smart Cycler thermocycler machine (Cepheid, Sunnyvale, CA) with the following conditions for the RT step (50^{o}C for 30 min and 94^{o}C for 15 min) and the PCR cycling protocol (94^{o}C for 15 sec and 60^{o}C for 20 sec for 45 cycles). Data were reported as the average cycle threshold (CT) value and compared to a standard curve to determine relative amounts of virus (EID_{50}/ml) present in the samples. The standard curve was generated by extracting viral RNA template from all sixteen LPAI viruses inoculated in experimental birds, making ten-fold serial dilutions of these RNAs and carrying out real time RT-PCR reactions on all samples, as described previously.

Histopathology

Histopathological examination was conducted on the following tissues: heart, lung, liver, spleen, pancreas, duodenum, jejunum, cecum, cecal tonsils, ileum, bursa of Fabricius, breast and

thigh muscle, thymus, nasal cavity and brain. Tissue samples were fixed in 10% neutral buffered formalin and routinely processed into paraffin. Thin sections were cut and processed for hematoxylin and eosin (H & E) staining.

Serum Collection and Serological Testing

Sera were tested for antibodies to AIV using a commercial ELISA kit, FlockChek™ Avian Influenza Virus Antibody Test (IDEXX, Portland, ME). In addition, samples were tested for antibodies via HI using 4 HA units of viruses with the highest HA titers representing H_4, H_6 and H_9 subtypes.

Table 3: Low pathogenic avian influenza wild bird isolates used in the study.

Isolate	Subtype	NVSL Accession #	Code	Passage
Mallard/MN/530/00	H_4N_6	355801	MN 00-530	P2
Mallard/MN/253/99	H_6N_5	199074	MN 99-253	P2
Mallard/MN/153/98	H_9N_2 (?)	182745	MN 98-153	P2
A/Ruddy Turnstone/ NJ/1148660	H_6N_8	1148660	AI 04-02	P2
Blue-Winged Teal/LA/69B/87	H_4N_8	-	LA 69B	P2
Mallard/MN/346233/2000	H_6N_3	346233	MN 00-38	P2
Red Knot/744/03	H_9N_2	1016450	AI 03-744	P?
Ruddy Turnstone/DE/1044/02	H_9N_9	650633	AI 02-1044	P2
RT/114/03	H_9N_2	1016395	AI 03-114	P2
Ruddy Turnstone/DE/1070/02	H_9N_4	650637	AI 02-1070	P2
Mallard/MN/263/99	H_4N_9	199076	MN 99-263	P2
Ruddy Turnstone/ NJ/1467/02	H_9N_9	650588	AI 02-1467	P2
Mallard/MN/198/99	H_4N_6	199059	MN 99-198	P2
A/Ring-billed Gull/GA/ 421733	H_6N_4	421733	AI 01-124	P2
Blue-Winged Teal/LA/B156/87	H_4N_6	-	LA B156	P2
A/Ruddy Turnstone/ NJ/ AI01-1407	H_4N_6	471604	AI 01-1407	P2
RT/452/03	H_9N_2	1016432	AI 03-452	P2
Blue-Winged Teal/LA/240B/88	H_4N_6	-	LA 240B	P2
A/Tx/828197/02	H_6N_4	828197	TX 02-260	P2
Mallard/MN/232/98	H_9N_2 (?)	182753	MN 98-232	P2
Mallard/MN/304/98	H_9N_2 (?)	182763	MN 98-304	P2
Mallard/MN/365/99	H_9N_2 (?)	199108	MN 99-365	P2
Ruddy Turnstone/DE/1523/02	H_9N_5 (?)	650593	AI 02-1523	P2
Ruddy Turnstone/ NJ/749/02	H_9N_9 (?)	650616	AI 02-749	P2

(?) Tested PCR (-) against H9 primers

CHAPTER 4

RESULTS

Virus and Virus Titrations

Embryo infectious dose titer of each virus using the Reed and Munch method are shown in Table 4. Infectious titers ranged from 1 x 10^3 to 1 x $10^{9.9}$ EID_{50}/ml. AI 02-1044 had the lowest mean EID_{50}/ml while LA 240B had the highest. Correlation of the lesions found in SPF chicken embryos with the hemagglutination test done on the allantoic fluid of surviving embryos noted that viruses with high titers caused more pronounced embryo lesions (e.g. stunting, curling) and hemagglutination was observed in a wider range of ten-fold dilutions of the virus (from 1 x 10^{-3} to 1 x 10^{-10}). In the quantitative hemagglutination test, on the other hand, viruses AI 02-749 had the highest HA titer while MN 98-232 had the lowest hemagglutination titer in a 1:2 dilution (Table 4). Among all these viruses, three with the highest HA titers were chosen to represent H_4 (LA B156), H_6 (TX 02-260) and H_9 (AI 03-452) subtypes for the HI test.

Clinical Signs and Gross Lesions

All 16 viruses did not cause significant clinical disease except for MN 99-263 in turkeys, which resulted in one death and one bird with moderate respiratory signs and depression. The first bird was found to be depressed a day after virus inoculation and was found dead the following morning. Examination of the carcass revealed post-mortem autolysis of internal organs, indicating that the bird may have died during the night.

The second bird, on the other hand, showed clinical signs of depression 10 days PI. It was found sitting in a corner of its isolator with its head bended down, both of its eyes closed and had swollen infraorbital sinuses. Necropsy of the bird the following day showed no gross lesions in its internal organs, which were sampled and submitted for histopathology.

RNA Extraction and Quantitative real-time RT-PCR

Analysis of the cloacal and oropharyngeal swabs taken from chickens and turkeys during the experiment (days 2, 4 and 7) are shown in Table 5. Negative control birds (non-inoculated) for each experimental trial were negative by qRRT-PCR (results not shown).

Among the 16 LPAI viruses inoculated into chickens, oropharyngeal (OP) shedding was detected as early as day 2 PI in groups of birds infected with 7 different viruses (MN 00-530, AI 02-749, LA 69B, AI 02-1070, MN 99-263, AI 01-124 and MN 99-198) while cloacal (C) shedding was detected in only two (AI 02-749 and MN 99-198) out of the seven previously mentioned. At day 4 PI, additional OP shedding was noted with 4 viruses (MN 99-253, AI 03-114, LA B156 and TX 02-260) while C shedding was detected in chickens inoculated with 3 viruses (MN 99-253, TX 02-260 and LA 69B). Most of the birds were negative for OP and C shedding at day 7 PI except for those inoculated with MN 00-530 (OP and C), MN 99-253 (OP only), LA B156 (C only), TX 02-260 (OP and C) and AI 02-749 (OP only).

In the case of turkeys, seven different viruses also caused OP shedding in birds at day 2 PI (TX 02-260, AI 03-452, LA 240B, MN 98-232, MN 99-263, AI 02-1070 and MN 99-198). Among these, the last three viruses also caused OP shedding in chickens (see previous paragraph). On the other hand, only one virus caused C shedding in birds (MN 99-198) at day 2 PI. An additional virus (AI 02-749) caused OP and C shedding at day 4 PI. Cloacal shedding was

detected in two out of the seven viruses previously mentioned (MN 99-198 and AI 03-452) while only one (AI 02-749) caused cloacal shedding at day 7 PI.

Fecal samples from both species were also collected at days 2, 4 and 7 PI. Shedding of three viruses were detected in samples collected from days 4 (MN 00-530) and 7 PI (MN 00-530, AI 02-749, LA 69B) (Table 6). Along with correlation coefficients, linear regression equations (Table 7) derived from the standard curve of each virus was computed and used to determine the relative amounts of virus (Tables 8, 9 and 10) present in the swab and fecal samples. Based on these results, SPF chickens were generally found to be shedding more virus from both the oropharynx and cloaca than commercial turkeys in this study.

Histopathological Lesions

Microscopic lesions found in the internal organs and tissues of infected SPF chickens collected at 3 days PI are shown in Table 11. Negative control birds did not demonstrate any significant lesions (data not shown). Most of the lesions observed were predominantly confined to the respiratory and gastrointestinal tract. In the nasal cavity, 10 out of the 16 viruses caused mild to severe forms of catarrhal rhinitis while 2 viruses (MN 99-198 and AI 03-452) caused lymphocytic rhinitis in chickens. Sloughing of the respiratory epithelium and the presence of a mild amount of mucin (catarrhal tracheitis) were noted in the tracheal lumen of birds infected with 9 different viruses while only LA 240B has caused acute degeneration of multiple areas in the tracheal epithelium. Excessive amounts of mucin/edema in the lamina propria of a secondary bronchus in the lung (edematous bronchitis) were found in a chicken infected with MN 00-530 while a mild to moderate form of catarrhal bronchitis was noted in birds inoculated with viruses MN 98-232 and LA B156, respectively. A mild to severe form of interstitial pneumonia (MN 99-253, AI 03-114 and AI 02-749), mild to moderate proliferation of bronchiole-associated

lymphoid tissues (BALT) (TX 02-260 and AI 02-749) and lymphocytic bronchiolitis (LA 69B and AI 01-1407) were also found present in infected birds.

A minimal amount of scattered myocardial fibers with shrunken nuclei and loss of cross-striations (myocardial degeneration) were noted in chickens infected with MN 99-263 while all skeletal muscle samples (breast and thigh) yielded negative for lesions. In addition, a minimal amount of lymphocytic infiltrates was seen to surround a small blood vessel in the cerebral part of the brain (lymphocytic encephalitis).

Lesions in the gastrointestinal tract were predominantly found in the ceca of chickens infected with 5 viruses wherein mild tonsil micro-hemorrhages and inflammation were present. Only one virus (AI 01-124), on the other hand, caused a focal moderate lymphocytic infiltration in the lamina propria of the small intestine (lymphocytic enteritis). Infection with five viruses (MN 99-253, TX 02-260, AI 01-124, MN 99-198 and LA 240B) produced a mild to moderate lymphocytic hepatitis while inoculation of AI 02-749 resulted to a mild increase in lymphocytic infiltrates in the periportal regions (lymphocytic cholangiohepatitis) of the liver. Likewise, mild hepatic necrosis was noted in liver tissues of birds infected with AI 01-1407 and LA 240B virus. A mild proliferation of gut-associated lymphoid tissues (GALT) was present in the duodenal samples of chickens infected with LA B156, AI 02-749 and MN 00-38 viruses. Also, a mild to severe form of lymphocytic pancreatitis was recorded in samples taken from birds infected with AI 02-749 and TX 02-260, respectively. On the other hand, mild necrosis was observed in the spleen of a chicken inoculated with MN 00-38 while four viruses (MN 00-530, LA B156, TX 02-260 and AI 02-749) caused an increased number of macrophages throughout its parenchyma (histiocytosis).

Mild to moderate lymphoid depletion (in 7 viruses) and lymphoid atrophy (in 2 viruses only) were noted in the bursa of Fabricius and thymus of infected birds while adrenal glands had no histologic lesions present upon examination. On the other hand, mild tubular degeneration, mild to moderate interstitial nephritis and a severe lymphocytic orchitis were also noted in kidney and testicular sections, respectively.

Current data available in this study showed that turkeys had fewer histopathological lesions than chickens although the predominance of lesions is the same with the latter (i.e. respiratory and GI tracts) (Table 12). Similar to negative controls in chickens, no significant lesions were found in non-inoculated turkeys (results not shown). In the respiratory tract, the virus TX 02-260 caused both moderate catarrhal rhinitis and mild proliferation of BALT. Mild to moderate lymphocytic rhinitis was noted in turkeys inoculated with 4 different viruses (MN 00-530, MN 99-253, AI 03-114 and LA B156), with the first two previously mentioned also producing mild catarrhal and heterophilic tracheitis, respectively.

Lymphocytic endocarditis was noted in the heart tissue sample of a bird infected with AI 03-114 while loss of cross striations and hypereosinophilia of myofibers (degenerative myopathy) were observed in skeletal muscle samples collected from TX 02-260-infected birds. There were no recorded microscopic lesions in the brain, pancreas, adrenal glands, kidneys and reproductive organs.

Mild to moderate forms of enteritis were noted mainly in the duodenum and small intestine of turkeys infected with all the viruses included in the current data. Moreover, degeneration/necrosis of some villus tips (enteric necrosis) and multiple areas of mild heterophils in the lamina propria of the small intestine were seen in birds inoculated with AI 03-114. Proliferation of GALT was also observed in the duodenum of MN 00-530-infected birds.

Multiple foci of mild heterophils in the lamina propria of the cecum (typhlitis) were found in histopathological samples obtained from both AI 03-114- and TX 02-260-infected turkeys. Mild to moderate microvesicular hepatopathy was noted in liver samples taken from all infected birds (i.e. only those viruses included in the current data) while specimens taken from turkeys infected with TX 02-260 showed moderate numbers of large clear vacuoles within the cytoplasm of hepatocytes (lipidosis). TX 02-260 caused an increased number of macrophages in the splenic parenchyma while mild lymphoid atrophy (MN 00-530, MN 99-253 and LA B156) and mild to moderate lymphoid depletion (MN 99-253, AI 03-114 and TX 02-260) in the bursa and thymus were also noted.

Serological Testing

Sera from negative control birds (non-inoculated) for each experimental trial did not show seroconversion in both tests (results not shown). In both tests, antibodies against LPAI viruses were not demonstrated in chickens inoculated with 10 different viruses (MN 99-253, AI 03-114, LA B156, TX 02-260, AI 02-749, AI 02-1070, AI 01-1407, AI 03-452, LA 240B and MN 98-232) while in two viruses (MN 00-38 and AI 01-124), only one sample was positive for both tests (Table 13). On the other hand, viruses MN 00-530, LA 69B, MN 99-263 and MN 99-198 demonstrated an inconsistent trend at the time of each serum collection. Likewise, a similar variable trend exists with all 4 viruses in terms of the HI test, as shown in the table. Geometric mean titers (GMT) of HI for viruses which caused seroconversion ranged from 6.3 (MN 00-530 day 7 PI) to 64.0 (MN 99-198 day 14 PI) (data not shown).

In turkeys, viruses MN 00-38, AI 01-1407 and MN 98-232 did not demonstrate seroconversion at all times of serum collection in both serological tests (Table 14). AI 03-114 had only 2 serum samples (collected at day 14 PI) that were positive for both ELISA and HI

tests. Other viruses such as AI 03-452, LA 69B, LA 240B, MN 99-263, TX 02-260, MN 99-198, AI 02-1070, LA B156 and MN 99-253 had only 1 up to 7 sero-positive samples (in either test or both) out of the maximum 21 samples collected for each virus at serum collection times. On the other hand, seroconversion was observed in approximately 50% of the serum samples collected for the following three viruses in both tests: 1) MN 00-530, 2) AI 02-749 and 3) AI 01-124. Similar to seropositive samples in chickens, a variable trend exists with these viruses in terms of the ELISA and HI test such that some showed: 1) increasing antibody titers until day 21 PI or 2) peaking of seroconversion at day 14 PI and lowering of antibody titers at day 21 PI. The HI geometric mean titers for viruses which caused seroconversion in the majority of samples ranged from 2.0 (MN 00-530 day 7 PI) to 95.1 (MN 00-530 day 21 PI) (data not shown).

Based on the above-mentioned results, more LPAI wild bird isolates used in this study caused seroconversion in commercial turkeys compared to SPF chickens.

Table 4: Egg Infectious Dose 50 (EID_{50}/ml) and Hemagglutination (HA) titer of 24 low pathogenic avian influenza wild bird isolates.

Isolate	Subtype	Titer (1 x 10^n EID_{50}/ml)	HA Titer [A]
MN 00-530	H_4N_6	8.4	512
MN 99-253	H_6N_5	8.5	512
MN 98-153 [B]	H_9N_2 (?)	6.5	512
AI 04-02 [B]	H_6N_8	5.8	512
LA 69B	H_4N_8	8.4	1024
MN 00-38	H_6N_3	7.6	128
AI 03-744 [B]	H_9N_2	6.5	512
AI 02-1044 [B]	H_9N_9	$\leq$3.0	2048
AI 03-114	H_9N_2	8.4	512
AI 02-1070	H_9N_4	7.4	512
MN 99-263	H_4N_9	8.7	128
AI 02-1467 [B]	H_9N_9	6.4	512
MN 99-198	H_4N_6	7.5	1024
AI 01-124	H_6N_4	8.5	2048
LA B156 [C]	H_4N_6	9.2	1024
AI 01-1407	H_4N_6	7.5	128
AI 03-452 [C]	H_9N_2	6.7	1024
LA 240B	H_4N_6	9.9	512
TX 02-260 [C]	H_6N_4	9.7	2048
MN 98-232	H_9N_2 (?)	6.8	64
MN 98-304 [B]	H_9N_2 (?)	5.4	128
MN 99-365 [B]	H_9N_2 (?)	5.4	512
AI 02-1523 [B]	H_9N_5 (?)	7.4	512
AI 02-749	H_9N_9 (?)	8.1	4096

[A] 1:2 dilution

[B] Not used for bird pathogenicity test

[C] Used for 4 HA units in HI test

(?) Tested PCR negative against H9 primers

Table 5: Viral shedding summary of oropharyngeal (OP) and cloacal (C) swabs from chickens and turkeys.

Virus	Sub-type	Titer (EID_{50}/mL)	Chicken [A]						Turkey [A]					
			Day 2		Day 4		Day 7		Day 2		Day 4		Day 7	
			OP	C	OP	C	OP	C	OP	C	OP	C	OP	C
MN 00-530	H_4N_6	$1 \times 10^{8.4}$	29.40	0.00	0.00	0.00	39.21	35.94	0.00	0.00	0.00	0.00	0.00	0.00
MN 99-253	H_6N_5	$1 \times 10^{8.5}$	0.00	0.00	37.68	35.64	37.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AI 03-114	H_9N_2	$1 \times 10^{8.4}$	0.00	0.00	37.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LA B156	H_4N_6	$1 \times 10^{9.2}$	0.00	0.00	36.34	0.00	0.00	38.64	0.00	0.00	0.00	0.00	0.00	0.00
TX 02-260	H_9N_2	$1 \times 10^{9.7}$	0.00	0.00	36.93	36.55	37.31	37.64	39.21	0.00	0.00	0.00	0.00	0.00
AI 02-749	H_9N_9 (?)	$1 \times 10^{8.1}$	40.89	36.08	35.28	36.68	39.73	0.00	0.00	0.00	32.91	29.15	0.00	25.53
LA 69B	H_4N_8	$1 \times 10^{8.4}$	30.34	0.00	32.11	26.72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MN 00-38	H_6N_3	$1 \times 10^{7.6}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AI 02-1070	H_9N_4	$1 \times 10^{7.4}$	38.20	0.00	0.00	0.00	0.00	0.00	38.85	0.00	0.00	0.00	0.00	0.00
MN 99-263	H_4N_9	$1 \times 10^{8.7}$	32.80	0.00	32.34	0.00	0.00	0.00	34.01	0.00	0.00	0.00	0.00	0.00
AI 01-124	H_6N_4	$1 \times 10^{8.5}$	32.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MN 99-198	H_4N_6	$1 \times 10^{7.5}$	29.66	32.11	29.78	0.00	0.00	0.00	31.42	40.97	36.87	0.00	35.70	0.00
AI 01-1407	H_4N_6	$1 \times 10^{7.5}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AI 03-452	H_9N_2	$1 \times 10^{6.7}$	0.00	0.00	0.00	0.00	0.00	0.00	37.24	0.00	0.00	0.00	34.81	0.00
LA 240B	H_4N_6	$1 \times 10^{9.9}$	0.00	0.00	0.00	0.00	0.00	0.00	35.22	0.00	33.18	0.00	0.00	0.00
MN 98-232	H_9N_2 (?)	$1 \times 10^{6.8}$	0.00	0.00	0.00	0.00	0.00	0.00	35.77	0.00	0.00	0.00	0.00	0.00

[A] Average CT values (numbers above 38 are considered negative for the test)

(?) Tested PCR negative against H9 primers

Table 6: Viral shedding summary of fecal samples from chickens and turkeys.

Virus	Subtype	Titer (EID_{50}/mL)	Chicken [A]			Turkey [A]		
			Day 2	Day 4	Day 7	Day 2	Day 4	Day 7
MN 00-530	H_4N_6	$1 \times 10^{8.4}$	-	28.88	22.35	0.00	0.00	0.00
MN 99-253	H_6N_5	$1 \times 10^{8.5}$	-	0.00	0.00	0.00	0.00	0.00
AI 03-114	H_9N_2	$1 \times 10^{8.4}$	-	0.00	0.00	0.00	0.00	0.00
LA B156	H_4N_6	$1 \times 10^{9.2}$	-	0.00	0.00	0.00	0.00	0.00
TX 02-260	H_6N_4	$1 \times 10^{9.7}$	-	0.00	0.00	0.00	0.00	0.00
AI 02-749	H_9N_9 (?)	$1 \times 10^{8.1}$	-	0.00	0.00	0.00	0.00	31.06
LA 69B	H_4N_8	$1 \times 10^{8.4}$	0.00	0.00	34.17	0.00	0.00	0.00
MN 00-38	H_6N_3	$1 \times 10^{7.6}$	0.00	0.00	0.00	0.00	0.00	0.00
AI 02-1070	H_9N_4	$1 \times 10^{7.4}$	0.00	0.00	0.00	0.00	0.00	0.00
MN 99-263	H_4N_9	$1 \times 10^{8.7}$	0.00	0.00	0.00	0.00	0.00	0.00
AI 01-124	H_6N_4	$1 \times 10^{8.5}$	0.00	0.00	0.00	0.00	0.00	0.00
MN 99-198	H_4N_6	$1 \times 10^{7.5}$	0.00	0.00	0.00	0.00	0.00	0.00
AI 01-1407	H_4N_6	$1 \times 10^{7.5}$	0.00	0.00	0.00	0.00	0.00	0.00
AI 03-452	H_9N_2	$1 \times 10^{6.7}$	0.00	0.00	0.00	0.00	0.00	0.00
LA 240B	H_4N_6	$1 \times 10^{9.9}$	0.00	0.00	0.00	0.00	0.00	0.00
MN 98-232	H_9N_2 (?)	$1 \times 10^{6.8}$	0.00	0.00	0.00	0.00	0.00	0.00

[A]Average CT values (numbers above 38 are considered negative for the test)

Note: Dashes denote missing data

(?) Tested PCR negative against H9 primers

Table 7: Linear Regression Equations (LRE) generated from standard curves of LPAI wild bird isolates.

Virus	LRE[A]	R^2 [B]	Standard Error of the Estimate[C]
MN 00-530	y = -0.287x + 14.539	0.997	0.092
MN 99-253	y = -0.293x + 14.881	0.993	0.143
AI 03-114	y = -0.293x + 15.115	0.990	0.151
LA B156	y = -0.298x + 15.797	0.984	0.156
TX 02-260	y = -0.287x + 15.906	0.982	0.203
AI 02-749	y = -0.306x + 14.474	0.989	0.193
LA 69B	y = -0.315x + 16.319	0.994	0.113
MN 00-38	y = -0.278x + 14.580	0.998	0.061
AI 02-1070	y = -0.301x + 15.024	0.950	0.263
MN 99-263	y = -0.325x + 16.460	0.990	0.164
AI 01-124	y = -0.313x + 14.828	0.979	0.242
MN 99-198	y = -0.298x + 13.797	0.992	0.140
AI 01-1407	y = -0.324x + 14.558	0.963	0.327
AI 03-452	y = -0.252x + 12.151	0.953	0.335
LA 240B	y = -0.273x + 16.257	0.990	0.135
MN 98-232	y = -0.295x + 13.514	0.994	0.098

[A] Dilutions used to determine LRE ranged from undiluted virus and 10^{-1} to 10^{-10}, with each dilution done in triplicate for all 16 viruses
[B] Correlation coefficient
[C] Standard deviation of the data about the regression line

Table 8: Average CT values and relative viral amounts detected in oropharyngeal (OP) and cloacal (C) swabs collected from chickens.

Virus	CT Value[A]						Relative Viral Amount (1 x 10^n EID_{50}/ml)					
	Day 2		Day 4		Day 7		Day 2		Day 4		Day 7	
	OP	C	OP	C	OP	C	OP	C	OP	C	OP	C
MN 00-530	29.40	0.00	0.00	0.00	39.21	35.94	6.10	-	-	-	3.30	4.20
MN 99-253	0.00	0.00	37.68	35.64	37.38	0.00	-	-	3.80	4.40	3.90	-
AI 03-114	0.00	0.00	37.45	0.00	0.00	0.00	-	-	4.10	-	-	-
LA B156	0.00	0.00	36.34	0.00	0.00	38.64	-	-	5.00	-	-	4.30
TX 02-260	0.00	0.00	36.93	36.55	37.31	37.64	-	-	5.30	5.40	5.20	5.10
AI 02-749	40.89	36.08	35.28	36.68	39.73	0.00	1.90	3.40	3.70	3.20	2.30	-
LA 69B	30.34	0.00	32.11	26.72	0.00	0.00	6.70	-	6.20	7.90	-	-
MN 00-38	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
AI 02-1070	38.20	0.00	0.00	0.00	0.00	0.00	3.50		-	-	-	-
MN 99-263	32.80	0.00	32.34	0.00	0.00	0.00	5.80	-	5.90	-	-	-
AI 01-124	32.30	0.00	0.00	0.00	0.00	0.00	4.70	-	-	-	-	-
MN 99-198	29.66	32.11	29.78	0.00	0.00	0.00	5.00	4.20	4.90	-	-	-
AI 01-1407	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
AI 03-452	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
LA 240B	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
MN 98-232	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-

[A]CT values above 38 are considered negative for the test

Note: Dashes on the relative viral amount column mean zero

Table 9: Average CT values and relative viral amounts detected in oropharyngeal (OP) and cloacal (C) swabs collected from turkeys.

Virus	CT Value[A]						Relative Viral Amount (1 x 10^n EID_{50}/ml)					
	Day 2		Day 4		Day 7		Day 2		Day 4		Day 7	
	OP	C	OP	C	OP	C	OP	C	OP	C	OP	C
MN 00-530	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
MN 99-253	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
AI 03-114	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
LA B156	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
TX 02-260	39.21	0.00	0.00	0.00	0.00	0.00	4.60	-	-	-	-	-
AI 02-749	0.00	0.00	32.91	29.15	0.00	25.53	-	-	4.40	5.50	-	6.70
LA 69B	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
MN 00-38	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
AI 02-1070	38.85	0.00	0.00	0.00	0.00	0.00	3.30	-	-	-	-	-
MN 99-263	34.01	0.00	0.00	0.00	0.00	0.00	5.40	-	-	-	-	-
AI 01-124	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
MN 99-198	31.42	40.97	36.87	0.00	35.70	0.00	4.40	1.60	2.80	-	3.20	-
AI 01-1407	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
AI 03-452	37.24	0.00	0.00	0.00	34.81	0.00	2.80	-	-	-	3.40	-
LA 240B	35.22	0.00	33.18	0.00	0.00	0.00	6.60	-	7.20	-	-	-
MN 98-232	35.77	0.00	0.00	0.00	0.00	0.00	3.00	-	-	-	-	-

[A]CT values above 38 are considered negative for the test

Note: Dashes on the relative viral amount column mean zero

Table 10: Average CT values and relative viral amounts detected in fecal samples collected from chickens and turkeys.

Virus	Chicken						Turkey					
	CT value[A]			Relative Viral Amount (1×10^n EID_{50}/ml)			CT value[A]			Relative Viral Amount (1×10^n EID_{50}/ml)		
	Day 2	Day 4	Day 7	Day 2	Day 4	Day 7	Day 2	Day 4	Day 7	Day 2	Day 4	Day 7
MN 00-530	x	28.88	22.35	x	6.20	8.10	0.00	0.00	0.00	-	-	-
MN 99-253	x	0.00	0.00	x	-	-	0.00	0.00	0.00	-	-	-
AI 03-114	x	0.00	0.00	x	-	-	0.00	0.00	0.00	-	-	-
LA B156	x	0.00	0.00	x	-	-	0.00	0.00	0.00	-	-	-
TX 02-260	x	0.00	0.00	x	-	-	0.00	0.00	0.00	-	-	-
AI 02-749	x	0.00	0.00	x	-	-	0.00	0.00	31.06	-	-	5.00
LA 69B	0.00	0.00	34.17	-	-	5.50	0.00	0.00	0.00	-	-	
MN 00-38	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
AI 02-1070	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
MN 99-263	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
AI 01-124	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
MN 99-198	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
AI 01-1407	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
AI 03-452	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
LA 240B	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-
MN 98-232	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00	-	-	-

[A]CT values above 38 are considered negative for the test

x - Denotes missing data

Note: Dashes on the relative viral amount column mean zero

Table 11: Average severity of histopathological lesions found in body organs and tissues of LPAI virus-infected chickens at 3 days PI.

Lesion[B]	Virus[A]															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Nasal cavity, trachea & lung																
- catarrhal rhinitis	+	-	-	+	-	+/+++	+	-	+	-	-	+	+/++	+	+	+/+++
- lymphocytic rhinitis	-	-	-	-	-	-	-	-	-	-	-	+	-	++	-	-
- catarrhal tracheitis	-	-	+	+	+	+	+	-	-	+	+	-	-	-	+	+
- degenerative tracheitis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
- edematous bronchitis	+++	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
- catarrhal bronchitis	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	+
- interstitial pneumonia	-	+	+	-	-	+++	-	-	-	-	-	-	-	-	-	-
- BALT hyperplasia	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-	-
- lymphocytic bronchiolitis	-	-	-	-	-	-	++	-	-	-	-	-	+	-	-	-
Heart, breast & thigh muscle																
- myocardial degeneration	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-
- lymphocytic myocarditis	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
Brain																
- lymphocytic encephalitis	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Gastro-intestinal tract																
- cecal tonsil micro-Hemorrhages	-	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-
- lymphocytic enteritis	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
- GALT hyperplasia	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-
- typhlitis	-	-	-	-	-	++	+	+	+	-	-	-	-	+	-	-
Liver and biliary structures																
- lymphocytic hepatitis	-	+	-	-	+	-	-	-	-	-	+	++	-	-	+	-
- lymphocytic cholangio-Hepatitis	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
- hepatic necrosis	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-

[A] 1 – MN 00-530; 2 – MN 99-253; 3 - AI 03-114; 4 – LA B156; 5 - TX 02-260; 6 - AI 02-749; 7 - LA 69B; 8 - MN 00-38; 9 - AI 02-1070; 10 - MN 99-263; 11 - AI 01-124; 12-MN 99-198; 13 - AI 01-1407; 14 - AI 03-452; 15- LA 240B; 16-MN 98-232

[B] - = no lesions; ± = minimal; + = mild; ++ = moderate; +++ = severe; M = missing

Table 11: Average severity of histopathological lesions found in body organs and tissues of LPAI virus-infected chickens at 3 days PI (continued).

Lesion[B]	Virus[A]															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Pancreas																
- lymphocytic pancreatitis	-	-	-	-	+++	+	-	-	-	-	-	-	-	-	-	-
Spleen																
- necrosis	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
- histiocytosis	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Bursa & thymus																
- lymphoid depletion	+	+	-	+	+	+	-	-	-	-	-	-	++	-	+	-
- lymphoid atrophy	-	-	-	+	-	-	-	-	-	-	-	-	++	-	-	-
Adrenal gland	-	-	-	-	M	-	M	-	-	-	-	-	-	-	-	-
Kidney & reproductive organs																
- tubular degeneration	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
- interstitial nephritis	-	-	-	-	-	-	-	-	-	+	++	+/++	-	-	-	-
- lymphocytic orchitis	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-

[A]1 – MN 00-530 3 - AI 03-114 5 - TX 02-260 7 - LA 69B 9 - AI 02-1070 11 - AI 01-124 13 - AI 01-1407 15- LA 240B
2 – MN 99-253 4 – LA B156 6 - AI 02-749 8 - MN 00-38 10 - MN 99-263 12-MN 99-198 14 - AI 03-452 16-MN 98-232

[B] - = no lesions; ± = minimal; + = mild; ++ = moderate; +++ = severe; M = missing

Table 12: Average severity of histopathological lesions found in body organs and tissues of LPAI virus-infected turkeys at 3 days PI.

Lesion[B]	Virus[A]															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Nasal cavity, trachea & lung																
- catarrhal rhinitis	-	-	-	-	++											
- lymphocytic rhinitis	++	+	+	+	-											
- catarrhal tracheitis	+	-	-	-	-						ND					
- heterophilic tracheitis	-	+	-	-	-											
- BALT hyperplasia	-	-	-	-	+											
Heart, breast & thigh muscle																
- lymphocytic endocarditis	-	-	+	-	-						ND					
- degenerative myopathy	-	-	-	-	+											
Brain	-	-	-	-	-						ND					
Gastro-intestinal tract																
- enteritis	+/++	+	+	+	+											
- typhlitis	-	-	+	-	+						ND					
- enteric necrosis	-	-	+	-	-											
- GALT hyperplasia	+	-	-	-	-											
Liver and biliary structures																
- microvesicular hepatopathy	++	+/++	+/++	- /++	++						ND					
- lipidosis	-	-	-	-	++											
Pancreas	-	-	-	-	-						ND					
Spleen																
- histiocytosis	-	-	-	-	+						ND					
Bursa & thymus																
- lymphoid atrophy	+	+	-	+	-						ND					
- lymphoid depletion	-	+	+	-	+/++											
Adrenal gland	-	-	-	-	-						ND					
Kidney & reproductive organs	-	-	-	-	-						ND					

[A] 1 – MN 00-530 3 - AI 03-114 5 - TX 02-260 7 – LA 69B 9 - AI 02-1070 11 - AI 01-124 13 - AI 01-1407 15- LA 240B
2 –MN 99-253 4 - LA B156 6 - AI 02-749 8 - MN 00-38 10 - MN 99-263 12-MN 99-198 14 - AI 03-452 16-MN 98-232

[B] - = no lesions; ± = minimal; + = mild; ++ = moderate; +++ = severe; M = missing; ND = no data available yet at the time of submission

Table 13: ELISA[A] and HI[B] test results of sera collected from LPAI virus-infected chickens.

Bird Number	MN 00-530 (H_4N_6)						MN 99-253 (H_6N_5)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.000	4	0.038	2	0.034	8	0.000	2	0.000	2	0.119	4
2	0.000	4	0.758	64	0.396	8	0.000	2	0.000	2	0.013	2
3	0.408	8	0.773	64	0.519	128	0.000	2	0.000	2	0.055	2
4	0.351	4	0.545	32	0.319	16	0.000	2	0.000	2	0.034	2
5	0.602	32	1.227	256	1.515	256	0.000	2	0.000	2	0.013	2
6	0.000	4	0.000	2	0.013	2	0.000	2	0.000	2	0.000	2

Bird Number	AI 03-114 (H_9N_2)						LA B156 (H_4N_6)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.000	2	0.000	2	0.000	2	0.000	2	0.000	2	0.000	2
2	0.000	2	0.000	2	0.119	2	0.000	2	0.000	2	0.153	2
3	0.000	2	0.000	2	0.000	2	0.000	2	0.000	2	0.051	2
4	0.000	2	0.000	2	0.136	2	0.000	2	0.000	2	0.136	2
5	0.000	2	0.000	2	0.021	2	0.000	2	0.000	2	0.021	2
6	0.000	2	0.000	2	0.000	2	0.000	2	0.270	2	0.000	2

Bird Number	TX 02-260 (H_6N_4)						AI 02-749 (H_9N_9)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.000	2	0.270	2	0.000	2	0.000	2	0.000	2	0.000	2
2	0.038	2	0.000	2	0.370	2	0.000	2	0.000	2	0.000	2
3	0.000	2	0.000	2	0.166	2	0.000	2	0.000	2	0.000	2
4	0.028	2	0.000	2	0.000	2	0.000	2	0.000	2	0.000	2
5	0.000	2	0.000	2	0.004	2	0.000	2	0.000	2	0.000	2
6	0.000	2	0.000	2	0.000	2	0.000	2	0.607	32	0.162	4

[A] S/P ratios (values greater than 0.5 are considered positive)

[B] Each serum sample tested 2x (titers greater than 32 are positive for the test)

Table 13: ELISA[A] and HI[B] test results of sera collected from LPAI virus-infected chickens (continued).

Bird Number	LA 69B (H_4N_8)						MN 00-38 (H_6N_3)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.050	2	0.090	2	0.083	8	0.084	2	0.020	2	0.002	2
2	0.314	32	0.748	64	0.514	64	0.053	2	0.031	2	0.000	2
3	0.359	64	1.216	128	0.761	128	0.014	2	0.084	2	0.761	128
4	0.078	64	0.387	2	0.197	8	0.028	2	0.006	2	0.000	2
5	0.014	2	0.322	2	0.131	16	0.000	2	0.020	2	0.000	2
6	0.000	2	0.003	2	0.000	2	0.008	2	0.003	2	0.000	2

Bird Number	AI 02-1070 (H_9N_4)						MN 99-263 (H_4N_9)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.067	2	0.042	2	0.018	2	1.308	128	0.916	128	0.232	32
2	0.062	2	0.359	2	0.028	2	0.905	64	1.311	128	0.326	8
3	0.031	2	0.053	2	0.000	2	0.487	16	0.028	2	0.000	2
4	0.140	4	0.084	2	0.031	2	0.258	8	0.468	16	0.184	4
5	0.050	2	0.238	2	0.011	2	0.227	8	0.104	4	0.000	16
6	0.132	4	0.028	2	0.000	2	0.014	2	0.373	8	0.155	16

Bird Number	AI 01-124 (H_6N_4)						MN 99-198 (H_4N_6)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.020	2	0.101	2	0.000	2	0.847	128	1.140	256	1.159	128
2	0.277	4	1.784	256	0.271	4	0.843	128	0.677	128	0.381	64
3	0.017	2	0.020	2	0.000	2	0.540	4	0.376	16	0.294	64
4	0.031	2	0.025	2	0.000	2	0.238	8	0.230	64	0.000	32
5	0.146	2	0.014	2	0.000	2	0.660	32	0.481	64	0.434	8
6	0.025	2	0.042	2	0.000	2	0.243	4	0.294	64	0.728	128
7							0.072	2	0.085	32	0.836	64

[A] S/P ratios (values greater than 0.5 are considered positive) [B] Each serum sample tested 2x (titers >32 are positive for the test)

Table 13: ELISA[A] and HI[B] test results of sera collected from LPAI virus-infected chickens (continued).

Bird Number	AI 01-1407 (H_4N_6)						AI 03-452 (H_9N_2)[C]					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.000	2	0.016	2	0.000	2	0.000	2	0.000	2	0.000	2
2	0.004	2	0.050	2	0.000	2	0.013	2	0.045	2	0.000	2
3	0.000	2	0.000	2	0.000	2	0.038	2	0.098	2	0.019	2
4	0.000	2	0.000	2	0.000	2	0.038	2	0.468	8	0.029	2
5	0.004	2	0.066	2	0.000	2	0.000	2	0.021	2	0.000	2
6	0.013	2	0.437	2	0.161	2	0.013	2	0.024	2	0.000	2
7	0.013	2	0.146	2	0.000	2	0.000	2				

Bird Number	LA 240B (H_4N_6)						MN 98-232 (H_9N_2)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.013	2	0.000	2	0.000	2	0.000	2	0.000	2	0.000	2
2	0.000	2	0.000	2	0.500	32	0.000	2	0.000	2	0.000	2
3	0.026	2	0.003	2	0.000	2	0.013	2	0.005	2	0.000	2
4	0.004	2	0.000	2	0.093	16	0.004	2	0.000	2	0.000	2
5	0.013	2	0.000	2	0.000	2	0.000	2	0.000	2	0.000	2
6	0.072	2	0.198	2	0.000	2	0.000	2	0.000	2	0.000	2
7	0.455	2	0.389	2	0.000	2	0.000	2	0.000	2	0.000	2

[A] S/P ratios (values greater than 0.5 are considered positive)
[B] Each serum sample tested 2x (titers greater than 32 are positive for the test)
[C] One bird died during blood collection on 12/24/07

Table 14: ELISA[A] and HI[B] test results of sera collected from LPAI virus-infected turkeys.

Bird Number	MN 00-530 (H_4N_6)						MN 99-253 (H_6N_5)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.146	2	1.614	128	1.167	64	0.086	2	0.460	8	0.154	4
2	0.149	2	0.266	8	1.216	64	0.417	4	0.294	4	0.154	4
3	0.129	2	0.540	4	0.782	64	2.091	256	0.726	128	0.329	8
4	0.086	2	0.440	4	1.572	128	1.149	128	0.726	64	0.302	8
5	0.137	2	0.597	64	1.975	256	0.317	4	0.454	8	0.191	4
6	0.157	2	0.523	64	1.708	256	0.546	8	1.931	256	0.374	8
7	0.129	2	0.477	4	0.163	32	0.657	64	4.374	256	0.591	32

Bird Number	AI 03-114 (H_9N_2)						LA B156 (H_4N_6)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.354	4	1.406	128	0.570	32	0.249	4	0.094	2	0.206	4
2	0.483	8	0.686	64	0.370	4	0.726	64	1.194	128	0.370	8
3	0.157	2	0.074	2	0.179	2	1.514	128	5.371	256	0.459	16
4	0.091	2	0.140	2	0.189	2	0.337	8	0.106	4	0.154	4
5	0.051	2	0.051	2	0.272	4	0.563	16	0.337	8	0.202	4
6	0.223	4	0.126	2	0.187	2	0.480	16	0.189	2	0.212	8
7	0.189	2	0.311	2	0.204	2	1.029	128	2.909	256	0.409	8

Bird Number	TX 02-260 (H_6N_4)						AI 02-749 (H_9N_9)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.769	64	2.611	256	1.319	128	0.008	2	0.096	2	0.025	2
2	0.274	8	0.091	2	0.407	4	0.000	2	0.207	2	0.093	2
3	0.680	64	0.214	2	0.370	4	0.165	2	0.889	128	0.915	128
4	0.066	2	0.089	2	0.582	32	0.079	2	0.504	32	0.831	64
5	0.211	2	0.120	2	0.181	2	0.526	16	2.563	256	1.839	256
6	0.406	2	0.134	2	0.200	2	1.315	128	2.637	256	4.381	256
7	0.240	2	0.074	2	0.296	2	0.339	4	0.941	64	1.729	256

[A] S/P ratios (values greater than 0.5 are considered positive) [B] Each serum sample tested 2x (titers >32 are positive for the test)

Table 14: ELISA[A] and HI[B] test results of sera collected from LPAI virus-infected turkeys (continued).

Bird Number	LA 69B (H_4N_8)						MN 00-38 (H_6N_3)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.399	8	0.189	4	0.015	2	0.198	2	0.000	2	0.037	2
2	0.165	2	0.000	2	0.015	2	0.206	4	0.000	2	0.022	2
3	0.523	8	0.417	64	0.696	64	0.222	2	0.000	2	0.022	2
4	0.263	2	0.000	2	0.126	4	0.173	2	0.024	2	0.067	2
5	0.290	2	0.417	64	0.533	16	0.187	2	0.000	2	0.015	2
6	0.313	2	0.000	2	0.096	2	0.169	2	0.000	2	0.015	2
7	0.160	2	0.016	2	0.037	2	0.148	2	0.071	2	0.007	2

Bird Number	AI 02-1070 (H_9N_4)						MN 99-263 (H_4N_9)[C]					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	2.366	256	1.181	128	1.319	128	0.403	8	0.362	8	0.252	16
2	0.340	8	0.654	32	0.400	8	1.899	256	0.874	64	0.519	64
3	0.543	32	0.882	128	0.607	64	0.477	8	0.417	32	0.496	32
4	0.506	16	0.677	64	0.526	32	0.586	16	0.827	32	0.889	64
5	0.325	8	0.016	2	0.281	8	0.253	4				
6	0.141	4	0.000	2	0.067	2						
7	0.259	8	0.079	2	0.119	4						

Bird Number	AI 01-124 (H_6N_4)						MN 99-198 (H_4N_6)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.776	32	1.480	128	0.807	64	0.000	2	0.015	2	0.000	2
2	0.200	4	0.661	16	0.993	128	0.236	4	0.607	256	0.331	32
3	0.230	4	0.449	8	0.496	8	0.094	2	0.452	16	0.144	4
4	0.276	4	1.787	256	2.437	256	0.165	2	0.844	256	0.347	32
5	0.183	2	1.118	128	1.185	128	0.299	2	2.333	256	0.576	64
6	1.788	256	1.693	128	1.304	128	0.076	2	0.415	256	0.000	2
7	0.368	8	0.646	32	0.459	8	0.076	2	0.067	16	0.042	2

[A] S/P ratios (values greater than 0.5 are Considered positive)

[B] Each serum sample tested 2x (titers >32 are positive for the test)

[C] One bird found dead before and 2 died after virus inoculation

Table 14: ELISA[A] and HI[B] test results of sera collected from LPAI virus-infected turkeys (continued).

Bird Number	AI 01-1407 (H_4N_6)						AI 03-452 (H_9N_2)					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.016	2	0.037	2	0.000	2	0.067	2	0.067	2	0.220	8
2	0.079	2	0.044	2	0.000	2	0.189	4	0.393	8	0.068	2
3	0.016	2	0.096	2	0.025	2	1.661	256	0.719	32	0.627	16
4	0.085	2	0.230	2	0.000	2	0.079	2	0.459	8	0.814	32
5	0.000	2	0.007	2	0.025	2	0.236	2	0.570	16	1.441	32
6	0.008	2	0.385	2	0.000	2	0.047	2	0.119	4	0.017	2
7	0.276	2	0.067	2	0.025	2	0.071	2	0.348	4	0.085	8

Bird Number	LA 240B (H_4N_6)						MN 98-232 (H_9N_2)[C]					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
1	0.047	2	0.074	2	0.236	16	0.016	2	0.044	2	0.000	2
2	0.126	2	1.148	32	0.636	32	0.079	2	0.156	4	0.000	2
3	0.047	2	0.519	32	0.432	8	0.008	2	0.037	2	0.000	2
4	0.008	2	0.022	2	0.042	2	0.008	2	0.126	4	0.178	2
5	0.000	2	0.474	8	0.449	32	0.189	4	0.576	16	0.237	4
6	0.197	2	1.274	64	0.695	64	0.016	2	0.341	2	0.127	2
7	0.354	4	1.356	128	1.178	64						

[A] S/P ratios (values greater than 0.5 are considered positive)
[B] Each serum sample tested 2x (titers >32 are positive for the test)
[C] One bird found dead and wounded 3/9/2008 (a day before virus inoculation)

CHAPTER 5

DISCUSSION

The objective of this research was to biologically characterize LPAI wild bird isolates thru bird pathogenicity, virus shedding and serologic response studies in SPF chickens and commercial turkeys. Clinical signs of AI infection can vary based on virus strain, host species, sex, age, immunity, concurrent infections and environmental factors (146). Clinical signs observed in our study are consistent with the previously reported signs (146) and ranged from no clinical signs to moderate depression, decreased activity and decreased food and water consumption, as seen in the case of MN 99-263 in turkeys, bird species that are considered to be the most susceptible among domestic poultry (4).

Avian influenza viruses are transmitted through excretion of the virus from nares, mouth, conjunctiva and cloaca of infected birds (146, 208). Tracheal and cloacal swabs are most often used to isolate/detect the virus (145). Our data shows that SPF chickens were generally found to be shedding more virus from the oropharynx than commercial turkeys (Table 5). This finding is in contrast with the study conducted by Tumpey and others (208), wherein the level of infectious virus recovered from the oropharynx of commercial turkeys was 20- to 158-fold higher than what was detected from the same anatomical site in SPF chickens. In this study, low or undetectable viral shedding from the cloaca was observed in both species at days 2, 4 and 7 PI. This may imply that these LPAI viruses replicate more efficiently in the respiratory tract than the gastro-intestinal tract (208).

Although the presence of PCR inhibitors in the cloacal swab samples was not proven due to the absence (unavailability) of an internal positive control (e.g transcribed RNA, inactivated virus) in the qRRT-PCR protocol utilized in this study, this could have possibly interfered with optimal virus detection (202). This may vary between chickens and turkeys and may have affected the outcome of the virus shedding data.

Histopathological lesions usually found following LPAI infection in poultry are mostly confined to the respiratory and gastro-intestinal tract, some of which include: 1) lymphocytic tracheitis and bronchitis and 2) pancreatitis (146). Our findings are consistent with these reports. Histopathological results in both species at day 3 PI showed the predominance of lesions in the respiratory and gastro-intestinal tract (Table 11 and 12), a finding which is consistent with the fact that these viruses are of low pathogenicity. In chickens, oropharyngeal shedding strongly correlated with the lesions found in the upper respiratory tract, which were mostly seen in the nasal cavity (rhinitis) and tracheal sections (tracheitis). However, viruses such as AI 01-1407, AI 03-452, LA 240B and MN 98-232 are notable exceptions wherein mild to severe catarrhal rhinitis was present but there was no OP shedding detected in chickens at all times of swab collection. On the other hand, OP shedding at day 2 PI was detected only in 2 out of 5 viruses (MN 00-530 and AI 02-749) that caused lesions in the lower respiratory tract (lung). Shedding of other viruses from the oropharynx was later detected during the course of infection as seen in Table 5.

Viral-induced changes in the gastrointestinal tract of chickens in this study include cecal tonsil micro-hemorrhages, GALT hyperplasia and typhlitis, which were the three most common GI lesions due to viral activity (e.g. multiplication and cell damage) (Table 11). Virus shedding was not detected in most cloacal swab samples at day 2 PI. It is possible that infection occurred

but the virus was not shed yet or the sampling technique, processing of swab samples, possible presence of PCR inhibitors in the cloacal swab samples and the sensitivity of the viral detection method utilized in this study played a role in the outcome of the data.

Although not all of the data are available at this time, our data of histopathological lesions in turkeys suggest that this species had fewer lesions in the respiratory tract than chickens but had more in the gastrointestinal tract (Table 12). These results do not correlate well with the oropharyngeal and cloacal viral shedding data for turkeys at day 2 PI wherein there had been more viral shedding in the oropharyngeal samples than in cloacal samples (Table 5). As mentioned previously, the presence of PCR inhibitors (202) in cloacal swab samples may have played a role in the results of viral shedding. On the other hand, one consistent lesion that was observed with the current turkey histopathological data presented was microvesicular hepatopathy. This histopathological lesion was most likely due to stress, which in this case may be due to virus infection and perhaps compounded with the irritants present in the environment (e.g. high ammonia levels inside isolators). This finding implies that commercial turkeys are susceptible to the LPAI wild bird isolates used in this study.

Antibodies (Ab) are serum proteins found in the blood or body fluids that are produced by the immune system in response to exposure to foreign agents such as viruses. Often, the first sign of LPAI infection in domestic poultry is seroconversion, which may be the only evidence of infection (i.e. no clinical signs present) with some subtypes of LPAI (82). The serological data in our study showed that commercial turkeys seroconverted to the majority of virus tested (13 out of 16) whereas SPF chickens only had 4 viruses which caused seroconversion in both tests. This indicates that turkeys are more susceptible to AI infection than chickens since seroconversion can only occur following infection (82).

CHAPTER 6

CONCLUSION

Sixteen low pathogenic avian influenza wild bird isolates were biologically characterized in specific pathogen-free chickens and commercial turkeys. Bird pathogenicity studies showed that all LPAI viruses except one did not cause significant clinical disease in both species while analyses of the oropharyngeal and cloacal swab samples collected from these birds via quantitative real-time RT-PCR demonstrated greater virus shedding from both anatomical sites in SPF chickens than in commercial turkeys. Moreover, histopathological results in both species have shown the predominance of lesions in the respiratory and gastro-intestinal tract, consistent with the fact that these viruses are of low pathogenicity. In serological tests, more LPAI wild bird isolates caused seroconversion in commercial turkeys compared to SPF chickens, an indication that turkeys are more readily infected with these viruses and hence, are more susceptible to AI infection.

In this study, most LPAI viruses of wild bird origin can infect and be shed by chickens and turkeys without causing overt clinical disease or mortality. Moreover, these viruses replicated in poultry but induced mild pathogenicity and poor seroconversion thus, would be difficult to detect in commercial poultry. These findings imply that LPAI viruses of wild bird origin may not be a significant disease threat to domestic poultry.

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