ABSTRACT

Title of Document: HOST MOLECULAR RESPONSES IN CHICKENS INFECTED WITH AN AVIAN INFLUENZA VIRUS.

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Doctor of Philosophy, 2008

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Avian influenza virus has a segmented RNA genome that allows the virus to evolve continuously and generate new strains. Wild birds serve as natural reservoirs of avian influenza virus and provide a potential source for emergence of new viruses, which traverse host barriers and infect new avian or mammalian species. The mechanisms involved in this process are not completely understood. Our main goal is to understand host-pathogen interactions involved in avian influenza pathogenicity. As part of our approach we studied the effect of pre-exposure of chickens to IBDV (infectious bursal disease virus) on host susceptibility to infection, disease progression, and host molecular responses to infection with a mallard H5N2 low pathogenic avian influenza (LPAI) virus.

We found that prior exposure of chickens to IBDV led to increased susceptibility to infection with the mallard H5N2 LPAI virus compared to normal chickens. This

increased susceptibility allowed us to further adapt the virus to chickens. After 22 passages (P22) in IBDV-pre-exposed chickens, the LPAI virus replicated substantially better than the wild-type (WT) mallard virus in both IBDV-exposed and normal chickens. Interestingly, the P22 virus showed similar levels of replication in the respiratory and intestinal tracts of both groups, although it caused exacerbated signs of disease and severe lesions in the IBDV-pre-exposed group. We suggest that prior IBDV exposure provides a port of entry for avian influenza in an otherwise resistant chicken population. Furthermore, adaptation of avian influenza (AI) in IBDV-exposed chickens may allow for the selection of AI virus strains with expanded tissue tropism. We also studied the effects of host response to H5N2 AI in normal and IBDV-infected birds using high-throughput gene expression analysis. We demonstrated that IBDV-exposed chickens showed less than optimal humoral responses to LPAI infection as well as alterations in local molecular pathways that eventually led to exacerbated disease and death. At the molecular level we found amino acid substitutions in the surface glycoprotein hemagglutinin (HA). Those changes suggest selection for a virus that binds to and replicates more efficiently in chickens. Taken together our results suggest that IBDV-pre-exposure may play a role in exacerbating AI-induced pathogenicity.

HOST MOLECULAR RESPONSES IN CHICKENS INFECTED WITH AN AVIAN INFLUENZA VIRUS

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2008

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Dedication

To my husband Ivan and my two kids Angelica and Sebastian.

You are my strength, my love and my life.

Acknowledgements

I want to express my sincere gratitude to my advisor Dr. Daniel Perez for giving me the opportunity to come to his lab and for his support during this time. I also thank all my committee members, Drs. Jeffrey DeStefano, Nathaniel Tablante, Vikram Vakharia and Xiaoping Zhu for their valuable support during the course of my studies.

I thank specially Dr. Siba Samal, chairman of the Veterinary Medicine Department for his support. My special thanks to Ireene Dryburgh-Barry for her kindness and help. She is the soul of our department.

I extend my sincere gratitude to my previous lab partners, Drs. Haichen Song, Hongquan Wan, Jaber Jossain and Mrs. Sharon Azogue. I also thank my lab partners specially Erin Sorrell, a wonderful and very determined woman, I thank you for being here; thank you to Danielle Hickman, I am proud of being your friend. I will not forget Matthew Angel, a very smart and sweet young man always eager to help. Thank you to Yonas Araya and Lindomar Pena. My gratitude goes also to Brian Kimble, my partner during the last year. It has been a pleasure working with him. Last but not least thank you to the other members of the lab: Dr. Subrat Kumar, Dr. Jiangqian Ye, Dr. Alicia Solorzano, Rangarajan Padmanabhan and Yibin Cai. My special thanks to Andrea Ferrero-Perez, you helped me in many different ways but specially with my family. You were always there when we needed you.

My thanks also to my fellow students and colleagues, and the faculty and staff of the Department of Veterinary Medicine for their cooperation.

I am deeply indebted to our collaborators. Thanks to Dr. Hyun S. Lillehoj and Dr. Chul Kim for allowing us to use their microarray system in our studies and to Dr. H. L. Shivaprasad for his expert contribution on histological analysis.

I thank also, the Fulbright Commission that honored me with a Fulbright Fellowship, the University of Maryland, and the Universidad Nacional de Colombia for sponsoring my studies.

I want to thank my parents, specially my mom; her life was an example of love and sacrifice and her memory inspires me always. Thanks to my brothers and sisters in Colombia for their love and support.

Finally, I want to thank my husband Ivan and my two kids Angelica and Sebastian. This has been a sacrifice for all of us but overall your love and support helped me to go through all this process. Thanks to God who always has been with us.

Thanks to all of you who made my dream come true.

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List of Abbreviations

aa	amino acid
AI	avian influenza
BHI	brain-heart infusion medium
AVIELA	Avian intestinal intraepithelial lymphocyte
bp	base pair
cDNA	complementary DNA
CID_{50}	chicken infectious dose 50%
dpi	days post-infection
EID_{50}	egg infectious dose 50%
HA	hemagglutination
IH	inhibition of hemagglutination
HPAI	highly pathogenic avian influenza
HPAIV	highly pathogenic avian influenza virus
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IVPI	intravenous pathogenicity index
LPAI	low pathogenic avian influenza
LPAIV	low pathogenic avian influenza virus
mRNA	messenger RNA
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction

P22	passage 22
RT	reverse transcription
SPF	specific-pathogen-free
WT	wild type

Chapter 1: **General Introduction**

1.1Introduction

The influenza A virus is a single-stranded, negative-sense segmented RNA virus that belongs to the family *Orthomyxoviridae*. Influenza A is comprised of eight genes, which encode at least eleven different proteins (Horimoto and Kawaoka, 2005; Steinhauer and Skehel, 2002; Subbarao and Joseph, 2007). The gene segments are encapsidated by a virally encoded nucleoprotein (NP) and the ribonucleoprotein (RNP) structures are associated with the three subunits of the viral polymerase: polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA) (Steinhauer and Skehel, 2002). Two surface proteins, hemagglutinin (HA) and neuraminidase (NA) are the main antigenic determinants of the virus to which neutralizing antibodies are made. A significant characteristic of the influenza virus, which makes it such an important threat, is its potential for variability. The virus can change gradually due to the accumulation of point mutations in the process known as antigenic drift. It is generally accepted that immunological pressure favors antigenic drift to a higher extent in humans but this phenomenon also occurs in avian influenza virus (Horimoto and Kawaoka, 2001). In addition, the segmented nature of the genome increases the opportunity for genetic reassortment; this leads to generation of different strains (Webster et al., 1997, Shortridge et al. 1997) in a process known as antigenic shift, which is a key factor in the emergence of pandemic strains. Influenza A viruses are further classified based on the antigenic properties of the

surface proteins HA and NA. Currently 16 hemagglutinin (H1-16) and 9 neuraminidase (N1-9) subtypes (Fouchier et al., 2005; Munster et al. 2005, Horimoto and Kawaoka, 2005) have been described. The primary host reservoir for all avian influenza viruses is wild aquatic birds (Munster et al., 2005; Perdue and Swayne, 2005; Webster et al., 1992). It has been shown that adaptation of avian influenza virus isolated from aquatic birds to chickens is the result of selective pressure, which leads to marked changes in both the HA and NA genes (Matrosovich et al., 1999). Type A influenza viruses are the agents of the most common and endemic infections and have been reported in swine, horses, wild birds – particularly the families Anseriforme (ducks, geese and swans) and Charadriiforme (shorebirds), domestic poultry, and humans (Fouchier et al., 2005; Horimoto and Kawaoka, 2005; Humberd et al., 2006). Sporadic infections have been reported in farmed mink, dogs, wild whales and seals, and captive populations of big cats (tigers and leopards) (Perdue and Swayne, 2005; Rimmelzwaan et al., 2006).

Avian influenza viruses can be separated into two major classes on the basis of virulence. The highly pathogenic avian influenza (HPAI) viruses cause severe systemic infection with birds dying soon (usually within one week) after infection and are associated with high rates of morbidity and mortality. The low pathogenic avian influenza (LPAI) viruses are associated with subclinical infections and produce only mild to moderate signs of disease in the field (Horimoto and Kawaoka, 2005). Only influenza viruses of subtypes H5 and H7 are categorized as HPAI viruses (Swayne and Suarez, 2000) and, among domestic avian species, chickens and turkeys are most

frequently involved in outbreaks of HPAI virus-related disease. Although there is no recognized wild-bird reservoir, HPAI viruses can be isolated from wild birds during outbreaks in domestic poultry (Swayne and Suarez, 2000). Phylogenetic analysis indicates that pathogenic viruses of the H5 and H7 subtypes may be derived from nonpathogenic lineages that are maintained in aquatic birds (Kawaoka et al., 1987; Rohm et al., 1995).

Influenza A virus pathogenicity is complex and of a polygenic nature (Steinhauer and Skehel, 2002) however, the glycoprotein HA is integral to the process (Horimoto and Kawaoka, 2005; Kuiken et al., 2006). Cleavage of HA into the subunits HA1 and HA2 (Lazarowitz and Choppin, 1975) is considered to be the main determinant of tissue tropism of avian influenza viruses. Differences in the tissue distribution of proteases and in HA susceptibility to these enzymes can determine the severity of virus infection (Horimoto and Kawaoka, 2005) and define the range of tissues where viral replication can occur. For certain avian strains of subtypes H5 and H7, the cleavage properties of HA are the most significant determinant of virus pathogenicity. The HAs of these viruses contain a series of basic amino acids (Kawaoka and Webster, 1988b; Wood et al., 1993), which are cleaved in the Golgi apparatus by ubiquitous intracellular proteases; the ubiquity of these proteases, such as furin or PC6 (Horimoto et al., 1994; Stieneke-Grober et al., 1992), facilitates the systemic spread of the virus (Steinhauer and Skehel, 2002). Conversion of avirulent avian influenza viruses to virulent strains has been associated with an increase in HA cleavability; this phenomenon was observed in Pennsylvania (H5N2)

in 1983 (Kawaoka et al., 1984), in Mexico (H5N2) in 1994 (Garcia et al., 1996; Horimoto et al., 1995), in Italy (H7N1) in 1997 (Banks et al., 2001), in Chile (H7N3) in 2002 (Suarez et al., 2004), and in Canada (H7N3) in 2004 (Hirst et al., 2004). In nonpathogenic viruses HA generally contains a single basic amino acid at the cleavage site; this basic amino acid is readily recognized and cleaved by extracellular trypsin-like proteases, limiting the effects of the virus to mild respiratory symptoms and a reduction in egg production (Bosch et al., 1979; Stieneke-Grober et al., 1992). The presence or absence of carbohydrate attachment sites adjacent to the cleavage loop can also affect HA cleavage properties. There are examples in which mutations that eliminate such glycosylation sites led to acquisition of a highly pathogenic phenotype (Deshpande et al., 1987; Kawaoka et al., 1984; Kawaoka and Webster, 1989).

Despite the availability of these data describing molecular events associated with pathogenicity, the mechanism(s) by which an avirulent avian virus becomes highly virulent in nature is poorly understood. However, the transition to pathogenicity should be considered a polygenic event, in which several gene segments can contribute to the pathogenic phenotype; this process depends on factors such as the genetic characteristics of the virus, the nature of the host, and the route of infection (Steinhauer and Skehel, 2002).

A very interesting area of research involves the role that host factors may play in AI viral infections. The host factors that determine differences in susceptibility to

avian influenza viruses in avian species are unknown (Horimoto and Kawaoka, 2005). Although avian influenza viruses typically demonstrate host specificity, interspecies transmission occasionally occurs (Perkins and Swayne, 2001). Following transmission, the influenza A virus must go through a process of adaptation to the new host species before efficient replication can be achieved. Replication efficiency is associated with expression of virulence (Perkins and Swayne, 2003); however, the factors that determine interspecies transmission and pathogenicity of influenza viruses remain poorly understood.

Many parameters of the role of host factors in determining differences in degree of virulence of influenza viruses are related to the immune response. Increased susceptibility to infection, as a result of genetic variation in viral receptors or other properties of host cells, might allow more rapid spread within tissues (Perdue and Swayne, 2005). Elucidation of the mechanisms by which influenza virus adapts in poultry, a non-natural host of avian viruses, is crucial for determining how the virus might potentially improve its transmissibility among humans (Campitelli et al., 2006).

Although other factors are involved, the genetically-determined diversity of the immune system is the major factor underlying differences between individuals in resistance to diseases of infectious origin. Infectious disease occurs when the immune system fails to protect the organism, due to an inadequate or abnormal immune response, from assault by the invading pathogen. Immune responsiveness

can be correlated with resistance or susceptibility to a pathogen (Zekarias et al., 2002). In this regard, infections caused by infectious bursal disease virus (IBDV) may exacerbate the outcome of diseases caused by other etiologic infectious agents, such as viruses and bacteria, and reduce the chicken's ability to respond to vaccination (Muller et al., 2003). Previous studies have shown that IBDV-induced immunosuppression causes a reduced post-vaccination immune response and greater susceptibility to challenge in viral respiratory diseases, such as Newcastle disease, avian infectious bronchitis, and avian infectious laryngotracheitis in chickens (Rosenberger and Gelb, 1978). Because host immune competence is a factor in determining predisposition to infectious agents and IBDV-induced immunosuppression increases susceptibility to viral respiratory infections (Muller et al., 2003), it is essential to determine the precise effects that pre-exposure to IBDV has on the host and how IBDV pre-exposure influences subsequent infection by avian influenza viruses in poultry (Fouchier et al., 2005).

IBDV is one of the major, economically important diseases of poultry globally (Sharma et al., 2000) due to its detrimental effect on the immune status of the host. Both broiler and layer flocks are vulnerable to the immunosuppressive effects of the virus (Saif, 1991). It is generally accepted that both humoral and cellular immune responses are compromised (Rosenberger and Gelb, 1978). The degree of immunosuppression depends on several factors but the age at which poultry are infected plays an important part, with greater impairment in younger chicks, infected at 1 day old, than in chicks infected at three weeks of age or later (Higashihara et al.,

1991). This is an important consideration because commercial chickens are typically exposed to IBDV early in life (Saif, 1991), giving other pathogens the opportunity to take advantage of a debilitated immune system.

1.2 Research Objectives

Determination of the susceptibility of chickens to A/mallard/12180/ Pennsylvania/84 (H5N2) influenza A virus using natural routes of inoculation.

Adaptation of A/mallard/12180 /Pennsylvania/84 (H5N2) influenza A virus in chickens that have been pre-exposed to IBDV. By successively passing the influenza virus in IBDV-infected chickens we expect to obtain new variants of the virus, which will adapt, replicate, and transmit more efficiently in birds/domestic poultry. This process will provide the basis for establishing the role of host immune status in the susceptibility of chickens to AIV.

Study the pathogenesis of the A/mallard/Pennsylvania/84 (H5N2) avian influenza virus after adaptation in chickens pre-exposed to IBDV. This study will provide data on changes in tissue tropism and/or pathogenicity after passing the H5N2 LPAI virus 22 times in IBDV-infected chickens.

Molecular characterization of wild-type and adapted viruses. Sequence analysis of the adapted and non-adapted viruses will provide data necessary to define the mutational variations that lead to the adapted phenotype.

Determination of global gene expression profile using microarray analysis. Analysis of the patterns of global gene expression of normal and IBDV-pre-exposed chickens inoculated with the adapted avian influenza virus will provide data necessary to understand the role of host factors in susceptibility to AIV infections.

Chapter 2: **Review of Literature**

2.1 Influenza Virus

2.1.1 Classification

Influenza virus belongs to the family *Orthomyxoviridae* and to the genus *Influenza virus A*. The genome of influenza virus is single stranded, negative-sense, segmented RNA (Subbarao and Joseph, 2007). Viruses in the genus *Influenza* are recognized for their ability to undergo genetic reassortment (Knipe, 2007).

Influenza A viruses are further classified according to their hemagglutinin (HA) and neuraminidase (NA) subtypes. Sixteen hemagglutinin (H1 to H16) and nine neuraminidase (N1 to N9) subtypes of influenza A viruses have been described (Halvorson, 2008; Knipe, 2007; Landolt and Olsen, 2007).

2.1.2 Virion

Influenza virus is pleomorphic and enclosed by a lipid envelope derived from the host cell. HA and NA project in a spike-like fashion from the surface of the virion and are anchored in the lipid membrane. The M1 protein is located within the envelope and the M2 protein within the envelope acts as an ion channel. The core of the virus particle contains the RNP (ribonucleoprotein) complex, which consists of the viral RNA (vRNA) segments, polymerase proteins PB1 (polymerase basic 1),

PB2 (polymerase basic 2) and PA (polymerase acid), and the nucleoprotein (NP) (Knipe, 2007; Landolt and Olsen, 2007).

2.1.3 Genome Organization

The Influenza A virus genome consists of eight RNA segments encoding 11 viral proteins. Specific viral proteins have been assigned to individual RNAs. Proteins M2 and NEP/NS2 are derived from the M and NS genes, respectively, and are produced as a result of mRNA splicing. The protein PB1-F2 has recently been identified (Suzuki, 2006).

2.1.4 Viral Proteins

Viral Polymerase Proteins. Approximately half of the total genome encodes the three viral polymerase proteins; segments 1, 2, and 3 encode PB2, PB1, and PA, respectively. These three proteins contain nuclear localization signals. In addition to the NP proteins, the three viral polymerase proteins constitute the minimum complement of proteins required for viral transcription and replication (Honda et al., 2002).

It has been suggested that polymerase basic protein 1 (PB1) is an RNA polymerase; it contains the four conserved motifs of RNA-dependent RNA polymerases (Poch et al., 1989). PB1 contains independent binding sites for PB2 and PA. This polymerase plays a role in the assembly of the three polymerase protein subunits and catalysis of RNA polymerization.

The polymerase basic protein 2 (PB2) is transported to the nucleus of infected cells where transcription and replication take place (Jones et al., 1986). PB2 has cap-binding and endonuclease activity and also contains a binding site for the PB1 protein and two regions for binding the NP protein (Poole et al., 2004).

PB2 has also been identified as a determinant of pathogenicity in some influenza A viruses and this property is associated with an amino acid mutation at position 627 (Hatta et al., 2001).

The polymerase acid protein (PA) is essential for viral transcription and replication, although a specific function for PA has not been described. It has been suggested that PA has helicase and ATP-binding activities and may be involved in the protease activity in infected cells (Sanz-Ezquerro et al., 1998).

Hemagglutinin (HA). HA (encoded by segment 4 of the genome) is a type I glycoprotein; the hydrophobic carboxy terminus of the protein is embedded in the viral membrane and the hydrophilic amino terminal end projects in a spike-like fashion away from the viral surface (Knipe, 2007). The primary function of HA is receptor binding via a binding site located within the globular head. HA binds to sialic (N-acetyl neuraminic) acid residues linked to galactose in an α2,3 or α2,6 conformation. Avian influenza viruses bind preferentially to sialic acid in the α2,3 conformation while human influenza viruses prefer an α2,6 linkage conformation (Palese, 2007; Subbarao and Joseph, 2007). This preference confers a role on HA in

maintenance of the species barrier of influenza viruses. Mutations in HA can result in changes that affect host specificity and, in this way, could affect the efficiency of transmission to humans. A second important function of HA is fusion under conditions of acid pH. HA is also involved in budding and particle formation.

In chickens, the HA gene is the main determinant of viral pathogenicity. In this regard, the accumulation of basic amino acids at the cleavage site of HA is a hallmark of avian influenza viruses having high pathogenic potential (Banks et al., 2001; Kawaoka et al., 1984). HA also plays a central role in the host immune response to influenza. HA is recognized by the adaptive host immune system and the host produces neutralizing antibodies against HA (Subbarao and Joseph, 2007).

Nucleocapsid protein (NP). Nucleocapsid protein (NP) (encoded by segment 5 of the genome) is an arginine-rich protein with RNA-binding activity and its primary role is in encapsidation. NP coats the RNA and is the major viral protein in the ribonucleoprotein (RNP) complex. During early stages of infection NP is localized predominantly in the nucleus, where it mediates the transport of incoming RNPs from the viral particle; in later stages it is found in the cytoplasm, reflecting the trafficking of RNPs during the virus life cycle (O'Neill et al., 1995).

Neuraminidase (NA). Neuraminidase (NA) (encoded by segment 6 of the genome) is a type II glycoprotein that plays a role in virion release, facilitating budding of progeny virions from cells. NA is a sialidase; its enzymatic activity

catalyzes the release of virus particles from the sialic acid-binding sites on the host cell (Palese, 2007). The NA monomer consists of four domains: a globular head, a stalk, and transmembrane and cytoplasmic domains. The NA enzymatic and antigenic sites are located in the globular head.

Antibodies specific to NA are not neutralizing but they interfere with virus replication by preventing the release of new virus particles (Gulati et al., 2002; Subbarao and Joseph, 2007; Webster et al., 1988).

Matrix protein (M). The matrix protein (encoded by segment 7 of the genome) of the influenza virus encodes two proteins M1 and M2. M2 is the result of mRNA splicing of segment 7.

M1 expression occurs at later stages of virus replication, consistent with its role in viral transcription and in exporting RNPs from the nucleus after replication (Martin and Helenius, 1991). M1 is the only viral product that has been shown to be an absolute requirement for assembly of virus particles (Gomez-Puertas et al., 2000). It has been shown that M1 is necessary and sufficient for the formation of virus-like particles, indicating a key role for M1 in the budding process (Gomez-Puertas et al., 2000).

M2 of influenza A viruses is a tetrameric type III integral membrane protein. M2 has been shown to possess ion channel activity and to participate in the uncoating

process (Pinto et al., 1992). M2 may also play a role in assembly and budding of virus particles. The ion channel activity of M2 is the target of the antiviral drug amantadine. M2 has also been considered a vaccine candidate because antibodies specific for M2 have been shown to be protective in *vivo* (Fiers et al., 2004; Treanor et al., 1990).

Nuclear structural protein (NS). The nuclear structural protein (NS) gene (segment 8 of the genome) of influenza A virus encodes two proteins: NS1, the only non-structural protein of influenza virus, and NS2, which is encoded by the spliced mRNA of segment 8. NS1 facilitates virus replication and is active early in the virus life cycle due to its central role in combating the host immune response (Squires et al., 2008). NS2 is also known as Nuclear Export Protein (NEP), due to its role in vRNP nuclear export (Neumann et al., 2000).

2.1.5 Influenza virus replication

Attachment. The first step in initiation of influenza virus replication is the interaction of HA in the globular head of the virus with sialic acid receptors on the surfaces of specific cells. Influenza viruses from different species show different preferences for sialic acid. Human viruses bind preferentially to sialic acid with an α2,6 linkage (SAα2,6Gal) whereas avian virus prefer sialic acid with an α2,3 linkage (SAα2,3Gal) (Knipe, 2007; Swayne, 2007). The presence of a specific receptor type determines the tissue tropism of an influenza virus. The different steps in the life cycle of influenza virus are represented schematically in Figure 1.

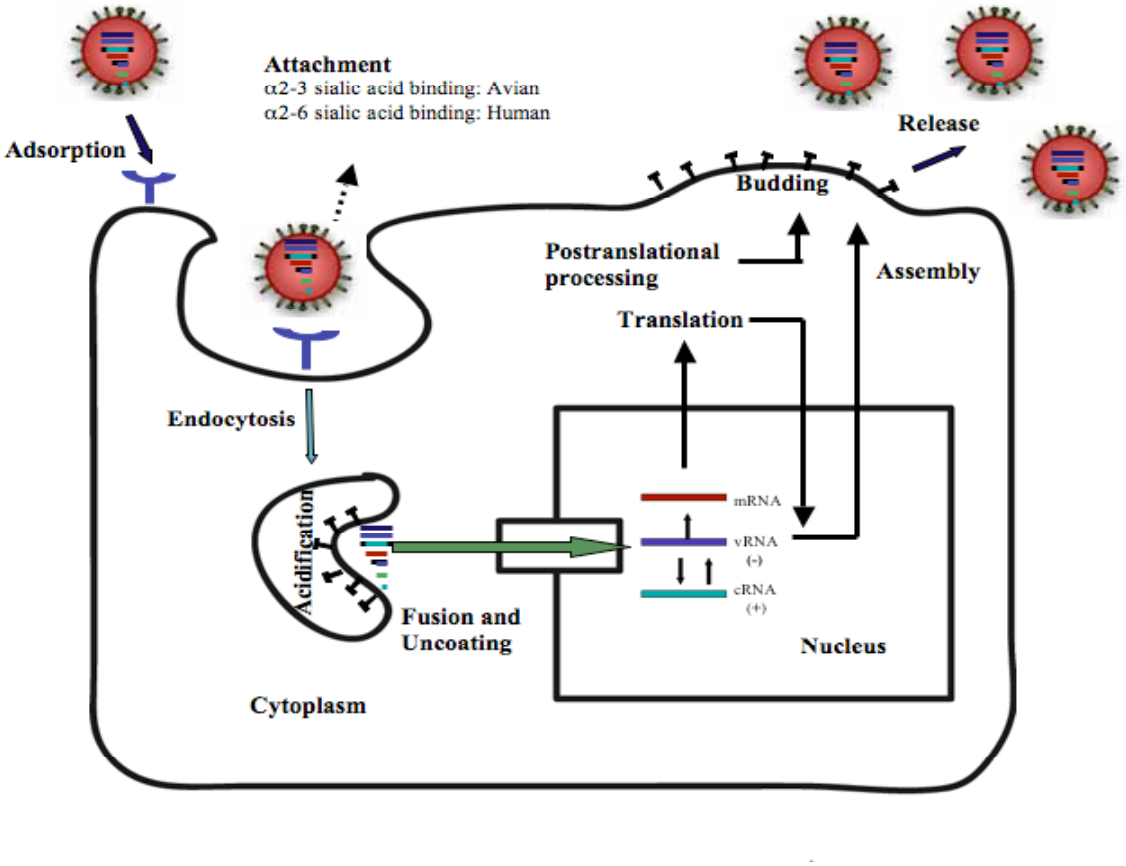

Figure 1. Life cycle of influenza virus. (Figure prepared, with modification, from Fields Virology, 5th edition, 2008). Influenza virus attaches to specific viral receptors on the cell surface via the glycoprotein HA and is then internalized by receptor-mediated endocytosis. In the acidic environment of the endosome, the virus undergoes conformational changes that lead to the fusion of the viral envelope and the endosomal membranes and activation of M2 ion channel activity. The vRNP segments migrate, via nuclear pores, to the nucleus, where transcription and replication of influenza virus takes place. The negative sense vRNA is transcribed into mRNA by a primer-dependent mechanism. Viral replication occurs via a two-step process catalyzed by the viral polymerase complex and mature RNPs are exported from the nucleus. The plus-strand RNA is transported into the cytoplasm for translation into early and late viral proteins. Following translation and posttranslational modifications, the virion is assembled and the newly formed viral particles bud from the cell and are released.

Entry. Influenza virus enters the cell by endocytosis in a low-pH environment. It has been proposed that the primary mechanism by which virus is internalized is receptor-mediated endocytosis associated with clathrin-coated pits (Skehel, 2000).

Fusion and Uncoating. The fusion of the influenza virus envelope and endosomal membrane requires a low pH. After a structural change in the HA precursor HA0, which results in the cleavage of HA0 into two subunits, HA1 and HA2, the fusion activity of HA is initiated (Swayne, 2007). As a consequence of cleavage, the fusion peptide is exposed at the N-terminus of the HA2 subunit, making interaction with the endosomal membrane possible. As a result of this structural change in multiple neighboring HA molecules, the content of the virion is released into the cytoplasm of the cell through a fusion pore.

The M2 protein participates in uncoating of influenza virus via its ion channel activity. HA-mediated fusion of the viral membrane with the endosomal membrane and release of the RNP, mediated by M-2, results in the appearance of free RNP complexes in the cytoplasm. The uncoating process is then complete (Palese, 2007).

Transcription and Replication. As noted earlier, the genome of influenza virus consists of single-stranded, negative-sense RNA. In contrast to other single-stranded, negative-sense RNA viruses, transcription and replication of influenza virus takes place in the nucleus of the cell. To initiate synthesis of its mRNA, influenza virus depends on cellular RNA polymerase II activity because the virus needs to "hijack" a

5'-capped primer from host pre-mRNA transcripts to initiate mRNA synthesis. To accomplish this, the virus uses the cap-binding function of the PB2 protein and the endonuclease function of the PB1 protein (Palese, 2007). Transcription is initiated by binding of the 5' end of the vRNA to the PB1 subunit. As a result, the PB2 protein is able to recognize and bind the cap structure on host pre-mRNAs. The polyadenylation of influenza virus mRNA is a host-independent process and is catalyzed by the same polymerase that is used for transcription (Plotch and Krug, 1977).

The vRNA serves as a template for both mRNA and cRNA synthesis. In contrast to mRNA synthesis, initiation of cRNA synthesis occurs without a capped primer and cRNAs are full-length copies of the vRNA. Complementary RNAs are not transported to the cytoplasm during viral infection. In further contrast to mRNA, newly synthesized cRNAs and vRNAs are encapsidated (Palese, 2007).

In the second step of replication, the positive-sense cRNA serves as the template for the synthesis of negative-sense genomic vRNA in a primer-independent reaction.

Virus Assembly and Release. Influenza viruses assemble and bud from the apical plasma membrane of polarized cells. This characteristic has an effect on viral pathogenesis and tissue tropism and explains why, in general, influenza virus has a more restricted tissue tropism than other viruses (Tucker and Compans, 1993).

2.2 Avian Influenza Infection

A wide variety of species of birds and mammals are susceptible to influenza A virus infection and the 16 HA and 9 NA subtypes have been identified in avian species (Alexander, 2000; Fouchier et al., 2005). Migratory birds, particularly ducks, allow circulation of influenza viruses, which maintains the virus in nature (Ito et al., 1995) and provides the opportunity for avian influenza virus to spread through contact of wild birds with chickens (Isoda et al., 2006).

2.2.1 Avian Influenza Virus Pathotypes

Based on the severity of clinical signs seen in birds, avian influenza viruses are classified into two pathotypes, high pathogenic (HPAI) and low pathogenic (LPAI) avian influenza viruses (Alexander, 2000; Subbarao and Joseph, 2007).

As noted earlier, in gallinaceous birds the HA protein is the primary mediator of pathogenicity; the amino acid sequence of the proteolytic cleavage site and the proximity of carbohydrates to the site determine differences in cleavability (Banks et al., 2000; Lee et al., 2007; Subbarao and Joseph, 2007). Differences in the protease cleavability of HAs are responsible for establishing tissue tropism and determining whether infection will be systemic (highly pathogenic) or restricted to the respiratory and enteric tracts (low pathogenic), based on which proteases recognize the sequence that is present (Lee et al., 2007). In general, HPAI virus HAs have multiple basic amino acid residues (arginine and lysine) at their cleavage sites. Cleavage of

precursor HA0 of HPAI viruses is achieved by ubiquitous intracellular proteases, such as furine and PC6 (Horimoto et al., 1994; Subbarao and Joseph, 2007), which are present in multiple cell types in several tissues and organs throughout the body. The ubiquity of the enzymes capable of cleaving HA0 allows infection of a wide range of tissues and viral replication throughout the organism; consequently, severe systemic disease can occur in the infected birds. LPAI viruses have a single arginine at the cleavage site and another basic amino acid at position 3 or 4 from the cleavage site. To replicate, LPAI viruses require the presence of extracellular host proteases such as trypsin-like enzymes, which are found in restricted anatomical sites, primarily in the respiratory and digestive tract (Banks et al., 2000; Halvorson, 2008; Horimoto et al., 1994; Subbarao and Joseph, 2007; Swayne, 2007) which restricts infections to these sites.

Only viruses of the H5 and H7 subtypes have been identified as HPAI viruses. Viruses are classified as HP if they have an intravenous pathogenicity index (IVPI) greater than 1.2 in 6-week-old chickens or cause at least 75% mortality in four to eight week-old chickens infected intravenously. If an H5 or H7 virus does not have an IVPI of greater than 1.2 or causes less than 75% mortality in an intravenous lethality test, the viral genome should be sequenced to determine whether multiple basic amino acids are present at the cleavage site of the HA molecule (OIE, 2006).

The process by which HPAIVs emerge is poorly understood. It has been suggested that these viruses are generated as result of mutations that occur after LPAI

viruses have been introduced into domestic poultry or the waterfowl reservoir, although most evidence supports the former (Banks et al., 2000). Proposed mechanisms for the generation of H5 and H7 HPAI viruses include insertion of extra basic amino acids into the HA cleavage site and substitution of non-basic with basic amino acids; this was shown for the H5N2 HPAI virus of Mexico in 1994 (Horimoto et al., 1995). Another possible mechanism involves the loss of a sugar group near the HA cleavage site; an example of this occurs in the H5N2 HPAI virus that caused an outbreak in 1983 in the United States (Kawaoka and Webster, 1985). A third possible mechanism is homologous recombination; this was demonstrated in Chile in 2002 when an insertion of 27 nucleotides from the NP gene was identified in the HA cleavage site of an H7N3 HPAI virus. Evidence of a similar recombination event was observed in the H7N3 HPAI virus, in which an insertion of 21 nucleotides from the M gene was identified in 2004 in Canada (Pasick et al., 2005; Suarez et al., 2004).

2.2.2 Disease in poultry.

Highly Pathogenic Avian Influenza Infection. The most virulent forms of highly pathogenic avian influenza (HPAI) are characterized by a highly fatal systemic infection that spreads to most organ systems, including the cardiovascular and nervous systems. Depending on the virus and species of bird, the appearance of clinical signs and the nature of gross lesions vary. Clinical presentation varies and depends on the extent of lesions to specific organs and tissues. In domestic poultry HPAI viruses produce severe depression, extreme decreases in feed and water consumption, high morbidity and mortality rates, sudden death, and, occasionally,

neurological symptoms if the animals survive the peracute phase. The incubation period is usually between three and seven days, depending on the virus isolate, and the age and species of the bird. If the animals survive longer than seven days, nervous system symptoms can appear (Sorrell et al., 2007; Swayne and Suarez, 2000). Common lesions include edema to necrosis of comb and wattles, edema of the head and legs, lungs filled with fluid and blood, and small hemorrhages on internal organs such as coronary fat. These lesions point to alterations in the cardiovascular system, principally affecting vascular endothelium, and the resulting viremia (Swayne, 2007).

Low pathogenic avian influenza virus. LPAI viruses emerged as a cause of respiratory disease and reduced egg production in turkeys in Canada and the United States during the early 1960's. Since then, LPAI viruses of subtype H5 have been isolated in Canada in 1966 and in the United States in1968.

Large surveys have resulted in the isolation of LPAI viruses from wild birds showing no signs of disease. Wild aquatic birds, primarily of the orders Anseriforme (ducks and geese) and Charadriiforme (shorebirds, gulls, terns, and auks), are considered reservoirs of AI viruses (Halvorson, 2008; Suarez, 2000). Infection with an LPAI virus can result in mild to moderate signs of disease or be asymptomatic. Domestic birds infected with an LPAI virus usually do not show signs of disease during the period when the virus is effectively transmitted, whether in live bird markets, backyard flocks, or large or small commercial poultry operations. LPAI infections are economically important. In domestic poultry clinical signs are seen in

the respiratory, digestive, urinary, and reproductive organs. Respiratory signs are usually mild to severe and include coughing, sneezing, rales, and excessive lacrimation. General symptoms include ruffled feathers, depression, decreased activity, lethargy, and decreased feed and water consumption (Saif, 2008).

Natural infection by avian influenza virus results in a wide range of clinical forms, depending on factors such as the virus strain, host species, age of the bird, and environmental factors. Based on mortality rates and clinical signs and lesions after infection with AI virus in the field, four clinical classes of the disease can be assigned: highly virulent, moderately virulent, mildly virulent, and avirulent. The highly virulent group results from infection with the H5 and H7 HPAI viruses; infection is characterized by systemic disease, with involvement of the cardiovascular and nervous systems. Morbidity and mortality are very high in this group and can reach 100%. The moderately virulent group is the result of infection with LPAI viruses and/or co-infection with other pathogens. The mortality rate ranges from as low as 5% up to 97% in young or severely stressed birds. Lesions may be found in the respiratory tract, reproductive system, kidneys, or pancreas. The mildly virulent class of disease results from infection with LPAI viruses; animals with this type of disease show mild respiratory symptoms or a drop in egg production and mortality is low - usually less than 5%. The avirulent group corresponds to birds infected with an LPAI virus in which no clinical signs or mortality are observed. During an outbreak it is possible to have viruses with different degrees of virulence (Saif, 2008).

2.2.3 Epidemiology

Avian influenza virus evolves continuously. New strains are generated as a result of the segmented genome, which allows reassortment, and the lack of proofreading during vRNA replication.

Host range restriction is considered polygenic but the specific role of each gene contributing to determination of host specificity is not known. Although there appears to be a considerable degree of species specificity, the virus has jumped the species barrier and can now be found in a broad range of species not previously reported (Morgan, 2006).

Wild waterfowl are considered the natural reservoirs of influenza A viruses. The virus replicates primarily in the gastrointestinal tract. Virus is shed into the feces, which allows transmission between wild birds as a result of fecal contamination of water (Webster et al., 1978). Geographic dissemination of virus by infected migratory birds is potentially very extensive (Morgan, 2006). Trade and illegal smuggling of poultry are also a means of disseminating the virus.

Outbreaks have occurred frequently over the last decade in North America, Europe, and Asia. In Asia, HPAI H5N1 influenza viruses have been recognized since 1996 (Isoda et al., 2006; Li et al., 2004; Sims et al., 2003; Xu et al., 1999). In 1997, HPAI viruses were directly transmitted from birds to humans in Hong Kong (Suarez et al., 1998). This first incidence of an H5 influenza virus being isolated from humans was accompanied by concern about the pandemic potential of H5 viruses.

The H5N1 isolates originated completely from an avian virus; this is in contrast to the 1957 and 1968 pandemic strains, which were reassortants (Claas et al., 1998; Subbarao et al., 1998).

Currently there is no evidence of HPAI strains, including H5N1, in the United States. Historically, there have been three outbreaks of infection with HPAI virus in poultry in the US. In 1924 an outbreak was caused by an H7 virus that originated in live-bird markets in the eastern part of the country and, in 1983-84, the outbreak was traced to an H5N2 in the northeastern US (Bean et al., 1985). In 2004 an outbreak caused by infection with H5N2 occurred in the southern part of the country; this virus was classified as HPAI based on the the amino acid sequence of the HA (Lee et al., 2005).

The impact of HPAI virus infections is not limited to the high mortality and economic losses, but also includes the possibility of origination of new viruses with wider host ranges and pandemic potential.

Pathogenicity of AI strains in chickens do not necessarily correlate with pathogenicity in other species, such as pigs, indicating that factors involved in host range restriction may reside in parameters other than the structure of the HA.

Low-pathogenic AI outbreaks have caused significant economic losses for poultry producers in general; these losses are associated with secondary viral or

bacterial infections as a result of a primary AI virus infection. Mortality, condemnation of carcasses, disinfection, and delayed placement of new birds also contribute to the economic losses (Halvorson, 2008).

2.2.4 Immune Response

Although the influenza virus has developed mechanisms to counteract the initial immune response to viral infection, elimination of the virus is carried out by a combination of innate and adaptive immune responses.

Infection with AI viruses, as well as immunization with vaccines, results in a humoral antibody response. HA and NA induce production of neutralizing and protective antibodies. Internal viral proteins may induce an immune response that, although it does not prevent clinical signs, could reduce the period of virus replication and shedding (Halvorson, 2008).

Influenza viruses have the ability to generate new strains through accumulation of mutations. The mechanism by which these changes affect the efficiency of the innate immune response is not completely understood. In this regard, the innate immune response plays a major role in counteracting the initial infection. Invasion of cytotoxic CD8-positive T cells into the lungs is critical for viral clearance. Cellular components of the innate response to influenza A virus infection includes NK (natural killer) cells, macrophages, and neutrophils. The role of macrophages in the immune response to influenza virus infection is complex.

Macrophages produce chemokines and cytokines during influenza A virus infection *in vivo,* resulting in a protective or deleterious effect during the infection (Wijburg et al., 1997). It is necessary to determine the contribution of macrophages, as opposed to other leucocytes or epithelial cells, to the immune response in influenza infections (Tecle, 2005).

The innate immune response must effectively control viral replication without inducing an excessive inflammatory response, which can potentially cause damage to the lungs (Tecle, 2005). It has been proposed that differences in the outcome of influenza A virus infections in humans could be related to innate immune response; outcomes would be influenced if the innate immune response wasimpaired or resulted in a severe inflammatory process. It has been proposed that influenza A virus infection activates the inflammatory responses of respiratory epithelial cells via a pathway involving TLR3 (Toll-like receptor 3) (Guillot et al., 2005). Influenza A virus induces IL-8 (Interleukin-8), IL-6 (Interleukin-6) and RANTES (regulated upon activation, normal T cell expressed and secreted) protein expression, as well as upregulation of ICAM-1 (intracellular adhesion molecule-1) (Tecle, 2005).

2.3 Infectious Bursal Disease (IBD)

Infectious Bursal Disease (IBD) is a highly contagious, immunosuppressive infection of young chickens. IBD is also known as Gumboro disease because the first recorded outbreak occurred in Gumboro, Delaware, USA in 1962 (Muller et al., 2003).

2.3.1 Classification

The etiologic agent of IBD is infectious bursal disease virus (IBDV), which belongs to the family *Birnaviridae* and the genus Avibirnavirus (Muller et al., 2003). The IBDV genome consists of two segments of double-stranded RNA enclosed in a non-enveloped icosahedral capsid (Hirai and Shimakura, 1974; Nick et al., 1976).

There are two serotypes of IBDV, which can be differentiated by a virus neutralization test (McFerran et al., 1980). Both serotypes are present in chickens but only strains of serotype 1 are associated with disease (Ismail and Saif, 1991).

2.3.2 Virion.

The virion consists of two segments of double-stranded RNA encoding five proteins. The small segment B encodes the vRNA polymerase VP1, and the large segment A encodes VP2, VP3, VP4, and VP5. VP2 and VP3 are the major structural proteins and VP2 contains the major neutralizing epitopes. VP4 is a nonstructural protein and has protease activity associated with cleavage of the progenitor polyprotein, which yields VP2 and VP3. A specific function for VP5 has not been

assigned but a regulatory function involved in virus release and apoptosis has been suggested (Liu and Vakharia, 2006).

2.3.3 Disease in chickens

Infectious Bursal Disease (IBD) is one of the most prevalent diseases in the poultry industry. This virus is restricted to chickens and has varying degrees of pathogenicity in the field; although turkeys and ducks can be naturally infected with IBDV, they do not develop signs of disease. Flocks infected with IBDV do not perform well, due to the immunosuppressive effects of this virus, and show reduced economic return and severe losses (Farooq, 2003; Lutticken, 1997; Saif, 1991; Saif, 2008).

IBD in chickens can appear in an acute lethal form but, depending on the host cell in which the virus replicates, disease with variable degrees of severity can occur. Clinical signs are nonspecific and include diarrhea, anorexia, depression, and ruffled feathers; the severity of the signs and degree of mortality are variable and depend on several factors in addition to the virulence of the virus strain.

IBDV primarily targets the lymphoid tissue in the bursa of *Fabricius*; this affects mainly humoral immunity, due to the virus's predilection for B lymphocytes or their precursors, but secondary lymphoid organs, such as the cecal tonsil and the spleen, as well as macrophage activity can also be affected (Kaufer and Weiss, 1980). IBDV infection can result in impaired humoral and local immune responses, with the degree of severity depending on the virulence of the virus strain and the age of the

infected bird. Chickens infected with IBDV early in life do not show clinical signs but have subclinical infections and compromised humoral and local responses (Balamurugan and Kataria, 2006). It has been shown that chickens infected at one day of age are deficient in serum immunoglobulin G and produce only monomeric IgM. The number of peripheral blood B cells is also reduced following IBDV infection (Ivanyi and Morris, 1976). Chickens are more susceptible to disease when the infection occurs between three and six weeks of age, resulting in clinical signs, severe bursal atrophy and immune suppression. In addition, IBDV infection can have a transient effect on cell-mediated immune responses but this is not as evident as the effect on the humoral immune response (Craft et al., 1990; Sivanandan and Maheswaran, 1980; Saif, 2008).

The effect of IBDV on the immune response was reported many years ago in relation to the reduced ability of infected chickens to respond to vaccination and demonstration of exacerbated infections with other pathogens; in contrast, the response to IBDV itself is normal (Bracewell et al., 1972; Faragher et al., 1974; Hirai et al., 1974; Rosenberger and Gelb, 1978; Muller et al., 2003; Fadly et al., 1976; Sharma, 1984).

Since 1986, when a variant of IBDV emerged as a consequence of antigenic shift in viruses belonging to serotype 1, IBDV has been classified as classical, variant or very virulent (Snyder et al., 1988). Antigenic variants, such as the GLS and Delaware strains, were isolated from vaccinated farms. In the mid 1990's very

virulent strains of IBDV, characterized by high mortality and lesions in organs such as the bone marrow and thymus, appeared in several European and Asian countries. The variants induce disease in chickens in the presence of antibodies to vaccine strains of serotype 1, also known as classical strains. The presence of these variant viruses is a challenge to control of IBD because classic serotype 1 viruses provide only partial protection against variants of these viruses. In contrast, variant viruses provide complete protection against both classical and variant viruses (Ismail and Saif, 1991; Wu et al., 2007).

IBDV vaccines are grouped on the basis of their residual virulence into mild, mild intermediate, intermediate, and intermediate plus or hot strains (Saif, 2008). IBDV intermediate vaccine strains vary in their virulence and can have an effect on the immune status of chickens with a detrimental effect on the immune response due to a suboptimal response to vaccination (Muller et al., 2003; Rautenschlein et al., 2007).

In general, variant viruses induce few or no clinical signs of disease but target primarily the bursa of *Fabricius*. Classical strains induce mortality rates between 2% and 15% and can reach 50%, showing clinical signs and lesions. Infection with very virulent strains can result in mortality rates between 50% and 100%, with clinical signs and lesions (Saif, 2008).

Chapter 3: **Adaptation of A/mallard/12180/Pennsylvania/84 H5N2 in IBDV-pre-exposed chickens.**

3.1 Abstract

Pathogenicity of avian influenza virus is related to both host and viral factors. Low pathogenic avian influenza virus (LPAIV) circulates in wild birds that serve as natural reservoirs of influenza viruses. It is generally accepted that those birds are a potential source for new viruses to emerge, cross host barriers, and infect new avian or mammalian species. During this process changes in pathogenicity occur, resulting in different disease outcomes in animals infected with the viruses. In this regard, the immunological status of the host is considered a predisposing factor that could contribute to differences in disease susceptibility and/or viral pathogenicity. In order to understand host factors involved in susceptibility to infection by avian influenza virus we study the effect of pre-exposure to IBDV (infectious bursal disease virus) on the adaptation of a mallard H5N2 LPAIV in chickens. We found that a dose of 5 x 10^6 EID_{50} (50% egg infectious dose) did not replicate at all in normal chickens and replicated inefficiently in IBDV-pre-exposed chickens; however, passage of the mallard H5N2 LPAIV in IBDV-pre-exposed chickens resulted in a virus that replicated efficiently, causing clinical signs and severe lesions in the respiratory tract of IBDV-pre-exposed chickens. When tested in other avian species the P22 (passaged 22 times) adapted virus replicated more efficiently than the wild-type (WT) virus, showing a two to three log increase in virus titer in lungs of turkey and

pheasants at 3 dpi. Results of this study showed that pre-exposure of the host to IBDV might play a role in adaptation and/or pathogenicity of avian influenza virus.

3.2 Introduction

Influenza A viruses can be assigned to two main pathotypes based on their virulence: the highly pathogenic viruses, which cause severe systemic disease in poultry, and the low pathogenic viruses which cause mild or no signs of disease. Influenza virus virulence is a polygenic trait but it is also influenced by host factors. The association of both virus genetic and host factors determines the outcome of influenza virus infection.

A major outbreak caused by an H5N2 HPAI virus (A/Chicken/Penn/83) occurred in the United States in 1984, killing approximately 17 million birds and infecting primarily chickens and turkeys (Halvorson, 2008; T. Horimoto, 1995; Wood, 1984). The origin of the virus has not been conclusively determined, however it is antigenically similar to viruses isolated from wild ducks in Canada and the United States. There is evidence that the virus was either circulating in wild ducks or that it was present in other water birds (Wood, 1984). It was also determined that H5N2 viruses isolated in 1985 and 1986 in the US were genetically related to the virus isolated in 1983-84 in Pennsylvania; those viruses originated from birds in live-poultry markets in New York City, New Jersey, and Miami, FL (Kawaoka and Webster, 1988a). Based on epidemiological and experimental data, it has been suggested that changes in host specificity and pathogenicity in chickens

probably occurred during the emergence of the HPAI virus. The H5N2 virus, which initially behaved as a low pathogenic avian influenza virus with a low mortality rate, underwent changes over a period of six months and became highly pathogenic in chickens, resulting in a virus with mortality increased up to 80% (Wood, 1984). As mentioned earlier, a LPAI virus is considered the ancestor of the H5N2 HPAIV and a selective mechanism of adaptation has been proposed (Bean et al., 1985).

Considering host factors and their effect on susceptibility to viral infection, host immune status is one parameter that has been associated with increased susceptibility to viral and bacterial infections. In this regard, infectious bursal disease (IBD) is a condition that affects immune status in chickens. IBD represents a major economic concern for the poultry industry due to its prevalence in most poultry-producing areas of the world (Sharma et al., 2000); the prevalence of IBD is the reason that most commercial chickens are exposed to IBDV early in life. Both broiler and layer flocks are susceptible to the immunosuppressive effects of the virus (Sharma et al., 2000), with humoral and cellular immune responses being compromised (Saif, 1991). The main consequence of IBDV infection is immunosuppression and this effect is greater when the chicks are infected at 1 day of age (Higashihara et al., 1991). However little is known of the potential effects of host pre-exposure to IBDV on susceptibility to subsequent AIV infection and on associated clinical signs, lesions, and virus shedding. For this reason, and due to the endemic nature of IBD in the poultry industry globally, we wanted to determine the

effect of pre-exposure to IBDV at an early age on the susceptibility of chickens to AIV infection and on the adaptation of AIV from wild birds into land-based poultry.

We wanted to determine if the virus used in this study, an H5N2 mallard virus, which is antigenically very similar to the H5N2/Pennsylvania/83 avian influenza virus that caused the outbreak in Pennsylvania in 1983 (Bean et al., 1985), was capable of replication in chickens without prior adaptation. To address this, we inoculated SPF (specific-pathogen-free) White Leghorn chickens with a dose of $5x10^6$ EID_{50} by ocular-nasal and tracheal routes. Interestingly, we did not see replication in the SPF chickens after several attempts.

In our effort to elucidate the mechanisms involved in the generation of pathogenic variants from low pathogenic influenza viruses and, considering IBDV infection a predisposing factor, we wanted to determine if pre-exposure to IBDV facilitates the adaptation and/or increases the pathogenicity of the mallard AI virus in chickens.

This chapter presents a description of the experimental methods used and results obtained during the adaptation of the A/mallard/Pennsylvania/84 H5N2 LPAIV in IBDV-pre-exposed chickens.

Analysis of host range restriction factors and virus adaptation mechanisms will aid in understanding the contribution of these factors to AI virus pathogenicity

and generation of new variants – an event with potentially deleterious consequences for the poultry industry because of the possibility that new variants will be more highly pathogenic than existing strains.

3.3 Materials and Methods

3.3.1 Viruses

Low Pathogenic Avian Influenza Virus: The A/Mallard/ Pennsylvania/12180/84 (H5N2) used in this study was obtained from the repository at St. Jude Children's Research Hospital, Memphis, TN, USA. The virus was propagated in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs and titrated to determine the 50% egg infectious dose (EID_{50}) by the Reed and Muench method (Reed, 1938).

Infectious Bursal Disease Virus: IBD E-Delaware variant virus was used to infect chickens at an early age in order to determine the effect of pre-exposure to IBDV on the susceptibility of chickens to avian influenza virus infection. Virus stock was prepared in 3-week-old SPF White Leghorn Chickens (Charles River Laboratories, Wilmington, MA, USA) that were infected by ocular, intranasal, and oral routes with 200 µl of IBDV stock diluted 1:10 in TPB (Tryptose Phosphate Broth, Sigma-Aldrich, St. Louis, MO, USA) containing Antibiotic - Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO, USA). Bursa and body weights were recorded to determine the bursa/body weight index. Bursas were collected at 24, 48, and 72 h post-infection, homogenized, and analyzed by Enzyme-Linked-

Immunosorbent-Assay (ELISA) (IBDV Antigen-capture ELISA Test Kit, Synbiotics Corporation, San Diego, CA, USA). The maximum concentration of viral antigen was obtained from bursa homogenate prepared at 48 h post-infection. The 50% chicken infectious dose ($CID_{50)}$ for the IBDV stock was determined.

3.3.2 Tissue Homogenates

3.3.2.1 Bursa homogenate. The bursa of *Fabricius* was extracted through an incision on the back of the chicken, washed in PBS containing Antibiotic – Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO, USA), and weighed. A sample was preserved in 10% buffered formalin for hematoxylin-eosin staining and histological analysis. The remaining tissue was cut into small pieces using scissors. An equal volume of antigen dilution buffer or TPB containing Antibiotic – Antimycotic (100X) (Sigma-Aldrich, St. Louis, MO, USA) was added and a layer of sterile laboratory sea sand (Fisher Scientific, Waltham, MA, USA) was added to the mixture. The sample was ground using a pestle. The homogenized tissue was frozen at -70 ºC and thawed three times, briefly vortex-mixed, and centrifuged at 1500 x g at 4 ºC for 10 min. The supernatant was collected, aliquoted into 1.5 ml microfuge tubes and stored at -70 ºC until use. Presence of the virus in the bursa homogenate was established by ELISA (IBDV Antigen-capture Elisa Test Kit, Synbiotics Corporation, San Diego, CA, USA) according to the manufacturer's instructions.

3.3.2.2 Lung Homogenate. Lungs were extracted and washed twice in PBS-Antibiotic - Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO) to remove particles and blood. Lungs were placed in petri dishes, weighed, and then transferred

to a mortar and cut into small pieces using scissors. A layer of sterile laboratory sea sand (Fisher Scientific, Waltham, MA, USA) was added to the tissue and the sample was ground using a pestle. A 10% (w/v) lung homogenate suspension was prepared by adding corresponding amount of Brain Heart Infusion (BHI) broth (BHI powder (BBL Cat. no. 211060) containing Gentamicin and Antibiotic - Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 1500 x g, at 4 ºC for 10 min and the supernatant was collected and filtered using a 0.22 µm filter unit. Aliquots of the homogenate were stored at –70^{o}C until use.

3.3.2.3 Soft Tissue Homogenates: Brain, Kidney, Intestine, Pancreas, Liver, Spleen. After collection the tissue was washed twice in PBS plus Antibiotic - Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO) and then placed in sterile tissue bags and weighed. A tissue homogenate was prepared using a laboratory blender (Seward Stomacher®80, Lab System). The samples were transported on ice and the machine was run for 1 - 2 minutes at a time until the tissue was completely homogenized. BHI medium (BHI powder (BBL Cat. no. 211060) Gentamicin, and Antibiotic - Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO) was added to make a 10% (w/v) homogenate. The tissue homogenate was centrifuged at 1500 x g at 4 ºC for 10 min and the supernatant was filtered using a 0.22 µm filter unit. Aliquots of the homogenates were stored at -70^{o}C until use.

3.3.3 Animals and Experimental Infections

SPF White Leghorn chickens (Charles River Laboratories, Wilmington, MA, USA) were used. Avian influenza virus was administered in doses of 1 ml at a concentration of 5 X 10^6 EID_{50}/ml and 1 X 10^8 EID_{50}/ml. Infection routes were through the nares, eyes, trachea, and cloaca. Animal feeding needles (Fisher Scientific, Waltham, MA, USA) were used to administer the virus. Tracheal and cloacal swabs were collected on alternate days and stored in glass vials containing 1 ml glycerol medium (50% sterile glycerol, 50% PBS, 1 ml/200 ml total volume Gentamycin, 10 ml 100X Antibiotic – Antimycotic (100X) (Sigma-Aldrich, St. Louis, MO, USA]) at -70 ^oC. Swab samples were tested for presence of virus by passing swab medium in 10-day-old embryonated chicken eggs. Three eggs were used for each swab collected and 200 µl medium was used to infect each egg. Infected eggs were incubated for 48 h and then chilled at 4 ^oC for no more than 24 h or at -20^oC for 30 min. Allantoic fluid was collected and a hemagglutination assay was performed, following World Health Organization (WHO) recommendations, to determine the presence of virus. Samples showing agglutination of chicken red blood cells were scored as positive.

Birds were observed and scored daily for clinical signs of disease and general well being. Animals were evaluated on the basis of appetite, activity, fecal output, and signs of distress or clinical illness, including ruffled feathers and respiratory distress. Birds were scored on a scale of 1 to 4. Table 1 shows the scoring sheet and scoring criteria for each parameter.

Experiments were carried out under BSL2+ conditions, with investigators wearing appropriate personal protective equipment, and were compliant with animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland, College Park, MD, USA and Animal Welfare Act (AWA) regulations.

3.3.4 H5N2 LPAI virus passages in IBDV- pre-exposed chickens

Two-day-old SPF chickens were infected with 100 CID_{50} of the E-Delaware IBD virus strain by oral and ocular routes. Adaptation experiments consisted of 22 passages of A/mallard/Pennsylvania/12180/84 (H5N2) virus in three-week-old IBDV-pre-exposed chickens. The dose for the first passage was a 1:10 dilution of H5N2 wild-type viral stock that had been grown once in embryonated chicken eggs. Subsequent passages were carried out by inoculation with a 10% pooled lung homogenate from the previous passage, with the exception of passages 16, 18, and 19, where a 1:10 dilution of allantoic fluid collected from the lung homogenate inoculation in chicken embryonated eggs was used as the inoculum for the next chicken passage. Tracheal and cloacal swabs were collected on days 1 and 3 post-infection and virus was detected using a hemagglutination assay. Doses of 1 ml were administered to the chickens. One milliliter aliquots of lung homogenate from each chicken were pooled and used as the dose for the following group.

Table 1. Scoring sheet for influenza virus and bird model study

Bird ID:

Date																						
Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Physical Appearance																						
Activity																						
Respirtory symptoms																						
Other symptoms																						
Body weight loss																						
Body weight (gms)																						
Comments																						
Initials																						

Explanation of Scoring Sheet for Birds:

Physical Appearance:
0 = Normal
1 = Ruffled feathers
2 = Light/Mild swelling of the face or legs
3 = Heavy edema and swelling of head, eyelids comb, wattles, and hocks

Activity:
0 = alert
1 = alert but only when personnel enters the room and/or works around the cage
2 = alert but only when stimulated
3 = inactive, not responding to stimuli

Respiratory disease symptoms:
0 = no symptoms
1 = light nasal discharge
2 = heavy nasal discharge/conjunctivitis
3 = mouth breathing/labored breathing/wheezing

Other disease symptoms/body weight loss
0 = no other symptoms/no weight loss

3.3.5 Dose preparation from lung homogenate

Lungs were collected on day 3 post-infection and a 10% (w/v) lung homogenate was prepared by adding corresponding amount of Brain Heart Infusion (BHI) broth as described in 3.3.2.2. The next chicken passage was infected with the 10% pooled lung homogenate as described in 3.3.4. Doses of 1 ml were administered using sterile animal feeding needles (Fisher Scientific, Waltham, MA, USA). Approximately 200 µl were administered in the eyes, 200 µl in the nares, 400 µl in the trachea, and the remaining 200 µl in the cloaca. Pooled lung homogenate was titrated in embryonated chicken eggs to determine virus concentration in each of the doses.

Allantoic fluid from pool lung homogenate inoculated in embryonated eggs was used for infection in passage 16, 18, and 19 was diluted 1:10 with PBS/Antibiotic - Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO).

3.3.6 Transmission of influenza virus in chickens

For transmission experiments, three SPF White Leghorn chickens, three to four week of age, were infected with virus at a concentration of 1 X 10^8 EID_{50} in a volume of 1 ml as described in 3.3.5. Three contact birds were introduced at 1 dpi into the cage with the infected chickens. Water and food, as well as cage liners, were changed in order to prevent transmission of virus via contamination. Tracheal and

cloacal swab samples were collected 1, 3, 5, 7, and 9 dpi and hemagglutination assays were conducted to determine extent of viral shedding.

3.3.7 Replication of the mallard H5N2 WT and P22 chicken-adapted LPAI virus in turkeys and pheasants.

In order to determine if adaptation of the H5N2 mallard virus in IBDV-pre-exposed chickens resulted in a virus that replicates more efficiently than in other avian species, we tested the replication of the WT and P22 adapted virus in the respiratory tract of turkeys and pheasants. To achieve this the avian influenza viruses were administered in doses of 1 ml at a concentration of 1 X 10^8 EID_{50}/ml to groups of two (WT) or three (P22) turkeys and groups of three (WT and P22) pheasants. Infection routes included the nares, eyes, trachea and cloaca, following the same procedure described previously in section 3.3.5 for chickens. Animal feeding needles (Fisher Scientific, Waltham, MA, USA) were used to administer the virus. Tracheal and cloacal swabs were collected on alternate days and stored in glass vials containing 1 ml glycerol medium (50% sterile glycerol, 50% PBS, Gentamycin (Sigma-Aldrich, St. Louis, MO), Antibiotic – Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO) at -70^{o}C. Swab samples were tested for virus by passing swab medium in 10-day-old embryonated chicken eggs, following the same procedure as described in 3.3.3. Lung homogenates were prepared from each animal at 3 dpi, following the same procedure as described in 3.3.2.2, and titrated to determine the EID_{50} using the Reed and Muench method (Reed, 1938).

Birds were observed and scored daily for clinical signs of disease and general well being using the same criteria as for chickens (Table 1). Experiments were carried out under BSL2+ conditions, with investigators wearing appropriate personal protective equipment, and were compliant with animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland, College Park, MD, USA and Animal Welfare Act (AWA) regulations.

3.4 Results

3.4.1 The mallard H5N2 LPAI virus replicates efficiently after passage in IBDV-pre-exposed chickens

Inoculation with a dose of 5 x 10^6 EID_{50} A/mal/Penn/84/H5N2 virus in 2 to 3-week-old normal SPF White Leghorn chickens resulted in no replication and no transmission of the virus. The same experiment was repeated four times with similar results. When the same dose of virus was used in IBDV-pre-exposed chickens there was poor transient replication of the LPAIV.

Different routes of inoculation and virus dose were evaluated. Initially only ocular-nasal and intratracheal routes of inoculation were attempted, but later an intracloacal route was added. Infection with a dose of 1x10^8 EID_{50} of the WT virus resulted in virus appearing in the lungs of infected chickens. Considering host immune status as a determinant for an animal's susceptibility to infectious agents, adaptation of H5N2 LPAIV was conducted in chickens that had been pre-exposed to IBDV. We found that, during the first four passages, the virus could only be isolated

from tracheal swabs. However, during and after passage five, virus was found not only in the trachea but also in the cloacae. At the same time there was an increase of approximately 1 $\log_{10}$ in virus titer in lung homogenate (Table 2).

3.4.2 Clinical signs and macroscopic findings suggest an increase in pathogenicity during adaptation in chickens pre-exposed to IBDV

Gross lesions found at necropsy included fibrinous airsacculitis after a low number of passages, with severity increasing after 10 passages. A similar pattern was observed in lung lesions and was consistent with clinical outcome. During the first 13 passages none of the virus-inoculated chickens presented clinical signs or died; at passage 14 one of three animals showed the first evidence of clinical signs - sneezing at days two and three post-infection. This and another animal from the same group showed cloudy eyes and periorbital edema. Respiratory signs were observed again in chickens infected at passage 16 and were present and very evident in 3/3 infected chickens in passage 18. Animals showed respiratory distress, sneezing, and cloudy eyes. With continued adaptation, mild respiratory signs became more severe after passage 22. There was an increase in virus titer in lung and in virus shedding in trachea. During adaptation the virus titer in the lung peaked at $10^{6.6}$ EID_{50}/ml (Figure 2). The chickens shed more virus from the trachea than from the cloaca, providing an explanation for the respiratory signs observed after passage 22 (P22) in IBDV-pre-exposed animals. As expected, a constant finding was atrophy of the bursa of *Fabricius* in IBDV-pre-exposed chickens.

Table 2. Virus shedding in trachea and cloaca and virus titers in pooled lung homogenate 3 dpi during adaptation of the mallard H5N2 virus in IBDV-pre-exposed chickens.

Passage/ Chicken ID	Shedding Trachea			Shedding Cloaca			Log_{10} EID_{50} Pooled lung homogenate
	I1	I2	I3	I1	I2	I3	
P1	+	+	+	-	-	-	4.5
P2	+	-	-	-	-	-	4.5
P3	-	+	-	-	-	-	4.5
P4	+	+	-	-	-	-	5.7
P5	+	+	+	+	-	-	5.5
P6	+	+	+	+	-	-	6.5
P7	-	+	+	+	+	+	5.5
P8	+	+	+	-	-	-	6.5
P9	+	+	-	+	+	+	5.5
P10	-	+	+	+	+	+	6.0
P11	+	+	+	+	-	-	6.2
P12	+	+	+	+	-	+	6.5
P13	+	+	+	-	+	-	6.2
P14	+	+	+	+	+	+	6.2
P15	+	+	+	+	+	+	6.2
P16	-	+	+	+	+	+	6.2
P17	+	+	+	+	+	+	6.2
P18	+	+	+	+	+	+	6.7
P19	+	+	+	-	+	+	6.6
P20	+	+	+	+	+	+	6.2
P21	+	+	+	+	+	+	6.2
P22	+	+	+	+	+	+	6.6

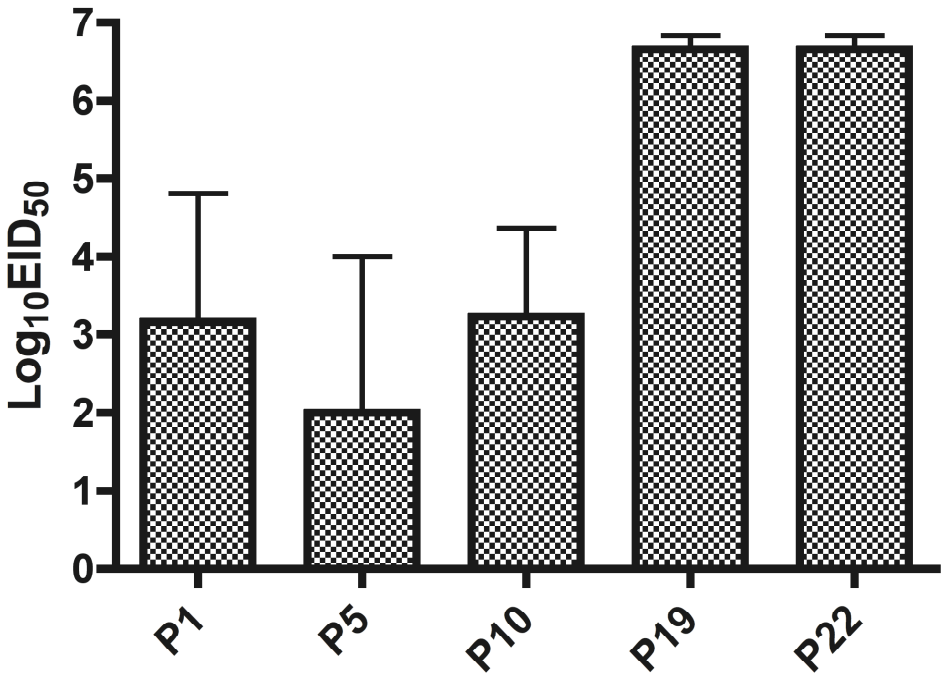

Figure 2. Virus titers in the lung during adaptation of a mallard H5N2 AI virus in IBDV-pre-exposed chickens. Groups of three chickens pre-exposed to IBDV were inoculated with a 1:10 dilution of pooled lung homogenate from the previous passage. Values represent the mean and standard deviation of virus titers in lung homogenates from three chickens per passage. Results are shown for passages (P) 1, 5, 10, 19, and 22.

3.4.3 Differences in the outcomes of replication and transmission studies were observed when comparing different passages

Macroscopic examination and clinical observation suggested an expansion of tissue tropism during the adaptation of the H5N2 AI virus and, on that basis, the range of tissues tested for virus isolation was expanded. Brain homogenate was evaluated from passages 10 to 19, resulting in 25/30 positive samples. Pancreas homogenates analyzed from passages 17 to 19 were 100% positive (9/9) for virus isolation. We then wanted to determine whether the increase in virus replication and broader tissue tropism were reflected in more efficient transmission to contact birds. With this goal, replication and transmission studies in IBDV-pre-exposed chickens were conducted at passage 10, 17, and 22. Results of this experiment showed isolation of virus from tracheal and cloacal swabs from infected animals until 7 dpi, as well as occasional transmission to contact animals, with virus isolated from tracheal and cloacal swabs only when chickens were infected with virus in lung homogenates from passage 17 (Table 3).

3.4.4 P22 AI virus replicates efficiently in turkeys and pheasants

Adaptation of the mallard H5N2 virus in IBDV-pre-exposed chickens resulted in a virus that replicates efficiently, not only in chickens, but also in other avian species such as turkeys and pheasants. Efficient replication in the respiratory tract was observed after infection with the WT and P22 viruses; however more than a two-$\log_{10}$ difference in virus titer was found in the lungs of both turkey and pheasant infected with the P22 compared to the WT virus (Figure 3).

Table 3. Replication and transmission studies in IBDV pre-exposed chickens at passages 10, 17 and 22.

		No. positive chickens /total No. of chickens					
		3 dpi		5 dpi		7 dpi	
Passage	Group	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
P10	Infected	3/3	2/3	1/3	1/3	0/3	0/3
	Contact	0/3	0/3	0/3	0/3	0/3	0/3
P17	Infected	3/3	3/3	3/3	2/3	1/3	0/3
	Contact	**1/3**	**2/3**	0/3	0/3	0/3	0/3
P22	Infected*	3/3	3/3	3/3	2/3	0/1	1/1
	Contact	0/3	0/3	0/3	0/3	0/3	0/3

***Two animal died, one at 6 and the other at 7 dpi

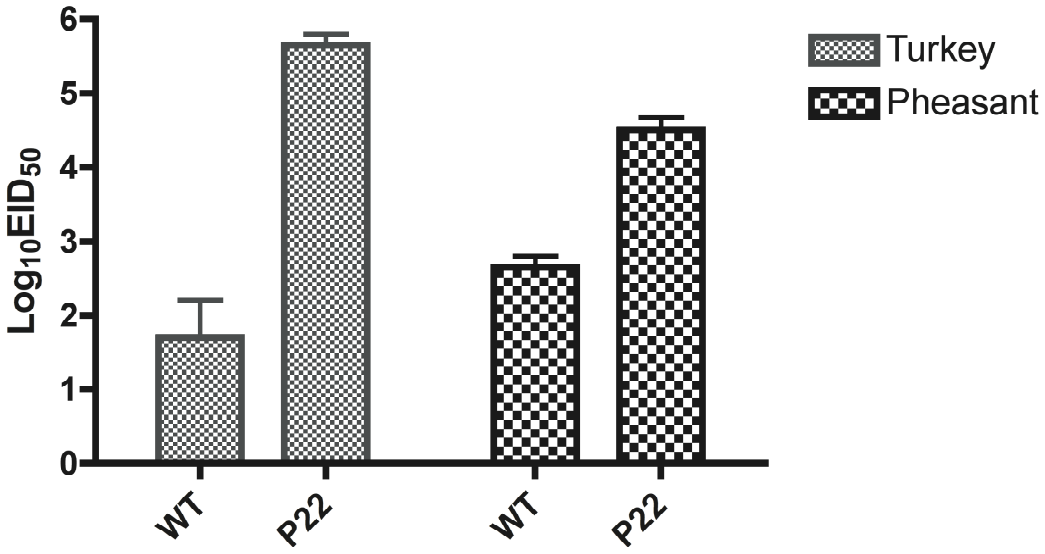

Figure 3. Replication of WT and P22 AI virus in the lungs of 3-week-old turkey and pheasant. Samples were collected from groups of three birds at 3 dpi with the P22 and WT viruses. Titration was performed in inoculated embryonated chicken eggs with EID_{50}. Values represent the average and standard deviation of the titer for individuals in each group of three birds. The results for WT in turkey represent the average and standard deviation of values for individuals in groups of two birds.

3.5 Discussion

Several factors must converge for a virus to become adapted and transmit into a new host. The route of exposure and dose of the virus should be appropriate to permit infection. A series of genetic changes in the virus, which increase the efficiency of infection, as well as secondary factors that enhance host susceptibility to infection, provide the opportunity for avian influenza virus to successfully adapt**.**

It has been shown that the immune status of the host influences the outcome of viral respiratory diseases in poultry (Rosenberger and Gelb, 1978). To elucidate the role of host immune status on the susceptibility of chickens to avian influenza virus, we analyzed a mallard H5N2 LPAIV in IBDV-pre-exposed chickens. We evaluated the effect of passing inoculation of the LPAIV in birds that had been pre-exposed to IBDV, using natural routes of infection, to determine whether this facilitates replication and transmission among susceptible chickens. Changes in virus shedding and replication were observed, with virus replicating preferentially in the respiratory tract of chickens. Although few or no clinical signs were observed in the birds monitored during 3 dpi throughout the 22 passages, when the animals were observed for a longer period of time disease signs were evident in IBDV-pre-exposed chickens after passage 22, indicating a change in virus pathobiology. Respiratory signs were observed in the infected animals and a significant increase in virus titer was observed 3 dpi. The appearance of clinical signs, efficient replication, and increase in virus titer after 22 passages in IBDV- pre-exposed chickens suggests adaptation of the virus.

The results of this study show that pre-exposure of animals to IBDV facilitated adaptation of LPAIV from wild birds into land-based poultry. Clinical signs and virus titers were consistent with a virus that had acquired not only a broader tissue tropism in chickens, but also the potential to expand its host range, as shown for a 2 to 3-$\log_{10}$ increase in virus titers in lung from turkeys and pheasants when compared the P22 and WT AI viruses.

Results of this research suggest an important role for immune status in the adaptation of LPAIV in terrestrial birds. Predisposing factors, such as IBDV infection at an early age, should be considered risk factors for generation of virus with increased pathogenicity when LPAI strains circulate in a population of birds with suboptimal immune status, which is likely to be a common event in nature.

Analysis of changes in pathogenicity as a result of adaptation of the H5N2 AI virus in IBDV-pre-exposed chickens, as well as host molecular factors involved in this process, will be discussed in separate chapters.

Chapter 4: **A mallard H5N2 low pathogenic avian influenza virus showed increased virulence and pathogenic effects after passing in IBDV-pre-exposed chickens.**

4.1 Abstract

In previous studies we found that passage of a mallard H5N2 low pathogenic avian influenza virus (LPAIV) in chickens that had been pre-exposed to infectious bursal disease virus (IBDV) resulted in a more efficiently replicating virus, suggesting that the adapted virus might also show an increase in pathogenicity in IBDV-pre-exposed compared to normal chickens. Therefore, we analyzed the outcomes of infection with wild-type (WT) and P22 (22 passages) adapted AI virus in normal and IBDV-pre-exposed chickens by examining the lesions caused by the two viruses at the microscopic level in a time-point study. Analysis of histological data from a comprehensive group of tissues showed that IBDV pre-exposure might play a role in determining pathogenicity of avian influenza virus. Detection of viral antigen by immunohistochemistry confirmed the presence of virus mainly in inflammatory infiltrates, primarily in the respiratory tract of P22 AIV- infected chickens. We also investigated changes in the virus at the molecular level by comparing the sequences of the open reading frames in the WT and the P22 AI adapted virus genomes. Amino acid substitutions occurred primarily in the surface glycoprotein HA, suggesting selection for a virus that has improved fit and replicates more efficiently in chickens.

4.2 Introduction

The factors that determine interspecies transmission and pathogenicity of influenza viruses are still poorly understood. A large variety of avirulent influenza viruses are maintained in wild birds, which are considered the principal reservoir of influenza virus (Humberd J et al., 2006; Ito et al., 2001; Rott et al., 1976; Vines et al., 1998). These birds provide a large reservoir from which new viruses can emerge and infect other mammalian or avian species (Perdue and Swayne, 2005; Silvano et al., 1997; Webby and Webster, 2001). Virulence is considered a polygenic property (Rott et al., 1979; Scholtissek et al., 1977a; Scholtissek et al., 1977b). In this regard, the surface glycoprotein hemagglutinin (HA) plays a central role determining host range while contributing to the virulence of avian influenza viruses (Ito et al., 2001; Rott et al., 1976; van der Goot et al., 2003). It has been shown that the internal genes coding for RNA polymerase (PB2, PB1, PA), nucleoprotein (NP), matrix protein (M1, M2), and nonstructural protein (NS1, NS2/NEP) also contribute to determining host range.

Most influenza virus infections in poultry are subclinical, producing only mild to moderate signs of disease. These avian influenza viruses (AIVs) have been categorized as nonpathogenic (NP) or mildly pathogenic (MP). However some avian H5 and H7 viruses cause severe systemic disease associated with high rates of morbidity and mortality; these strains are classified in the category of high pathogenicity avian influenza (HPAI) virus (Bean et al., 1985; Horimoto et al., 1995; Murphy, 1986; Capua I and DJ, 2004; Garcia et al., 1996).

It has been proposed that highly pathogenic avian influenza viruses may be derived from nonpathogenic avian strains that have acquired mutations which render the HA cleavable by intracellular proteases (Banks et al., 2000; Donatelli et al., 2001; Rohm et al., 1995). In nature, when low pathogenic avian influenza virus (LPAIV) strains are transmitted from avian reservoir hosts to highly susceptible poultry species, which then support several cycles of infection and replication, these strains may undergo a series of mutational events resulting not only in adaptation to their new hosts but also mutation into highly pathogenic forms (Banks et al., 2000; Perdue and Swayne, 2005; Rohm et al., 1995). Although much attention has been focused on HPAI viruses, the importance of LPAI viruses can not be overlooked. These viruses have major impact not only with regard to the economic losses suffered by poultry producers (Halvorson, 2008) but also because of the potential of LPAI viruses of the H5 and H7 subtypes to become HPAI viruses, breach transmission barriers, and infect other species, including humans. Expansion of host species range, with its implications for public health, make the understanding of mechanisms related to pathogenicity and host range restriction of AI viruses very important issues (Halvorson, 2008; Perkins and Swayne, 2003). Combined pathology-based and molecular approaches, such as the one described here, will provide useful information about the molecular determinants of replication and pathogenicity of the H5 virus in chickens.

4.3 Materials and Methods

4.3.1 Viruses

Low Pathogenic Avian Influenza Viruses: Two LPAI viruses were used in this study, referred to as WT and P22 AI viruses. The A/Mallard/Pennsylvania/12180/84 (H5N2) wild type (WT) virus was obtained from the repository at St. Jude Children's Research Hospital, Memphis, TN, USA. This virus was adapted by 22 serial passages of virus from lung in chickens that had been pre-exposed to IBDV, herein referred as P22 LPAI virus.

The viruses were propagated in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs and titrated to determine the 50% egg infectious dose (EID_{50}) using the Reed and Muench method (Reed, 1938).

Infectious Bursal Disease virus (IBDV): IBD E-Delaware variant virus, prepared as described in 3.3.2.1, was used to infect 2-day-old SPF White Leghorn Chickens (Charles River Laboratories, Wilmington, MA, USA). The IBDV-pre-exposed chickens were housed for three weeks before inoculation with the WT or P22 AI viruses.

4.3.2 Animals and experimental infections

To investigate the effect of adaptation in IBDV-pre-exposed chickens on the pathogenesis of the H5N2 LPAI virus, we evaluated histological changes after infection with the WT and the P22 AI virus in normal and IBDV-pre-exposed White

Leghorn chickens. Four groups of nine 3-week-old chickens, including two groups of IBDV-pre-exposed and two groups of normal chickens, were infected with 10^8 EID_{50} of either WT (one normal and one IBDV-pre-exposed group) or P22 (one normal and one IBDV-pre-exposed group) AI virus. Two additional groups of chickens were used as controls: one group of IBDV-pre-exposed chickens and one of normal chickens that had been mock infected using PBS containing Antibiotic – Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO) as inoculum.

Chickens were infected via ocular-nasal, intratracheal, and intracloacal routes with a total dose of 1 ml. Tracheal and cloacal swabs were collected 1, 3, 5, and 7 days post-infection (dpi) in buffered glycerol (1:1 glycerol, phosphate buffered saline with Antibiotic – Antimycotic solution (100X) (Sigma-Aldrich, St. Louis, MO)) and stored at -70° C until use. The birds were observed and scored daily for clinical signs of disease. Groups of three birds were euthanized and necropsied at 3, 5, and 7 dpi for macroscopic examination and collection of organs for histological analysis.

4.3.3 Transmission Experiments with the P22 LPAI virus in normal and IBDV pre-exposed chickens

To determine if viral replication and pathogenicity were related to improved transmissibility we evaluated transmission in normal and IBDV-pre-exposed chickens after infection with the P22 LPAI virus. In order to do this, three IBDV-pre-exposed uninfected chickens were placed in direct, aerosol, and fecal contact with three IBDV-pre-exposed inoculated chickens 1 dpi with the H5N2 P22 virus. Transmission

studies were also conducted without IBDV-pre-exposure (normal chickens). Chickens one and three weeks of age were tested. Throughout the experimental period, feed and water were provided *ad libitum.* Tracheal and cloacal swabs were collected in buffered glycerol at 1, 3, 5, 7 and 9 dpi, inoculated in 10-day-old embryonated chicken eggs, incubated at 35^{o} C, and tested by hemagglutination assay 2 dpi.

Animal experiments were carried out under BSL2+ conditions, with investigators wearing appropriate personal protective equipment, and were compliant with animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland, College Park, MD, USA and Animal Welfare Act (AWA) regulations.

4.3.4 Histological examination

To study differences in the pathogenesis of the H5N2 WT and P22 AI virus in chickens, a time-point evaluation of histological changes was conducted in tissues collected from groups of three chickens sacrificed systematically at 3, 5, and 7 dpi as described in 4.3.2. Tissues, including lung, trachea, nasal sinus, conjunctiva, kidney, pancreas, spleen, liver, heart, thymus, intestine, brain, and bursa of *Fabricius* were collected and fixed in 10% buffered formalin followed by embedding in paraffin. Sections of 5 µm were prepared and stained with hematoxylin-eosin for microscopic examination. Scoring of lesions was carried out by an avian pathologist, without prior knowledge of samples, using the following scoring criteria: +++, severe; ++,

mild; +, moderate; +/-, light; -, no lesions. A value from 1 to 4 was assigned according to severity of lesions with 1 corresponding to light lesions and 4 to severe lesions.

4.3.5 Immunohistochemistry

AI viral antigen-positive cells were identified using an anti-avian influenza nucleoprotein (NP) monoclonal antibody (Synbiotics, Co.). Sections of formalin-fixed, paraffin-embedded tissue were incubated with a 1:150 dilution of the anti-NP monoclonal antibody overnight at 4 ºC. Slides were then washed four times with 1X PBS and incubated at 37º C for 1 h with anti-mouse IgG peroxidase-conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:200 according to the manufacturer's recommendations. Slides were then incubated for 10 min at room temperature with the substrate 3-amino-9-ethylcarbazole (AEC) and counterstained with hematoxylin. AI virus-positive cells were visualized under the microscope at magnifications of 20X, 40X and 100X.

4.3.6 Virus sequencing

Total RNA was extracted from allantoic fluid containing virus using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out with the Uni12 primer (5' – AGCAAAACGAAGG- 3') and AMV reverse transcriptase (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) amplification was performed using universal primers described by Hoffmann et al. (Hoffmann et al., 2001) as well as

specific primers. The PCR products were sequenced using the Big Dye Terminator Protocol v3.1 (Applied Biosystems, Foster City, CA, USA). Complete genome sequences, with the exception of regions recognized by the 5'- and 3'-end universal primers, were determined.

4.4 Results

The P22 H5N2 virus showed more efficient replication and caused clinical signs and severe lesions in the respiratory tract in IBDV-pre-exposed chickens. Results of previous experiments showed that passage of the mallard H5N2 LPAIV in IBDV-pre-exposed chickens resulted in efficient replication and suggested an increase in virus pathogenicity (Chapter 3). We then wanted to determine if those changes would result in changes in the pathogenicity of the adapted virus in normal and IBDV-pre-exposed chickens compared to the WT virus; we addressed this by analyzing histological changes in a large group of tissues.

4.4.1 Histological analysis confirmed the presence of pathological lesions primarily in the respiratory tract of P22 H5N2-infected chickens

Based on clinical, virological, and macroscopic findings we observed a change in pathogenicity of the P22 H5N2 AI virus in this study. In order to generate additional evidence in support of this finding, a time-point study was designed in which a comprehensive group of organs was analyzed by microscopy with the purpose of assessing the presence and severity of lesions. The analysis showed that the P22 is more pathogenic than the WT AI virus. Macroscopic findings showed

lesions primarily in the respiratory tract, with increased severity in IBDV-pre-exposed, P22 AI virus-infected chickens. Lungs were congested and edematous; areas of pneumonia were evident, primarily in the ventral region. Histological analysis of the lungs of infected animals confirmed that the P22 AI virus caused significantly more severe pathology in chickens than the WT virus. The P22 virus caused severe lesions in lung and trachea in 5/9 chickens and mild to moderate lesions in 4/9 normal infected chickens. In contrast, the WT virus caused only mild to minimal lesions in lung; with the exception of one animal that had minimal lesions in the trachea the other tissues were normal (Figure 4). The severity of lesions caused by the P22 virus in IBDV-pre-exposed chickens was also greater in lung and trachea, varying from severe to moderate in comparison to lesions seen in animals infected with the WT virus, which caused mild to minimal lesions in the same organs (Figure 5).

The P22 virus caused lesions in organs other than the respiratory tract, including the kidney, pancreas, liver, thymus, and heart while the WT virus caused minimal or no lesions in these organs in either normal or IBDV-pre-exposed infected chickens (Figure 4, Figure 5, Figure 6). Taken together, our results show that IBDV-pre-exposure influences the severity of lesions caused by AI, particularly in combination with the P22 AI virus strain.

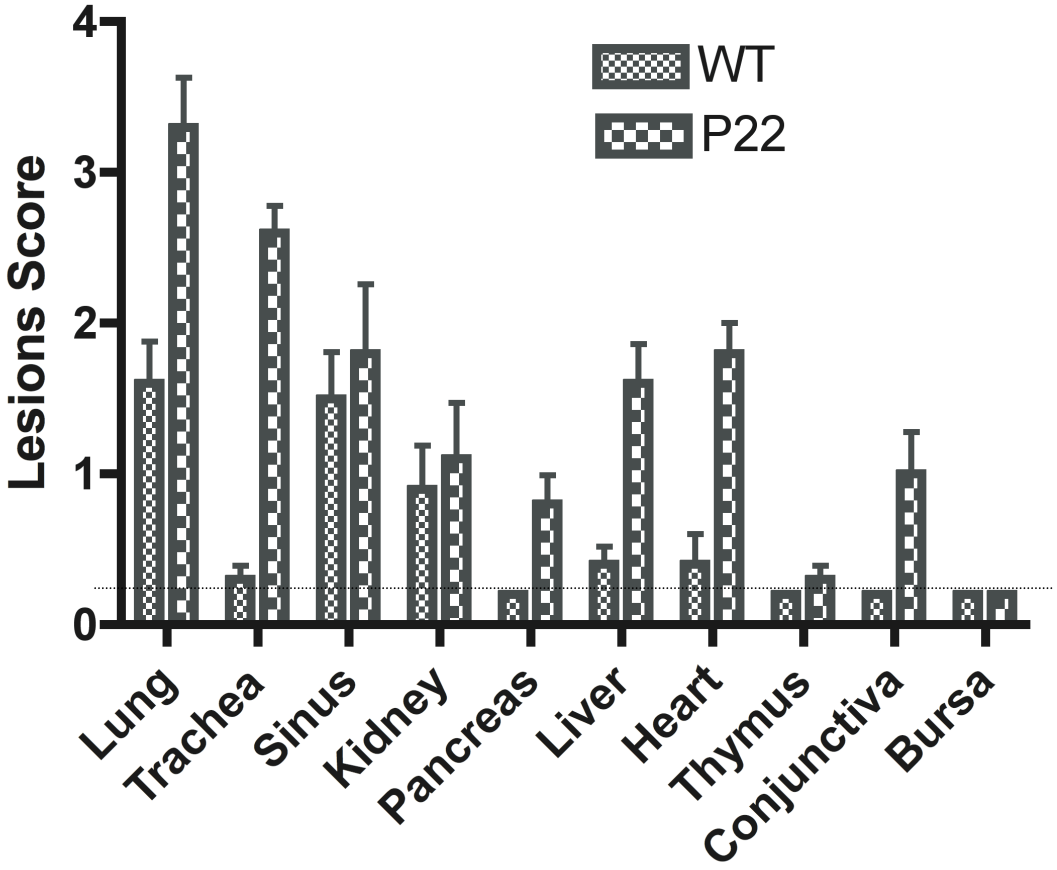

Figure 4. Histological lesions in normal chicken infected with the WT and P22 chicken-adapted H5N2 virus. Groups of nine chickens were inoculated with 10^8 EID_{50}/ml of the H5N2 AIV. Tissue samples collected from groups of birds (3/group) at 3, 5, and 7 dpi. A value of 1 to 4 was assigned according to severity of lesions as follows: 4: Severe; 3: moderate; 2: mild; 1: minimal; 0.2: no lesions. Each bar represents the average of lesions score per group of nine chickens.

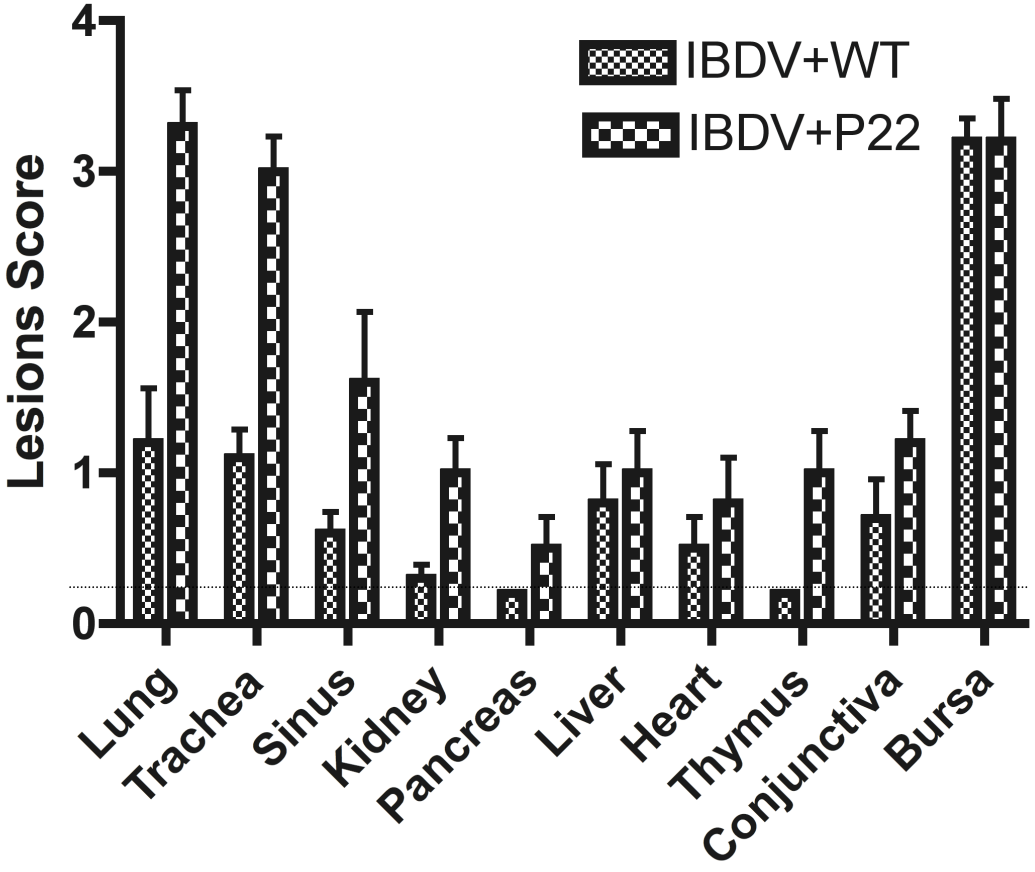

Figure 5. Histological lesions in IBDV-pre-exposed chicken infected with the WT and P22 chicken-adapted H5N2 virus. Groups of nine chickens were inoculated with 10^8 EID_{50}/ml of the H5N2 AIV. Tissue samples collected from groups of birds (3/group) at 3, 5, and 7 dpi. A value of 1 to 4 was assigned according to severity of lesions as follows: 4: severe; 3: moderate; 2: mild; 1: minimal; 0.2: no lesions. Each bar represents the average of lesions score per group of nine chickens.

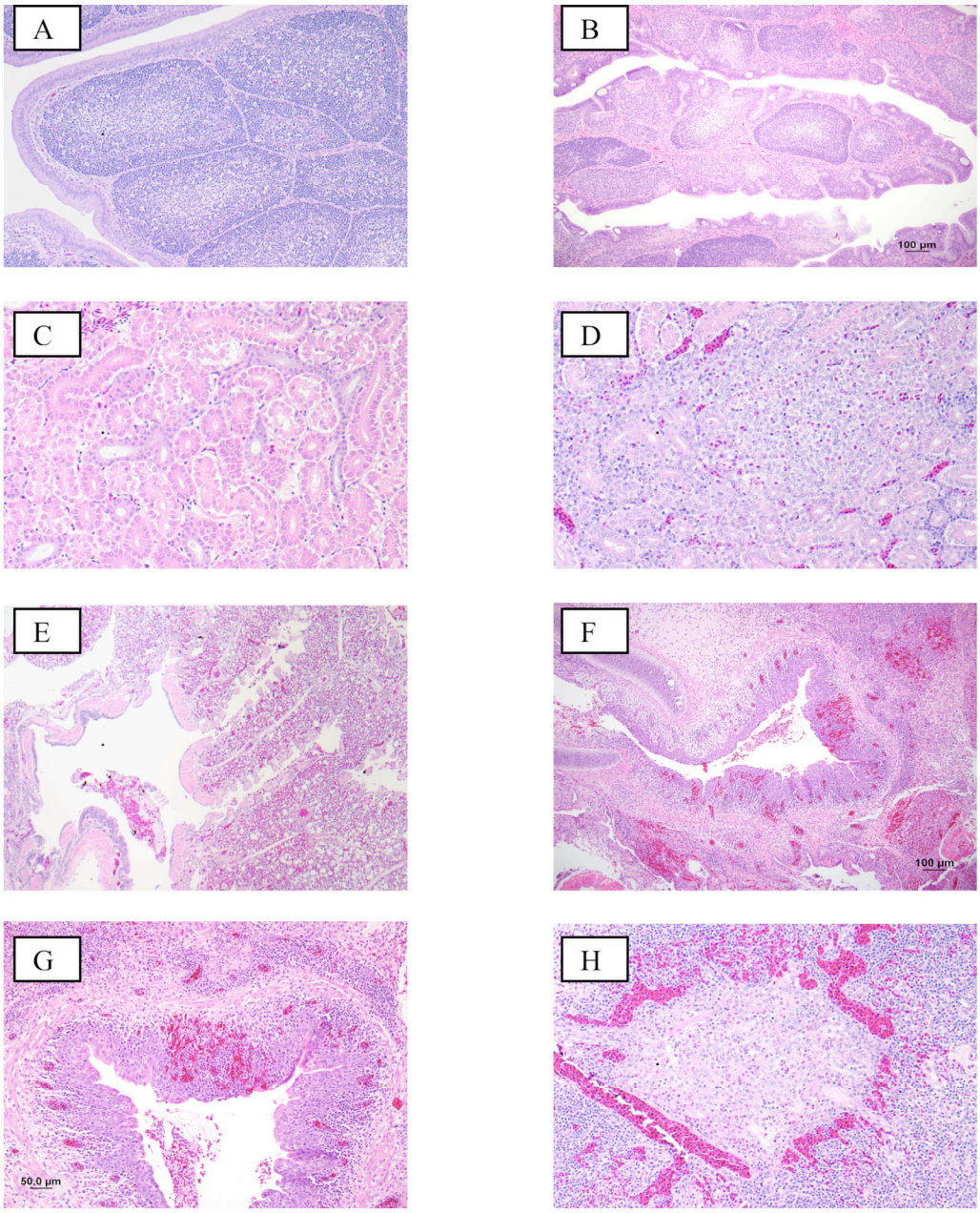

Figure 6. Histological lesions. Tissue samples collected from IBDV-pre-exposed chickens 3 dpi with P22 H5N2 virus. Panels A, C, and E correspond to normal tissue. Panel B shows severe lymphoid depletion in the bursa of *Fabricius*. Figure D shows interstitial nephritis and tubule necrosis. Panels F, G, and H show pneumonic lesions and inflammatory infiltrate in the lung of infected chickens.

4.4.2 Viral antigen was identified primarily in inflammatory infiltrates in lungs of infected chickens

Consistent with the knowledge that monocytes and macrophages are fully susceptible to influenza A virus infection in mammals (La Gruta et al., 2007; Perrone et al., 2008) we found that P22 H5N2 virus causes a severe infiltration of inflammatory cells in the respiratory tract of infected chickens. Immunohistochemical analysis demonstrated the presence of viral antigen in the respiratory tract, particularly in inflammatory infiltrates and most likely in macrophages, in the lungs of infected chickens (Figure 7). Viral antigen was also detected in the spleen, kidneys, and occasionally in the liver (Figure 7). These findings are consistent with the results of the histological analysis.

4.4.3 Increased pathogenicity and severity of lesions are not sufficient for efficient transmission of the P22 H5N2 virus in chickens

We wanted to determine if the increase in virus replication and severity of lesions caused by the P22 H5N2 virus was associated with efficiency of transmission. We were also interested in exploring whether the age of the birds would influence the results. To address these issues we determined transmissibility in normal and IBDV-pre-exposed chickens inoculated with the P22 H5N2 virus at one and three weeks age.

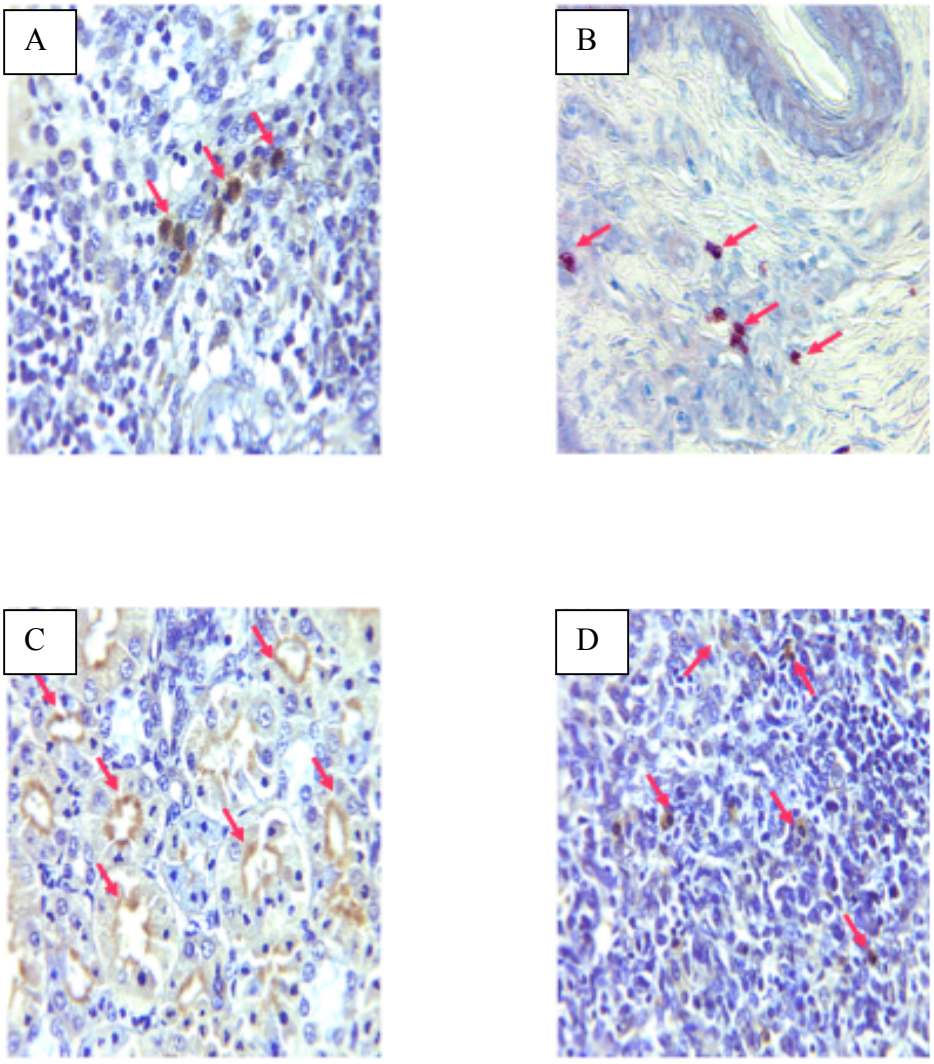

Figure 7. Detection of AI-viral antigen-positive cells. H5N2 P22 AIV antigen positive reaction is evident in the lung (Panel A), upper respiratory tract (Panel B), kidney (Panel C) and spleen (Panel D) of chickens 3 dpi with the P22 LPAI virus. Images show immunohistochemical detection using anti-avian influenza nucleoprotein (NP) monoclonal antibody as primary antibody, immunoperoxidase labeled secondary antibody, hematoxylin counterstained.

Virus was isolated from tracheal swabs up to 7 dpi in the groups of one- and three-week-old IBDV-pre-exposed chicken. It is important to note that, in the three-week-old IBDV-pre-exposed infected chickens, two out of three animals showed respiratory symptoms and died at 6 and 7 dpi; the experiment was repeated once with similar results. At necropsy, macroscopic findings included severe airsacculitis and pneumonic lesions; despite these findings, virus was not detected in tracheal or cloacal swabs from the contact animals (Table 4).

When the same approach was carried out in normal chickens, virus was isolated from tracheal and cloacal swabs in 3-week-old infected chickens up to 3 dpi. The animals appeared clinically normal and virus was isolated from tracheal swabs in one out of three contact animals at 3 dpi. In 2-day-old normal chickens the virus was isolated from tracheal and cloacal swabs up to 7 dpi in infected chickens. In this experiment two out of three chickens died at 5 and 6 dpi and the virus was isolated from tracheal swabs in one out of three contact animals at 5 dpi. When 1-week-old normal chickens were infected with the same virus, the virus was isolated from tracheal and cloacal swabs up to 3 dpi only in infected chickens and there was no transmission to contact birds (Table 5).

4.4.4 Amino acid substitutions occurred in the surface glycoproteins of the LPAIV H5N2 during passage of the virus in IBDV-pre-exposed chickens

Due to an increase in pathogenicity observed in our study and knowing that surface glycoproteins play a role in the pathogenicity of avian influenza viruses (Neumann and Kawaoka, 2006), we wanted to determine the molecular properties of

Table 4. Replication – Transmission studies of P22 chicken-adapted H5N2 AI virus in IBDV pre-exposed chickens

		No. positive chickens /total No. of chickens					
		3 dpi		5 dpi		7 dpi	
Age	Group	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
1 week	Infected	3/3	3/3	3/3	0/3	1/3	0/3
	Contact	0/3	0/3	0/3	0/3	0/3	0/3
3 weeks	Infected∗	3/3	3/3	2/3	2/3	0/1	1/1
	Contact	0/3	0/3	0/3	0/3	0/3	0/3
3 weeks[1]	Infected∗	3/3	0/3	3/3	0/3	0/1	0/1
	Contact	0/3	0/3	0/3	0/3	0/3	0/3

[1]No cloacal route of inoculation

∗In the 3-week group one animal died at 6 dpi; a second animal died at 7 dpi.

Table 5. Replication – Transmission studies of P22 chicken-adapted H5N2 AI virus in normal chickens.

		No. positive chickens /total No. of chickens					
		3 dpi		5 dpi		7 dpi	
Age	Group	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
2 days	Infected*	3/3	2/3	2/2	1/2	2/2	1/2
	Contact	0/3	0/3	**1/3**	0/3	0/3	0/3
1 week	Infected	3/3	3/3	0/3	0/3	0/3	0/3
	Contact	0/3	0/3	0/3	0/3	0/3	0/3
3 weeks	Infected	3/3	3/3	0/3	0/3	0/3	0/3
	Contact	**1/3**	0/3	0/3	0/3	0/3	0/3

*One animal was sacrificed due to severe respiratory distress

the glycoproteins HA and NA that allowed the mallard H5N2 virus to become better adapted to chickens. The H5 and H7 subtypes of AI have the potential to mutate into highly virulent strains by different mechanisms. One mechanism involves mutations in the amino acid sequence of the surface glycoproteins; e.g., a single amino acid mutation in HA of the H5N2 virus was identified in virus responsible for an epidemic in 1993 in the USA (Silvano et al., 1997). Others have shown that point mutations affecting glycosylation sites in HA have been associated with regulation of virulence (Deshpande et al., 1987). We looked for these types of modifications by comparing deduced amino acid sequences from the complete open reading frames of the WT and P22 virus genomes. Comparative sequence analysis revealed several amino acid substitutions, which occurred primarily in HA; only one substitution was identified in NA (Table 6). None of the changes occurred in the HA cleavage site and there were no apparent changes in the glycosylation sites. The HA gene of the P22 virus differed from that of the WT virus in HA1 region at positions 24, 77, 118, 158, 216, and 230 (H3 numbering). In the HA2 region the P22 virus differed from the WT virus at position 106, 139, and 183 (H3 numbering). Based on examination of GenBank sequences, all amino acid substitutions identified in our analysis have been identified in at least one other strain of influenza A viruses from birds or in the H5N1 influenza virus isolated from humans in Asia. The one exception is the N77D amino acid substitution, which has not been previously identified. The E24K mutation is found primarily in chicken virus; interestingly, this amino acid substitution is present in strains from Central America as well as in the chicken Pennsylvania LPAI virus that

Table 6. Amino acid substitutions in the genome of the mallard H5N2 AIV after passage in IBDV pre-exposed chickens

Gene	Position	WT	P22
HA1*	24	E	K
	77	D	N
	118	F	L
	158	N	**D**
	216	E	V
	230	M	I
HA2	106	R	I
	139	E	K
	183	T	I
NA	370	S	L
PB2	624	A	K
PB1	59	T	**I**
NP	105	V	M
	373	S	F
	377	N	S
	402	S	F
M	93	M	I
NS1	166	M	I
	183	G	S

*Amino acid position in the HA protein corresponds to H3 numbering

circulated in 1983. The F118L substitution is found in a H5 virus from Guatemala in 2003. The N158D mutation is present in avian viruses from North America and, more frequently, in viruses from Asia, as well as in H5N1 virus isolated from humans. The amino acid substitution E228V is found in chicken viruses from Texas in 2002 and 2004. The M230I mutation has been identified in virus from chicken and other birds. Regarding changes in the HA2 region, R106I is found in a turkey H5N2 virus and the T183I mutation is found in chicken, other birds, and several H5N1 viruses from Asia. Interestingly, the E139K mutation is found only in a H5N1 virus isolated from humans.

A single amino acid substitution was observed in the NA protein at position 370 (S370L). This position is in a region that determines specific activity (Kobasa et al., 2001); previous studies have shown that individual amino acid mutations at residue 370 lead to increased NA activity (Kobasa et al., 2001). This amino acid is highly conserved among avian influenza viruses (Ser370). Interestingly, a Ser370Leu substitution was found in the NA protein of the P22 H5N2 virus and a Leu is present in this position in the human virus A/Ann Arbor/6/60. Position 370, as well as positions 367 and 400, is also important for NA hemadsorption activity. This is a characteristic of all NA subtypes in avian viruses but has been lost in human virus NAs (Kobasa et al., 1997).

Comparison of the complete genome sequences of the H5N2 WT and P22 adapted viruses showed amino acid substitutions in internal genes (Table 6). We found two amino acid substitutions in the M1 protein at positions 93 and 121. The change in amino acid 121 (T121A) is located in a position that is considered specific for either avian or human influenza strains (Buckler-White et al., 1986). This is one of three sites that possibly define an avian or human class of M1 protein, which are clustered within the center of the molecule at residues 115, 121, and 137 (Buckler-White et al., 1986).

There were no significant amino acid changes in the polymerase genes; the PA gene was unchanged; there was a single amino acid substitution in position 624 in the PB2 gene and one in position 59 of the PB1 gene.

Two amino acid substitutions were identified in the NS1 protein; one was in position 166 and the other in position 183. The NP protein showed amino acid substitutions in positions 105, 373, 377, and 402.

The implications of these kinds of changes for the adaptation and/or pathogenicity of influenza viruses will require further study.

4.5 Discussion

It has been shown that there is a relationship between the virulence of avian influenza strains and the pathogenicity of the viruses for various organs (Hooper et

al., 1995). It has also been suggested that the pattern of disease will vary with the virulence of the avian influenza virus and the resistance of the host (Hooper et al., 1995). Comparing the replication efficiency of the H5N2 WT and P22 LPAI viruses in normal and IBDV-pre-exposed chickens we found that, in contrast to previous studies showing a general predilection of nonpathogenic or low-pathogenic duck viruses to replicate in kidney and digestive tract tissues in chickens (Condobery and Slemons, 1992), the H5N2 LPAIV investigated in this study showed a predilection to replicate in the respiratory tract; this is consistent with data on influenza virus infection in mammals (Neumann and Kawaoka, 2006).

The results of these experiments indicate that, under the conditions of this study, IBD-pre-exposure favors adaptation and plays a role in the pathogenesis of AI in poultry. The replication efficiency, and the severity of gross and microscopic lesions, increased for the WT virus used in this study after passage of the H5N2 LPAIV in IBDV-pre-exposed chickens. According to virus titers found in tracheal swabs and tissues (primarily lung) following inoculation via natural routes, passage of the H5N2 LPAIV in IBDV-pre-exposed chickens apparently favors replication in normal as well as in IBDV-pre-exposed birds and, based on the number and severity of macroscopic and microscopic lesions, increases pathogenicity of the AIV in IBD-pre-exposed chickens. These properties are indicative of a virus that has been selected for during the adaptation process. As a result, the virus is a better fit than the WT virus and is able to take better advantage of its host, leading to increased pathogenicity.

Evidence of viral antigen in inflammatory cells is significant because these cells are involved in the host response to clear the infection but they can also contribute to aggravating the clinical outcome of the lung infection. This situation could be occurring in IBDV-pre-exposed chickens infected with the P22 H5N2 virus, where we observed a more severe inflammatory response in addition to the respiratory signs.

We found differences in viral replication between groups, with all infected animals shedding virus in trachea and/or cloaca. There was a remarkable difference in clinical signs after P22 infection in three-week-old IBDV-pre-exposed chickens compared to normal chickens; two out of three birds died after infection with the P22 AI virus and there was no transmission to contact birds in this group. Interestingly, the three-week-old normal chickens did not show clinical signs, while younger birds did and transmission of AIV to contact birds occurred only sporadically in the normal group. These results confirm that factors other than pathogenicity and level of virus shedding are involved in transmission of influenza virus in chickens. It will be interesting to determine the role of age in the susceptibility to AI infection in chickens.

Passage of the mallard H5N2 AI virus in IBDV-pre-exposed chickens resulted in changes at the molecular level, particularly in the surface glycoproteins and most notably in the glycoprotein HA. The number of changes and their distribution

between the HA1 and HA2 regions agrees with the proportion of changes occurring during evolution of influenza virus in nature, where change usually occurs at a three-fold higher rate in the HA1 region than in HA2. This difference could be the result of different immune pressures (Nakajima et al., 2003).

Our results suggest that amino acid substitutions such as those identified in this study might contribute to favoring replication of the H5N2 virus in chickens. Changes such as the one at position 158, located near the receptor binding site, have been implicated in changes in receptor-binding affinity as a result of differences in oligosaccharide interactions, which could potentially have an effect on the host or cells within the host that are prone to infection by the virus (Ha et al., 2002).

Arginine, a basic amino acid, was replaced in the WT virus by the non-polar aliphatic amino acid, isoleucine, in the P22 virus at position 106 of the HA2 region. Whether this change will have an effect on ionization during cleavage of the precursor HA0 at low pH is not known (Ha et al., 2002). A solvent-restricted site occurs in the structure of the H5 HA near the fusion peptide and the HA2 position 106 amino acid is located in this area (Ha et al., 2002). Furthermore, the amino acid located at position 106 is one of the residues implicated in the low-pH-induced refolding of HA that is required for membrane fusion.

The NA stalk region, where a deletion has been identified in some H5N1 viruses, particularly those from land-based birds and from most humans (Mase et al.,

2005) remained intact in the P22 chicken adapted AI virus. However, a single amino acid substitution in the NA glycoprotein occurred in a region that is highly conserved among avian influenza viruses, acquiring an amino acid that is present in a human influenza virus. The potential contribution of this change to the generation of the new virus phenotype needs to be determined.

Although it is generally accepted that changes in the glycoprotein HA contribute preferentially to increased virulence, it is clear that the virulence of influenza virus is polygenic and that other, non-genetic factors are involved. Additional studies will be necessary to determine the contribution of the changes in amino acid residues to the virulence of AI virus in chickens and to establish their role in the adaptation and/or pathogenesis of H5 virus in land-based birds.

Understanding the molecular changes that occur during adaptation of influenza A virus to a new host will provide important insight into the requirements for productive viral replication and cross-species transmission. The results presented here show that pre-existing conditions, such as pre-exposure to IBDV, contribute to the mechanism of adaptation and generation of new, more highly pathogenic viruses in the field.

Chapter 5: **Exacerbation of clinical signs and altered host responses to low pathogenic H5N2 avian influenza virus in chickens previously exposed to infectious bursal disease virus**

5.1Abstract

We investigated the effects of viral-induced immunosuppresion in chickens and their susceptibility, disease progression and host molecular responses against infection with a H5N2 low pathogenic influenza virus (LPAI). Infectious bursal disease virus (IBDV) is a powerful immunosuppressive pathogen that preferentially infects and destroys B-cells. Prior exposure to IBDV lead to increased susceptibility to infections with a mallard H5N2 LPAI virus compared to normal chickens, although the infection did not cause overt signs of disease in either group. The increased susceptibility to the mallard H5N2 LPAI virus allowed us to further adapt the virus to chickens. After 22 passages (P22) in IBDV-pre-exposed chickens, a LPAI virus was obtained that replicated substantially better than the WT mallard virus in both IBDV-pre-exposed and normal chickens. Interestingly, the P22 virus showed similar levels of replication in the respiratory and intestinal tracts of both groups although it caused exacerbated signs of disease and excess mortality in the IBDV-pre-exposed group. Thus, we investigated if the expanded tissue tropism or altered local gene expression were associated with the different disease outcome. We suggest that prior IBDV exposure provides a port of entry for avian influenza in an otherwise resistant chicken population. Furthermore, adaptation of AI in IBDV-pre-exposed chickens may allow for the selection of AI virus strains with expanded tissue tropism, particularly in the

brain. We also demonstrate that IBDV-pre-exposed chickens show less than optimal humoral responses to LPAI infection and altered local molecular pathways that eventually lead to an exacerbated disease and death. These findings are particularly important when considering vaccination campaigns against IBDV and AI.

5.2 Introduction

Influenza A virus infections have been responsible for important disease outbreaks in birds, livestock, and wildlife (Perdue and Swayne, 2005). Introduction of avian influenza virus (AI) into poultry species is usually associated with outbreaks of varying intensities (Kwon et al., 2008). Most influenza virus infections are sub-clinical in poultry, producing only mild to moderate signs of disease. These AI viruses have been categorized as low pathogenic (LPAI) or mildly pathogenic (MPAI). AI strains that cause severe systemic disease, associated with high morbidity and mortality, are known as highly pathogenic AI viruses (HPAI).

The factors that determine virulence and interspecies transmission of influenza viruses are still poorly understood. The likelihood of a virus becoming endemic in a new host (recipient species) depends on two major factors: at the macro level, the interaction between donor and recipient animal species, and at the molecular level, the intricate interactions between the host and virus components (Kuiken et al., 2006). A wealth of knowledge exists with respect to the molecular markers of virulence of influenza viruses; thus, virulence is considered a polygenic property (Rott et al., 1979; Scholtissek et al., 1977a; Scholtissek et al., 1977b). The

accumulation of basic amino acids at the cleavage site of the HA protein is a hallmark of the generation of AI with high pathogenic potential. Only AI viruses of the H5 and H7 subtypes have shown the potential to become highly pathogenic (Ito et al., 2001; Rott et al., 1976; van der Goot et al., 2003). There is also evidence that the internal components of the virus including the RNA polymerase complex (PB2, PB1, PA), nucleoprotein (NP), matrix protein (M1, M2), and nonstructural proteins (NS1, NS2/NEP) also contribute to the host range, although little is known with respect to the role of these components for interspecies transmission of AI viruses among birds (Wasilenko et al., 2008).

Much less is known regarding the host's immune status as a potential facilitator in the cross-species transmission and virulence of AI (Swayne, 1997). Despite substantial differences among birds and mammals, effective elimination of AI viruses require a functional immune system. The nature of the host response to AI in birds is largely unknown. Any compromising conditions such as immunosuppression could enhance disease susceptibility to AI. In this regard, infectious bursal disease virus (IBDV) constitutes a major economic threat to the poultry industry because it adversely affects the immune status of poultry. IBDV is a RNA virus that causes severe immunosuppression. The immunosuppression is mostly at the humoral level due to the virus's preferential site of infection, the B cells in the Bursa of *Fabricius* (Kim et al., 1999; Saif, 1991; Withers et al., 2005). More importantly, IBDV live attenuated vaccines are used throughout the world, many of them with poorly characterized long-term effects on the bird's immune competence.

In this study, we investigated the effects of prior IBDV exposure on disease susceptibility or pathogenesis to infection with a LPAI H5N2 strain. Our results indicate that IBDV-induced immunosuppression exacerbates AI-induced pathogenicity and a prior IBDV exposure leads to a defective humoral immune response against LPAI virus in chickens. Microarray gene expression analysis revealed that IBDV-pre-exposed chickens show higher expression of genes associated with local inflammatory responses and cell death following AI infection compared to normal chickens.

5.3 Materials and Methods

5.3.1 Viruses

The A/Mallard/Pennsylvania/12180/84 (H5N2) virus was adapted by serial lung passages (22 times, herein referred as P22) in chickens previously exposed to IBDV. The P22 H5N2 chicken-adapted influenza virus was propagated in 10-day old embryonated chicken eggs and titrated by egg infectious dose 50 (EID_{50}) using the Reed and Muench method (Reed, 1938). The IBDV E-Delaware variant virus was used to induce immunosuppression in chickens. Specific-pathogen-free (SPF) White Leghorn chickens (3-week old, Charles River Laboratories) were infected with IBDV and bursas were collected 48 hours post-infection (hpi) and homogenized to prepare virus stocks. The chicken infectious dose 50 (CID_{50}) of the IBDV in bursa homogenates was determined.

5.3.2 Animals and experimental infections

Two-day old SPF chickens were infected with 100 CID_{50} of the E-Del IBDV strain by the oral and ocular routes to induce immunosuppression. Two to 4 weeks later, normal and IBDV-pre-exposed chickens were separated into groups of 3 and infected with either 5 x 10^6 EID_{50} or 1 x 10^8 EID_{50} of the P22 H5N2 LPAI virus as indicated. Chickens were infected via the oculo-nasal, intra-tracheal and intra-cloacal routes with 1 ml of AI virus *inoculum*. Mock-infected chickens were used as controls. Tracheal and cloacal swabs were collected at 1 and 3 days post-infection (dpi) in buffered glycerol (1:1 glycerol, phosphate buffered saline with Antibiotic - Antimycotic solution (100X), Sigma-Aldrich, St. Louis, MO) and stored at -70° C until use. Birds were euthanized and necropsied at 3 dpi. Homogenates from lungs were prepared in brain heart infusion medium (BHI), and stored at -70° C until use. To determine the chicken infectious dose 50 (CID_{50}) of the WT and P22 virus, groups of 3 SPF White Leghorn chickens (3-week old) were infected with serial ten fold dilutions of the virus and lungs were collected at 72 hpi and homogenized to determine presence of virus. Swabs samples and tissue homogenates were titrated for virus by the Reed and Muench method (Reed, 1938). Undiluted samples that were positive for hemagglutination but did not show hemagglutination at the 10^{-1} dilution in EID_{50} assays were scored as positive with the notation of "<1.0 EID_{50}/ml." The birds were observed and scored daily for clinical signs of disease. Experiments were carried out under BSL2+ conditions with chickens housed in isolators under negative air pressure. Investigators wore appropriate protective equipment. All experiments were compliant with animal protocols approved by the Institutional Animal Care and

Use Committee of the University of Maryland, College Park and under the Animal Welfare Act. Throughout the experimental period, feed and water were provided *ad libitum*.

5.3.3 Antibody determination

Serum samples collected from chickens after P22 H5N2 infection were tested for the presence of H5 AI and IBDV antibodies by ELISA tests. Commercially available, USDA licensed antibody test kits for the detection of antibodies to AI virus (Avian Influenza Virus Antibody Test Kit, ProFLOKPlus®, Synbiotics Co., San Diego, CA) and infectious bursal disease virus (Infectious Bursal Disease Virus Antibody Test Kit, ProFLOKPlus®, Synbiotics Co., San Diego, CA) were used following the manufacturer's recommendations. In addition, hemagglutination inhibition (HI) tests (following WHO recommendations) were performed using serum samples collected at 14 dpi to detect the presence of antibodies against H5 AI. Before HI test, serum samples were pretreated with receptor-destroying enzyme as recommended by the World Health Organization (WHO).

5.3.4 Microarray analysis

Gene expression analysis was carried out as described (Kim, 2008). Total RNA was isolated from tissues collected at 3 dpi using TRIzol (Invitrogen®, Carlsbad, CA), treated with Turbo DNase (Ambion®, Austin, TX), and then purified using RNeasy Mini RNA Purification Kit (Qiagen®, Valencia, CA) according to the

manufacturer's protocols. The concentration and purity were determined using a spectrophotometer. RNA quality was examined by 28S and 18S RNA band visualization following gel electrophoresis and ethidium bromide staining. Total RNA (3 μg) was used to generate Alexa Fluor 555 or Alexa Fluor 647 (Invitrogen) labeled aRNA probes. Amplified amino allyl RNA synthesis, dye coupling, and labeled aRNA purification were performed using the Amino Allyl Message Amp II aRNA Amplification Kit according to the manufacturer's instruction (Ambion, Austin, TX). Labeled aRNA (15 μg) from each infected group or uninfected control was used for hybridization to compare the uninfected vs. the infected by a reference design (i.e. control vs. WT, control vs. P22, control vs. IBDV, control vs. IBDV-WT, and control vs. IBDV-P22). The 10K element AVIELA microarray was constructed as previously described (Kim, 2008). All hybridizations were performed twice to make a technical replicate and control RNA from uninfected chicken was used as reference RNA (Dobbin et al., 2003). Hybridization was performed as following; washing with 1% SDS in 3X SSC (1X SSC is .015 M NaCl plus 0.015 M sodium citrate) at 40~50°C for 5 minutes with moderate agitation, washing 3 times with distilled water at 40~50°C for 5 minutes with mild agitation, boiling at 100°C for 3 min and then chilled in ice-cold 100% ethanol, and centrifuging at 1,000 rpm for 2 min to dry in capped 50 ml centrifuge tubes. For two-color hybridization, the aRNAs were incubated at 60°C for 10 min with HybIt hybridization buffer (Telechem International, Sunnyvale, CA) and aRNAs were applied to pre-warmed microarray slides and incubated at 50°C in a Hybridization Cassette Plus (Telechem) overnight. Slides were sequentially washed for 15 min in 0.5X SSC plus 0.01% SDS, 15 min in

0.06X SSC plus 0.01% SDS, 0.06X SSC, and 0.01X SSC buffers with moderate stirring using a High-Throughput Wash Station (Telechem), followed by centrifugation at 1,000 rpm for 2 min to dry slides.

Microarray images were acquired by laser confocal scanning using a ScanArray Lite microarray analysis system (Perkin-Elmer, Boston, MA) at a resolution of 10 μm. A 16-bit TIFF image was generated for each channel (Alexa Fluor 555 and Alexa Fluor 647). The scanned microarray images for each channel were overlaid and quantified to determine the fluorescent intensities of the two dyes for each spot by using the ScanArray Express analysis software version 3.0 (Perkin-Elmer). Spots were detected using an adaptive circle algorithm in QuantArray program and all spots were visually examined (Szretter et al., 2007).

The MIDAS 2.19 software of TM4 microarray analysis package (http://www.tigr.org) was used to produce qualified and normalized array data. Briefly, each median spot intensity (Alexa Fluor 555 or Alexa Fluor 647) was determined by subtracting the median local background from median signal intensity values, and then flag information was applied to filter bad spots. One bad channel tolerance policy was stringent and signal to noise threshold was 2. Two-step normalization was performed (Sioson et al., 2006); total intensity and block LOWESS (locally-weighted regression and smoothing scatter plots) method, followed by standard deviation (SD) regularization of block and slide SDs. The qualified and normalized array data were imported into the GeneSpring GX 7.3

software (Silicon Genetics, Redwood, CA) for statistical and fold change analyses. To generate Ch2/Ch1 intensity ratios, signal channel values (infected group, Ch2) were divided by control channel values (control group, Ch1). The elements that were modulated >2 fold change with statistical significance ($p < 0.05$) were filtered using Volcano plot method (Jin et al., 2001).

5.3.5 Bioinformatic analysis

All data files including DNA sequences and Gene Ontology (GO) data were obtained from NCBI (http://www.ncbi.nlm.nih.gov). Avian IEL cDNA sequences on the AVIELA chip were identified against reference RNA and protein sequences (formatted database for Blast, Dec. 2007) using the stand-alone Blast program (version 2.2.13). The acceptance criteria of Blast results were alignment length ≥ 100 nucleotides and e-value ≤ $1e^{-100}$ for DNA and alignment length ≥ 30 amino acids and e-value ≤ $1e^{-10}$ for protein. Entrez gene and Homologene data (Dec. 2007) from NCBI were used to gather gene information (identification number, symbol, name). GO annotations were extracted from GO data of Entrez gene in NCBI. Statistical and narrow-down analyses of GO mappings were performed using a stand-alone EASE (Expression Analysis Systematic Explorer) program and the criteria for acceptance was ≥ 2 genes with FDR < 0.1 at level 4 of biological processes of GO (Hosack et al., 2003). Python (version 2.2.4) and SQL scripts were used to process and manipulate Blast results and data processing, respectively (Hosack et al., 2003).

5.3.6 Real Time PCR validation

Total RNA was prepared as described above and cDNA synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche®) according to the manufacturer's instructions. Primers for real-time analysis were designed based on Genbank sequences using Oligo Perfect Primer software (Invitrogen®) (Table 7). Real-time PCR was performed using SYBR Green I Master (Roche®) and was normalized to the GAPDH housekeeping gene. The results were run on a 480 light cycler RT PCR machine (Roche®). Data analysis was carried out using relative quantification software, and relative fold differences were determined using the ΔΔCt method.

5.4 Results

5.4.1 Chicken-adapted H5N2 P22 influenza virus replicates at similar levels in the respiratory tract of normal and IBDV-pre-exposed chickens

Previous studies in our laboratory showed that prior exposure of chickens to IBDV early in their life (2-day old) increases their susceptibility to AI (Chapter 3). This increased susceptibility to AI virus allowed us to adapt an H5N2 influenza virus isolated from a mallard, A/mallard/Pennsylvania/84 (H5N2) virus, in IBDV-pre-exposed chickens. After 22 serial lung passages in IBDV-pre-exposed chickens, we obtained a mutant H5N2 virus (P22) which replicated better in IBDV-pre-exposed and normal chickens than the WT H5N2 mallard virus (Fig 8). The WT mallard H5N2 virus caused no signs of disease in IBDV-exposed chickens and further *in vivo*,

Table 7. Primers used in Relative Quantitative Real Time RT-PCR

Gene symbol	Gen Bank Accession No.	Forward	Reverse
CCL4	NM_001030360	5'-GTTCATCACCAGGAAGGGC-3'	5'-TGGCAGGGCCTGCAAGTGG-3'
CD3d	NM_205512	5'-CCTGCAATGTCAAGAAAGCA-3'	5'-GCCTCTGGGATCATCGTAAA-3'
B_2M	NM_001001750	5'-GCAGGTGTACTCCCGCTTCC-3'	5'-CCCACTTGTAGACCTGCGG-3'
IFI35	XM_418132	5'-GCTGCAAAAGGACAAGGAAG-3'	5'-TGGAGGGACACCTTTTCATC-3'
NK-lysin	NM_001044680	5'-GATGGTTCAGCTGCGTGGGATGC-3'	5'-CTGCCGGAGCTTCTTCAACA-3'
TAP	XM_425302	5'-CAGGGAAGAGCACTCTGGTG-3'	5'-GCACAGGTAGGAGTGCTGGT-3'
CREB3	XM_424990	5'-GCTGGGTCCTGCTCTTACTG-3'	5'-CATGCACAGCTTGGAAAGAA-3'

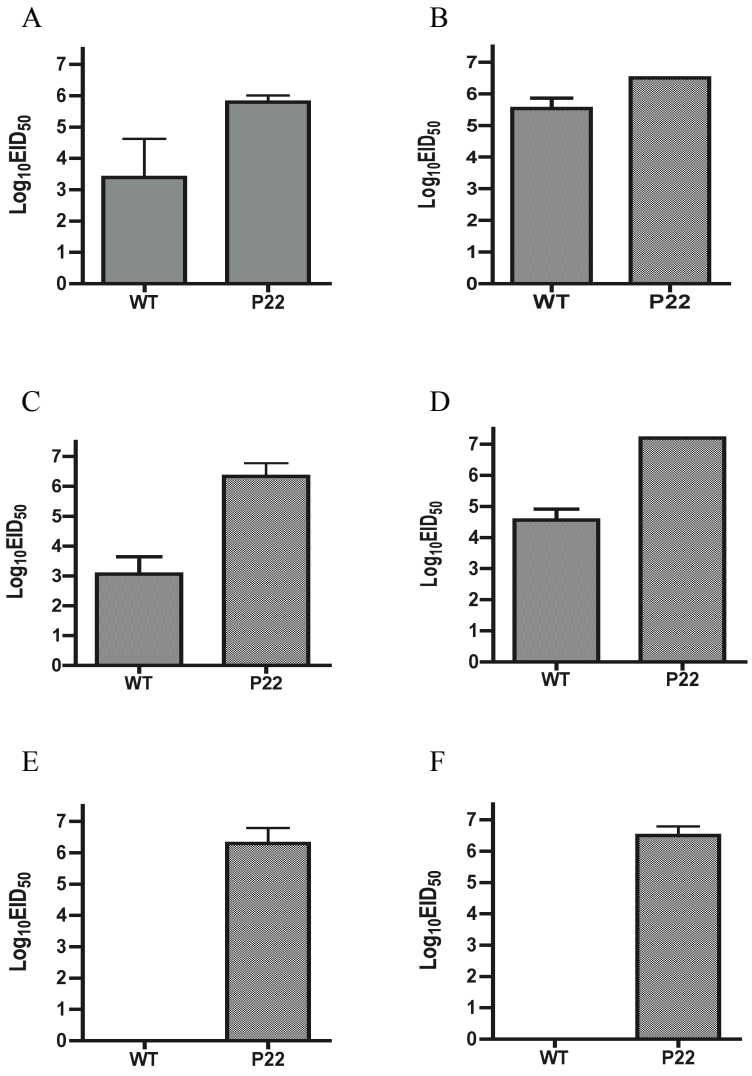

Figure 8. Replication of H5N2 AI virus in the respiratory tract of chickens. Normal and IBDV-pre-exposed chickens (3/group) were inoculated with H5N2 virus P22 and WT. Lung homogenates and tracheal swabs collected at 3 dpi were inoculated into embryonated chicken eggs and titrated by EID_{50}. Each bar shows the average titer per group. A, B, C and D show results after inoculation with $1X10^8$ EID_{50} of the WT or the P22 virus. A) and B) show the results of titers in trachea and lung in IBDV-pre-exposed chickens, respectively. C) and D) show the results of titers in trachea and lung in normal non-exposed chickens. E) and F) show results of virus shedding in trachea and virus replication in lung of normal chickens after inoculation with $5X10^6$ EID_{50} of the WT and P22 virus.

in vitro, and molecular analysis revealed that the P22 virus was, like its parental strain, a LPAI virus (Chapter 4). In order to better understand the effects of IBDV-pre-exposure on the susceptibility to the P22 LPAI virus, we compared the P22 and WT H5N2 virus infections in normal and IBDV-pre-exposed chickens infected with 1 x 10^8 EID_{50} of virus *inoculum*. Initial analysis using tracheal swabs and lung homogenates prepared at 3 dpi showed that the P22 virus replicates better than the WT mallard H5N2 virus in normal and IBDV-pre-exposed chickens (Fig 8); however, no statistical differences were observed in the amount of P22 virus shedding between the normal and IBDV-pre-exposed chickens. In contrast, the WT mallard H5N2 virus showed slightly better replication in IBDV-pre-exposed than in normal chickens (Fig 8). The improved adaptation of the P22 virus for chickens was better appreciated when a lower dose of virus was used: Normal chickens infected with 5 x 10^6 EID_{50} of the WT virus showed no virus shedding at 3 dpi from either the trachea or the lungs (Fig 8 E and F). In contrast, the same dose of P22 virus yielded an average of approximately 6 $\log_{10}$ EID_{50} of virus, a ~1 million fold improvement compared to the WT virus (Fig 8 E and F). The results are also consistent when CID_{50} were calculated for both viruses, indicating that adaptation of a LPAI virus in IBDV-pre-exposed chickens can indeed select for a strain better adapted for immunocompetent chickens (Table 8). These results suggest that IBDV-induced immunosuppression of birds provides an environment for the adaptation of mallard AI viruses; as a result these adapted viruses have the potential to infect immunocompetent poultry.

Table 8. CID_{50} for WT and P22 H5N2 AI viruses

Virus	Log_{10} CID_{50}/ml	Log_{10} EID_{50}/ml/ Log_{10} CID_{50}/ml
WT	2.5	6.5
P22	6.0	3.2

5.4.2 Previous IBDV exposure and the opportunity for LPAI brain tissue tropism.

We examined whether the P22 virus show altered tissue tropism in IBDV-exposed chickens compared to normal chickens. At 3 dpi, we determined virus titers in the respiratory and intestinal tracts, and in the brain (Fig 9). Several important observations were made after examining the tissue tropism of the P22 H5N2 virus: 1) Previous IBDV exposure resulted in both the P22 and WT viruses to be consistently isolated from the brain of infected chickens. To our knowledge, this is a novel feature of LPAI infections in chickens, and suggests that previous IBDV exposure may provide an opportunity for LPAI viruses to change their phenotype, tissue tropism or become better adapted to poultry, 2) Viremia was also occasionally observed in IBDV-pre-exposed chickens. Viremia was observed in 8 out of 33 chickens during the adaptation of the H5N2 LPAI virus in IBDV-pre-exposed chickens. This finding suggests that previous IBDV exposure allows the virus to reach organs that would normally not be affected in a LPAI infection, and 3) The P22 virus was consistently isolated from the respiratory and intestinal tracts and the brain in normal chickens. This is an indication that P22 LPAI extended its tissue tropism in an immune competent host. Our studies also suggest that the P22 virus replicated better in IBDV-pre-exposed and normal chickens than the WT H5N2 virus. More importantly, except for the presence of viremia in the IBDV-P22 group, the amount of P22 virus produced in the lungs and virus shedding in trachea was similar between the IBDV-pre-exposed and normal groups.

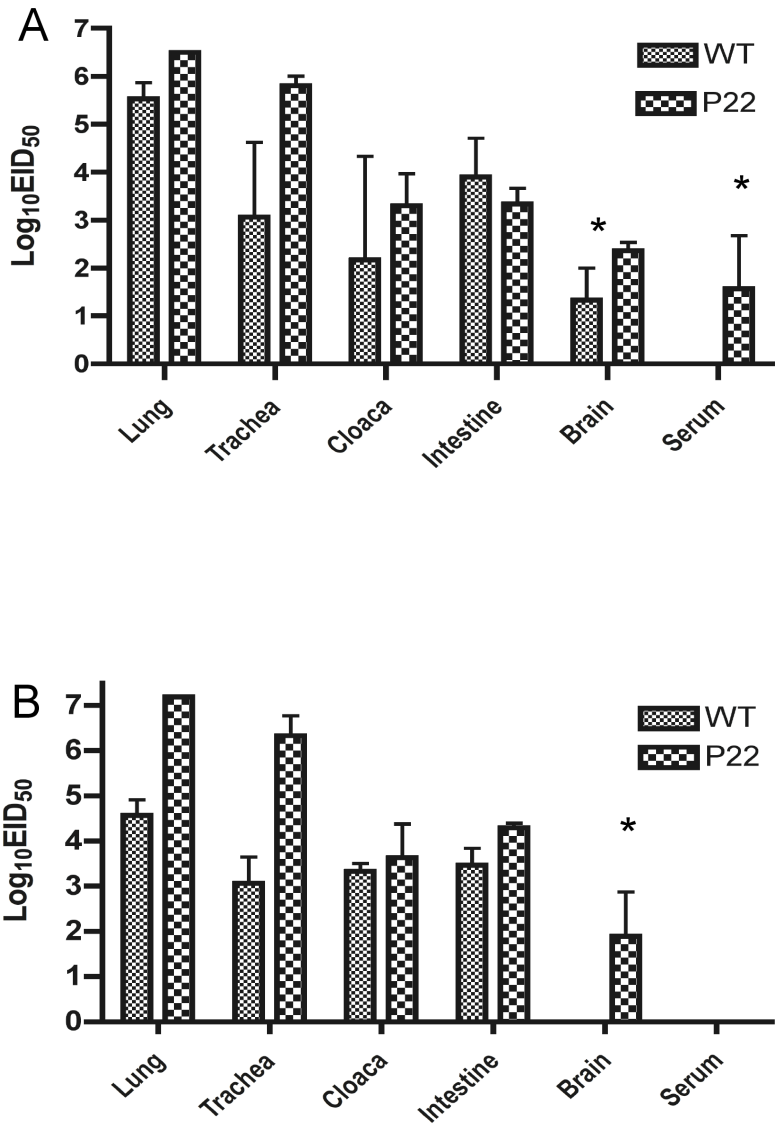

Figure 9. Replication of H5N2 AI virus in 3-week-old SPF chickens**.** Samples collected from groups of birds (3/group) at 3 days after inoculation of the P22 or WT viruses. Titration performed in inoculated embryonated chicken eggs by EID_{50}. Each bar shows the average titer for each group of 3 chickens. Figure A shows results in IBDV-pre-exposed chickens. Figure B shows results in normal chickens. *(Represents average of 2 positive chickens)

5.4.3 Previous IBDV exposure leads to a defective humoral immune response against LPAI

As expected from our initial studies, a constant finding was atrophy of the bursa of *Fabricius* in IBDV-exposed chickens (Chapter 3). Thus it was not surprising that these chickens would have a defective humoral immune response. In order to test the early humoral response to LPAI, IBDV-pre-exposed and normal chickens were challenged at 3 weeks of age (3 weeks post IBDV infection) with the P22 virus. At 0, 3, 5, and 7 dpi, blood samples were collected for IBDV and AI antibody titers. ELISA tests showed an antibody response to IBDV in the IBDV-infected group but not in the normal group (Table 9). In contrast, only the normal chicken group, not the IBDV-exposed group, showed positive antibody response against AI virus by ELISA and HI assays. Chickens of 1 and 3 weeks of age either naïve or previously infected with IBDV were challenged with the P22 virus and serum samples collected at 14 dpi (Table 10). Naïve animals seroconverted after P22 H5N2 LPAI virus infection, showing higher levels of antibodies than the chickens that were previously exposed to IBDV. These results suggest that infection with LPAI in chickens previously exposed to IBDV may result in a delayed or impaired humoral response. This observation has profound implications when considering vaccination to control the spread of AI and suggests that a poor humoral response in immunocompromised hosts could contribute to a failure to overcome AI infection and promote the spread of the disease.

Table 9. AIV-specific serum antibody titers in normal and IBDV-pre-exposed chickens

Chicken ID	Antibodies against IBDV			Antibodies against AIV					
	3 dpi	5 dpi	7 dpi	3 dpi		5 dpi		7 dpi	
	Elisa	Elisa	Elisa	HI	Elisa	HI	Elisa	HI	Elisa
1 N+P22	-			-	-				
2 N+P22	-			-	-				
3 N+P22	-			-	-				
4 N+P22	-	-		-	-	40	208		
5 N+P22	-	-		-	-	40	218		
6 N+P22	-	-		-	-	-	-		
7 N+P22	-	-	-	-	-	40	301	40	2083
8 N+P22	-	-	-	-	-	-	-	40	703
9 N+P22	-	-	-	-	-			40	1237
1 IBD+P22	+			-	-				
2 IBD+P22	+			-	-				
3 IBD+P22	+			-	-	-	-		
4 IBD+P22	+	+		-	-	-	-		
5 IBD+P22	+	+		-	-	-	-		-
6 IBD+P22	+	+		-	-	-	-	-	-
7 IBD+P22	+	+	+	-	-	-	-	-	-
8 IBD+P22	+	+	+	-	-	-	-	-	-
9 IBD+P22	+	+	+	-	-	-	-	-	-

Three-week-old chickens were inoculated with 10^8 EID_{50} of P22 H5N2 LPAI virus. Two sets of 9 chickens, one group previously exposed to IBDV at 2 days of age and a second group non-IBDV infected, were divided into groups of 3. Three chickens from each set were sacrificed at 3 dpi, 5 dpi and 7 dpi. Tissue samples and blood were collected from each individual. ELISA tests (Synbiotics®) were conducted for each sample. In addition to ELISA test HI test was carried out also for AI.

Table 10. Humoral immune response to P22 H5N2 AIV in normal and IBDV-pre-exposed chickens 14 dpi

IBDV + P22 AIV			**Ab titer 14 dpi**	
Age	**ID**	**Comments**	**HI**	**ELISA**
3 wks	I1	Normal	40	2407
3 wks	I2	Dead 6 dpi	NA	NA
3 wks	I3	Dead 7 dpi	NA	NA
3 wks	I4	Dead 6 dpi	NA	NA
3 wks	I5	Respiratory distress 6-7 dpi. Recovered 8 dpi.	20	1567
3 wks	I6	Dead 6 dpi	NA	NA
1 wk	I7	Severe respiratory distress. Sacrificed 7 dpi	NA	NA
1 wk	I8	Normal	<10	0
1 wk	I9	Normal	20	659
P22 AIV				
3 wks	I10	Normal	160	4111
3 wks	I11	Normal	40	4946
3 wks	I12	Normal	40	3501
1 wk	I13	Normal	80	1671
1 wk	I14	Normal	20	681
1 wk	I15	Normal	20	2705

5.4.4 Previous IBDV exposure leads to exacerbated signs of disease after infection with LPAI.

Surprisingly, the levels of P22 virus replication in the two groups were similar despite our initial observation showing exacerbated signs of disease in the IBDV-pre-exposed group. IBDV-pre-exposed chickens were lethargic and depressed upon infection with the P22 virus. Four out of 6 IBDV-pre-exposed chickens inoculated with the P22 virus (results of two independent studies) showed labored breathing, and died between 6 and 7 dpi (Table 10). When examining macroscopically various organs of infected birds, lesions with different degrees of severity were observed. In the respiratory tract, lesions compatible with pneumonia were evident, although these lesions were considerably more severe in IBDV-pre-exposed chickens. A constant finding was severe air sacculitis and occasional presentation of petechial hemorraghes in the thymus only in IBDV-pre-exposed-P22 infected chickens. Histological lesions found in lungs after P22 AI virus infection were also more severe in IBDV-pre-exposed birds compared to naïve chickens (Chapter 4). These observations indicate that host factors or the host's immune status might play a role in the severity of lesions found in the respiratory tract of IBDV-pre-exposed-P22 infected chickens.

5.4.5 Altered host responses as correlates of disease outcome to LPAI P22 virus infection

In order to better understand the effect of IBDV-mediated immunosuppression on LPAI infection, global gene expression analysis was performed using an avian cDNA microarray on lung tissues from normal and IBDV-pre-exposed chickens.

Although our gene expression study included detailed comparative analysis of host responses between normal and IBDV-pre-exposed chickens infected with the WT H5N2 and the P22 virus, only results with the P22 virus are shown in this chapter. IBDV-pre-exposed or normal chickens were infected with the P22 virus at 3 weeks of age. At 3 dpi, RNA samples from lungs were prepared as previously described. Following AI infection, the avian intestinal IEL cDNA microarray (AVIELA) elements that were up- or down-regulated more than 2-fold ($p<0.05$) in each infection group compared to non-infected chickens were analyzed using the Volcano plot. Following P22 virus infection in normal chickens, we identified 384 transcripts that were significantly changed in the lungs ($p<0.05$) (201 increased, 183 decreased). In IBDV-pre-exposed chickens, infection with P22 virus resulted in significant changes in 382 transcripts in the lungs (236 increased, 146 decreased). To identify elements, which are commonly altered in both P22 and IBDV-P22, infection groups, Veen Diagram analysis were applied. There were 148 up-regulated elements and 107 down-regulated elements that were shared between the P22 and IBDV-P22 groups (Figure 10).

The majority of commonly induced genes were related to the innate immune response including interleukins and IL receptors (IL-16, IL-18, IL2RG, IL-7R, IL-12, and IL-8). These genes were induced in both P22 and IBDV-P22 groups (Appendix I). Commonly down-regulated genes in both infection groups were related to various other cellular functions (Appendix II). To identify genes, which are differentially modulated, the array data of the IBDV-P22 group was compared to those of the P22

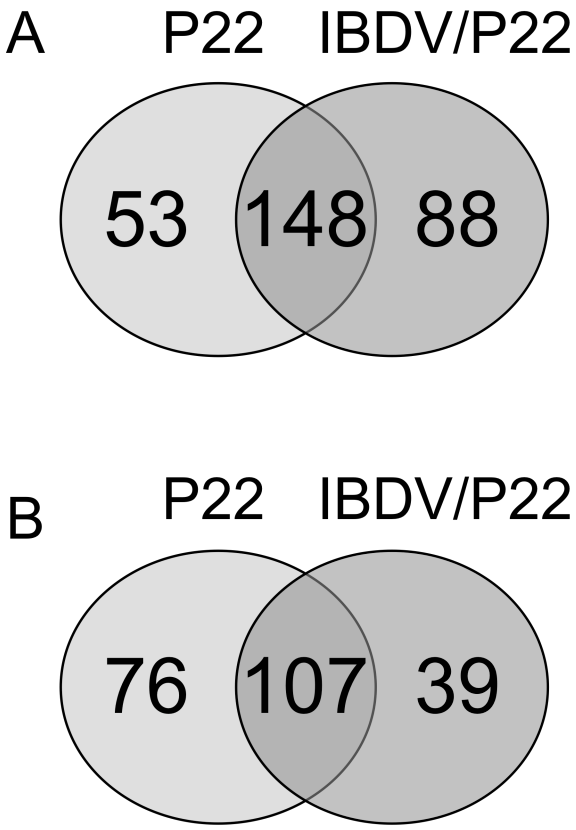

Figure 10. Host elements commonly and differentially regulated in chicken lungs following P22 and IBD-P22 virus infections. Veen Diagram analysis, A) 148 elements were commonly up-regulated and B) 107 elements were commonly down-regulated in the lungs of P22 and IBDV-pre-exposed-P22 AI virus groups.

group using the Volcano plot method. The genes that were altered more than 1.5-fold ($p<0.1$ with false discovery rate) were selected. Out of 35 genes, 14 immune genes were selected due to their differential expression in the IBDV-P22 infection group (Table 11). Real time RT-PCR was used to validate 7 of these genes (Fig 11). Our analysis revealed that the pro-inflammatory cytokine CCL4 (MIP-1β) was induced in both groups; however, it was induced 1.6- fold higher in the IBDV-P22 group (Table 11 and Fig 11). Since CCL4 release is involved in the recruitment of different leucocyte populations to the site of inflammation acting preferentially on mononuclear cells (Kaufmann et al., 2001; Kobasa et al., 2004; Wareing et al., 2004), this may explain the higher amount of cell infiltration of inflammatory cells and severe lesions observed in the lungs of chickens in the IBDV-P22 group. It has been previously shown that a strong inflammatory response is associated with the pathogenicity of influenza viruses in mammals (Cheung et al., 2002; Szretter et al., 2007).

In our study we also found up regulation of the B_2M and the TAP genes, with a relatively higher increase in the IBDV-P22 group than in the P22 group (Table 11 and Fig 11). These genes are related to the processing and presentation of antigen peptides in the context of the Major Histocompatibility Complex (MHC) Class I molecules. An efficient presentation of antigenic peptides to T lymphocytes in chickens depends in part on their transport to the cell surface. An effective and functional MHC class I/B_2M peptide complex in equilibrium with the transported

Table 11. Differentially expressed genes (>1.5 fold with p<0.1 FDR) in IBDV-pre-exposed-P22 compared with P22-infected chicken lungs

Gene symbol and description	GenBank Accession ID	Function	Fold change		Ratio of IBDV-P22/ P22[a]
			P22	IBDV-P22	
CD3D (CD3d molecule, delta)	NM_205512	T cell development	1.5~1.8	2.7~3.2	1.5~2.1[b]
LOC693257 (NK-lysin)	NM_001044680	Immune response	1.56	3	1.92
B2M (Beta-2-microglobulin)	NM_001001750	MHC I antigen processing	1.5	2.6	1.73
TAP (Transporter associated with antigen processing)	XM_425302	MHC I antigen processing	1.45	2.5	1.72
CREB3 (cAMP responsive elements binding protein 3)	XM_424990	Virus reactivation	1.2	2	1.66
TNFSF10 (TRAIL) (TNF (ligand) superfamily member 10)	NM_204379	Apoptosis	0.35	0.58	1.65
CCL4 (CC chemokine ligand 4) (MIP1β)	NM_001030360	Inflammation	1.8	2.9	1.61
ITGAV (Integrin, alpha V)	NM_205439	Inflammation	0.57	0.92	1.61
RAC2 (ras-related C3 botulinum toxin substrate 2)	XM_001237031	IFN alpha induction	1.25	2	1.6
IFI35 (Interferon-induced protein 35)	XM_418132	IFN signaling	0.6	0.95	1.58
GSN (Gelsolin)	NM_204934	Inflammation	0.9	0.6	0.66
IGJ (Immunoglobulin J polypeptide, linker protein)	NM_204263	IgG & IgA assembly and secretion	4	2.65	0.66
BLNK (B-cell linker)	NM_204908	B cell development	3.2	2	0.62
MCL1 (Myeloid cell leukemia sequence 1)	XM_001233734	Apoptosis	3.4	1.8	0.53

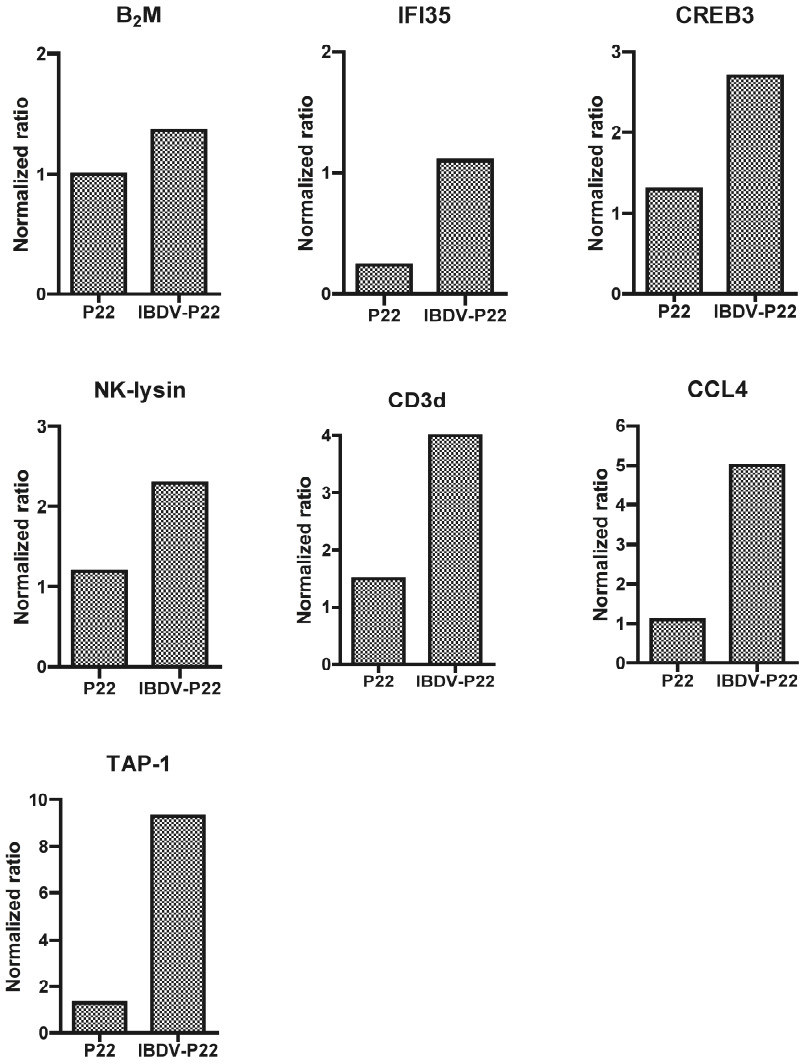

Figure 11. Relative Quantitative Real Time RT-PCR**.** Calibrator Normalized Relative Quantification (software-based) by Real Time RT-PCR of selected gene products differentially expressed in the lung of P22 H5N2 infected chickens. Total RNA was prepared from lungs of P22 H5N2 normal and IBDV-pre-exposed chickens 3 dpi. Fold changes were calculated based on the difference in ct values for a particular gene in uninfected (target calibrator) and infected (target unknown) lung, which were normalized against GAPDH (Glyceraldehide-3-phosphate-dehydrogenase) levels (reference). Results are expressed as the target/reference ratio of each sample divided by the target/reference ratio of a calibrator. The figure shows normalized ratio of fold changes in expression levels for each of these genes.

associated with antigen processing (TAP) is needed in order to present efficiently peptides derived from the pathogen by the MHC I molecules to the T cells (Kaufman, 2000; MacDonald, 2007; Riegert et al., 1996). Our results suggest that an increased level of expression of these genes in the IBDV-P22 group is perhaps a direct consequence of inefficient or altered antigen processing and/or presentation, which results in exacerbation of the clinical and pathological signs observed in this group.

We also observed a higher induction of NK-lysin in the IBDV-P22 group (Table 11 and Fig 11) that could be associated to an increased cytotoxic T cell response. It has been shown that NK-lysin is expressed predominantly by CD8+ T and NK lymphocytes (Hong et al., 2006; Ruysschaert et al., 1998). CD8+ cytotoxic T lymphocytes play a major role in the defense against viral infections (Stenger et al., 1998). In particular, populations of cytotoxic T cells have been detected in the lung of mice infected with influenza (Ennis et al., 1978; Mozdzanowska et al., 2000). Although the exact role for NK-lysin has not been determined in influenza virus associated pneumonia, the enhanced level of NK-lysin in the lung may reflect the increased infiltration of inflammatory and increased infiltration of inflammatory and T-cell activity seen during acute inflammation and pneumonia.

IFN pathway genes were differentially regulated in the two groups, with higher induction in the IBDV-P22 group. These genes included the IFN-induced 35 kDa protein and CD3d (Table 11 and Fig 11), which have been previously shown to be differentially expressed in studies with a H9 influenza virus (Degen et al., 2006). Chicken CD3d

protein shares homology with mammalian CD3γ and CD3δ (Hu et al., 2007). CD3d is essential for T cell development and along with the other members of the TCR-CD3 complex contributes to T-lymphocyte activation after the initial recognition steps. It can be speculated that a defective humoral response in birds leads to an exacerbation in the release of these cytokines to compensate for important missing signals or responses. This latter argument is in agreement with the up-regulation of genes related to the B cell development in the P22 group and a defective response (or down-regulation) in the IBDV-P22 group. Genes involved in IgG and IgA assembly and secretion, including BLNK and IgJ (Immunoglobulin J polypeptide, linker protein), were up-regulated in the P22 group; however, they were comparatively down-regulated in the IBDV-P22 group (Table 11). These observations could explain the poor antibody response seen against the P22 virus in the IBDV-exposed chickens. As mentioned above, such effect carries important implications when considering vaccination against LPAI as overall herd immunity is crucial to properly prevent AI infection and spread. Chickens that have been previously exposed to IBDV may not respond to the vaccine therefore herd immunity may be less than optimal and/or unsatisfactory. It is also important in the context of IBDV vaccination efforts as some of these vaccines have been shown to affect bursa morphology and cause immunosuppression (Rautenschlein et al., 2007; Rosenberger, 1989; Saif, 1991; Withers et al., 2005).

5.5 Discussion

The emergence of AI viruses with increased pathogenicity that are capable of infecting a wide range of land-based birds emphasizes the need to better elucidate the molecular mechanisms that modulate AI infections in bird species. Virulence is the result of a complex interaction between the host and the pathogen and plays a major role in host adaptation. In order to better understand the fundamental mechanisms involved in host response to AI virus, we used a LPAI virus, which showed enhanced virulence upon serial lung passages in a IBDV-pre-exposed host. In our original hypothesis, we speculated that immunocompromised chickens might provide an environment where a LPAI H5N2 virus could change to a HPAI virus. After 22 lung passages (P22) in the exposed host, we obtained a virus with increased virulence but it was not a HPAI virus. Sequencing and *in vivo* studies revealed that the P22 virus was more virulent than the WT in birds but still maintained all the features of a LPAI virus. Our results provide some insights on the role that the status of host immunity plays in viral pathogenicity, but the current findings also suggest that the host-pathogen interactions during AI infection are very complex.

IBDV is an economically important disease that affects poultry worldwide and causes severe immunosupression, weakening particularly humoral responses (Rautenschlein et al., 2007; Rautenschlein et al., 2003). Because IBDV infection primarily impairs the bursa suppressing host humoral antibody response, this experimental system was used to evaluate the effect of immunosuppression on influenza infection with a chicken-adapted LPAI H5N2 virus. The results of our study suggest that IBDV-pre-exposure favors the replication of the P22 H5N2 virus

and enhances its virulence. Virus titers of P22 H5N2 found in tracheal swabs and tissues (mainly in lung) (Figure 8) were elevated compared to the WT virus. Isolation of AI virus from organs other than the respiratory and gastrointestinal tracts is considered a rare event when animals are infected with LPAI. Nevertheless, in this study we showed that the P22 H5N2 virus acquired increased tissue tropism, caused severe lesions in lungs and was able to produce severe clinical respiratory symptoms in IBDV-pre-exposed chickens. The potential impact of this occurring in the field could be indeed devastating considering the possibility of AI infection in a large number of exposed, susceptible birds in which multiple "passages" can occur in a very short period of time. This type of scenario could easily contribute to expanding the host range of LPAI viruses and the generation of a new AI strain with novel phenotypes.

Despite many clinical and pathological reports of AI infections, our understanding of disease mechanisms and the nature of host factors influencing the viral virulence and/or lung pathogenicity are still unknown. Our study was focused on identifying the nature of immune-related factors that are involved in increased viral pathogenicity in P22 virus-infected chickens. Using newly developed tissue-specific AVIELA microarray, this study provides a new opportunity to assess mucosal responses to AI virus since this array contains 10,000 EST, which were derived from the chicken gut lymphocytes. Because the complete genome sequences of chickens have been deciphered (Consortium, 2004; Wallis et al., 2004), high-throughput gene expression analysis provides new avenues to investigate global expression of genes controlling local immune response to complex pathogens like AI virus. The results of our study indicate the

complex interactions between the host and the virus that are evident when analyzing respiratory tract lesions and gene expression profiles.

Recent studies in the mammalian systems clearly indicate important roles that cytokines and chemokines play in host innate response to AI and the resulting immunopathology following infection with HPAI viruses. Experimental evidence indicates strong association between increased cytokine production and enhanced immunopathology following infection with influenza viruses (Cheung et al., 2002). It has been previously shown that pro-inflammatory cytokines such as CCL4/MIP-1β are up-regulated after *in vitro* infection with influenza A virus (La Gruta et al., 2007). CCL4/MIP-1β expression in particular has been induced after influenza virus infection of human monocytes, macrophages, or bronchiolar epithelial cells *in vitro* (Wareign, 2004). Our study shows that P22 virus induces up-regulation of CCL4/MIP-1β *in vivo* and that the level of this cytokine is further up-regulated in IBDV-P22 infected chickens. The up-regulation of pro-inflammatory cytokines such as CCL4/MIP-1β could indeed be related to the increased pathogenicity in lungs observed in this study. CCL4/MIP-1β is also involved in inducing the recruitment of different leukocyte populations to the site of inflammation and acts preferentially on mononuclear cells (Kaufmann et al., 2001; Kobasa et al., 2004; Wareing et al., 2004). In this regard, our studies are consistent with this notion since a large amount of inflammatory cells was observed in the lung of P22 H5N2 AI virus-infected chickens. The up-regulation of CCL4/MIP-1β may also explain the higher infiltration observed in lungs infected with the P22 H5N2 virus in the IBDV-pre-exposed group than in the normal chickens. Higher infiltration will in turn result in exacerbated local inflammatory responses resulting in more

severe disease, as it has been associated with HPAI H5N1 infections (Cheung et al., 2002; La Gruta et al., 2007).

Although we concentrated in this report only on differentially expressed genes between the P22 and IBDV-P22 infected chickens, we found that many immune related genes including interleukins and IL receptors (IL-18, IL-16, IL-12, IL-2RG, IL-7R) were commonly up-regulated after P22 infection in both normal and IBDV-infected chickens. In particular, increased induction of transcription of IL-16 and IL-18 has been associated to avian influenza exposure (Keeler et al., 2007). This may be how the pro-inflammatory role of cytokines differentially expressed after P22 AI virus infection could be related with increased severity of lesions and respiratory signs. Even though pro-inflammatory cytokines will favor the development of antiviral immunity, an exacerbated response may lead to harmful tissue damage (Julkunen et al., 2000).

In contrast to a large amount of gene expression studies done in mammalian models, the availability of global gene-expression profiles associated with LPAI viruses is limited in birds. Taken together, our findings demonstrate different types of immune responses induced by the WT and P22 viruses. Viral titers in the respiratory tract did not correlate with pathological lesions and clinical respiratory signs. However, our studies showed that the host's immune status clearly played a role in determining the outcome of humoral response and clinical outcome of AI virus infection in chickens following infection with WT and P22 influenza viruses. We must note that we have used a very complex system with an outbred chicken population and a viral immunosuppressive agent to induce selective immunosuppression of humoral immune response

in chickens. However, exposure to a LPAI strain in normal and IBDV-pre-exposed chickens resulted in differential and consistent different responses that we could easily observe at the macro and molecular levels. Further *in vivo* studies to examine the role of differentially up-regulated genes in viral pathogenicity will facilitate the development of logical control strategy against avian influenza infection.

Chapter 6: Conclusions and future prospects

6.1 Conclusions

Influenza A viruses exhibit varying degrees of host adaptation, which affects infectivity and transmissibility. In order to study the role of host factors involved in these processes, we studied the effect of IBDV-pre-exposure, a condition very likely to happen in nature, on the susceptibility and pathogenesis of a mallard H5N2 LPAI virus in chickens.

Normal chickens infected with 5 x 10^6 EID_{50} of the WT virus showed no virus shedding from either the trachea or the cloaca or replication in the lungs at 3 dpi. In contrast, the same dose of P22 virus produced an average of approximately 6 $\log_{10}$ EID_{50} of virus, a ~1 million fold improvement compared to the WT virus. The results are also consistent when CID_{50} were calculated for both viruses, indicating that serial passages in IBDV-pre-exposed chickens of a mallard LPAI virus can indeed select for a strain better adapted to chicken.

This study supports the role of host immune status in the adaptation of avian influenza viruses. The adaptation did not favor transmission, however the virus gained increased pathogenicity. Isolation of virus from serum samples and other organs apart from the respiratory and digestive tract are remarkable for a LPAI virus. Macroscopic and microscopic findings demonstrated increased severity in lesions caused by the H5N2 P22 adapted virus in both normal and IBDV-pre-exposed chickens when compared to the WT virus. Thus our results suggest that

the adaptation of a mallard virus in IBDV-pre-exposed chickens could potentially lead to the emergence of influenza strains that can cause clinical disease in chickens and infect other species with increased replication efficiency.

Complete sequence analysis indicated amino acid changes occurring mainly in the surface glycoprotein HA. However changes were also found in PB2, PB1, NP, NA, NS and M. Thus multiple different amino acid mutations seem to be associated with virulence in chickens.

Global gene expression analysis indicated that important immune related genes were differentially regulated in lung after P22 H5N2 virus infection. It also showed that in agreement with clinical signs, macroscopic and microscopic findings, the P22 virus behaves as a respiratory pathogen in chickens. Apparently, there was no evident difference in gene regulation when samples from intestinal tissue of infected chicken were evaluated. This demonstrates that different host-pathogen interactions mediate avian influenza pathogenesis and disease outcome.

Poultry production is rapidly increasing worldwide, although this increase is not necessarily accompanied by improvements in biosecurity or quality of poultry farming and this situation becomes critical, particularly in developing countries. In this scenario the potential impact of a situation like this occurring in the field is highlighted. A large population of susceptible birds could potentially support a large number of passages in a very short period of time, generating new AI strains with different phenotypes that could contribute to expand the host range of LPAI viruses with unpredictable consequences.

6.2 Future prospects and perspectives

The following studies are needed:

1) Use reverse genetics system for influenza virus to map specific amino acid residues involved in the molecular changes and disease production after 22 passages of the AI virus in IBDV-pre-exposed chickens.

2) Identify specific cell populations participating in the inflammatory response in the respiratory tract of chickens and determine its role in the pathogenicity of AIV. Clarify mechanisms involved in the virulence of avian influenza virus particularly of the H5 subtype is important due to its potential to generate HPAI virus and its threat for human health.

Our studies highlight the complexities of influenza infections as they occur in the field: IBDV infections remain a worldwide problem in commercial poultry. Vaccination strategies against IBDV should be reconsidered as a result of the potential down side effects for increase susceptibility to other pathogens. Vaccination strategies against influenza should consider the overall health status of the flock and previous exposure or vaccination against other pathogens particularly immunossupressive agents.

Based on host molecular responses found in this study and having the ability to detect differences in the chemokine and cytokine response to AIV in chickens, we are now able to compare and contrast at the transcriptional level the immune responses to different subtypes of

AI in chickens and other avian species. Further *in vivo* studies to examine the role of differentially up-regulated genes in viral pathogenicity will facilitate the development of logical control strategies against avian influenza infection.

Appendix I. The AVIELA genes commonly up-regulated in chicken lung following P22 and IBDV-P22 infections

Clone ID	Fold changes		Gene symbol	GenBank Accession ID	Gene description
	P22	IBD-P22			
18Jul01_J02_1GAL85_033	11.63	6.803	AVD	CD729634	mature avidin
18Jul01_O16_1GAL85_074	7.655	7.877	LOC430511	CD729703	similar to MGC80493 protein
18Jul01_C18_1GAL85_092	6.613	7.433	LOC427453	CD729705	similar to RIKEN cDNA 5133401N09
16May01_D24_1GAL55_094	6.52	6.036	LOC420559	CD738021	similar to KIAA2005 protein
14Feb01_D20_1GAL25_026	5.642	3.92	CD83	CD740590	CD83 molecule
20Mar01_N12_1GAL32_099	5.279	4.554	c-ets-1	CD733068	c-ets-1 oncogene
14Feb01_B24_1GAL25_083	4.802	3.467	SURF1	CD740580	surfeit 1
28Jan01_D08_1GAL2_026	4.706	4.619	LOC421921	CD735098	similar to LOC129607 protein
24Dec00_F16_1GAL22_056	4.528	4.765	LOC768447	CD740058	similar to immunoglobulin-like receptor CHIR-B4
2Feb01_O14_1GAL23_031	4.515	2.262	LOC396429	CD740333	min alpha-trop
20Mar01_A12_1GAL32_084	4.434	3.672	LOC417472	CD733118	similar to Myotubularin related protein 4
14Feb01_K09_1GAL25_046	4.013	4.606	IGM	CD740539	Mu immunoglobulin heavy chain C region
22Mar01_D17_1GAL50_077	3.989	2.65	IGJ	CD733638	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides
22Mar01_I21_1GAL50_040	3.95	3.333	SLA	CD733715	Src-like-adaptor
19Mar01_B14_1GAL45_021	3.923	3.352	LCP1	CD737041	lymphocyte cytosolic protein 1 (L-plastin)
21Aug01_D04_1GAL69_060	3.865	3.211	LOC418295	CD729503	Complement component 1, r subcomponent
20Jul01_I11_1GAL57_072	3.801	3.496	LOC424357	CD738207	Glutaredoxin 2
19Jan01_L18_1GAL6_094	3.793	5.188	PCMTD1	CF074910	protein-L-isoaspartate(D-aspartate)O-ethyltransferase domain containing 1

27Jan01_G14_1GAL1_027	3.763	2.967	LAPTM4A	CD734951	lysosomal-associated protein transmembrane 4 alpha
20Apr01_O20_1GAL31_042	3.725	3.066	IL18	CD732584	interleukin 18
14Feb01_H06_1GAL25_093	3.689	3.366	LOC416431	CD740536	similar to ubiquitin specific protease 42
2Feb01_O12_1GAL23_095	3.653	2.469	LOC422903	CD740332	Hypothetical LOC422903
14Feb01_C02_1GAL25_025	3.51	2.501	PFKP	CD740581	phosphofructokinase, platelet
20Jul01_K18_1GAL57_096	3.485	3.196	ITGA4	CD738169	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
20Jul01_I15_1GAL57_040	3.482	2.89	CD8A	CD738220	CD8a molecule
21Aug01_B12_1GAL69_084	3.474	2.305	SCYL2	CD729537	SCY1-like 2 (S. cerevisiae)
27Jul01_L09_1GAL56_058	3.406	2.729	RCJMB04_18k24	CD734860	similar to Arfaptin 1 (ADP-ribosylation factor interacting protein 1)
7Mar01_O24_1GAL51_102	3.303	4.117	P20K	CD734452	quiescence-specific protein
3Jul01_H15_1GAL91_048	3.246	2.824	RBMS1	CD738530	RNA binding motif, single stranded interacting protein 1
18Aug01_P16_1GAL96_074	3.243	3.986	LOC421921	CD739412	similar to LOC129607 protein
21Aug01_B11_1GAL69_068	3.234	2.455	VPS4B	CD729535	vacuolar protein sorting 4 homolog B (S. cerevisiae)
7Jan01_J01_1GAL10_006	3.206	2.681	APOA1	CD730554	apolipoprotein A-I
28Jan01_C17_1GAL2_073	3.204	3.52	LOC416706	CD735088	similar to mitochondrial ribosomal protein S5
20Apr01_O21_1GAL31_058	3.183	2.826	M6PR	CD732585	mannose-6-phosphate receptor (cation dependent)
27Jul01_B11_1GAL56_069	3.176	2.008	BLNK	CD734780	B-cell linker
4Feb01_C01_1GAL28_009	3.111	2.868	IGLV	CD726893	Ig light chain variable region
2Feb01_F03_1GAL23_036	3.064	2.963	RASSF5	CD740323	Ras association (RalGDS/AF-6) domain family 5
13Jul01_K21_1GAL89_048	3.064	2.613	STK17B	CD730147	serine/threonine kinase 17b
pat.pk0062.b10.f	3.04	2.326	LOC770612	XM_001233949	hypothetical protein LOC770612
19Mar01_B12_1GAL45_085	3.023	2.667	CEP192	CD737039	centrosomal protein 192kDa
pmp1c.pk006.o10	3.002	3.458	NOS2A	NM_204961	nitric oxide synthase 2A (NOS2A)

19Mar01_C24_1GAL45_092	2.989	2.656	CCNG1	CD737084	cyclin G1
27Jan01_P13_1GAL1_016	2.986	3.449	COPG	CD734909	coatomer protein complex, subunit gamma
19Mar01_D02_1GAL45_029	2.955	2.637	GLUD1	CD737087	glutamate dehydrogenase 1
11Dec00_L20_1GAL18_041	2.892	3.05	BTG1	CD733882	B-cell translocation gene 1, anti-proliferative
21Jul01_M06_1GAL68_098	2.89	2.108	TRIO	CD729389	triple functional domain (PTPRF interacting)
27Apr01_E13_1GAL52_006	2.848	3.332	RCJMB04_22p10	CD734510	Sal-like 4 (Drosophila)
18Jul01_E02_1GAL85_022	2.821	2.343	CD300L-B1	CD729711	CD300 antigen-like family member
14Jun01_M24_1GAL42_099	2.798	2.046	PIK3AP1	CD736671	phosphoinositide-3-kinase adaptor protein 1
18Jul01_F21_1GAL85_039	2.792	2.381	WDR3	CD729631	WD repeat domain 3
6Jul01_N11_1GAL84_084	2.784	3.259	ADAM28	CD729273	ADAM metallopeptidase domain 28
7May01_A05_1GAL38_069	2.776	2.843	PHGDHL1	CD731464	phosphoglycerate dehydrogenase like 1
3Jul01_A24_1GAL91_085	2.763	2.158	LOC770109	CD738676	similar to Cathepsin H
3Jul01_G05_1GAL91_079	2.761	3.016	ISG12-2	CD738578	putative ISG12-2 protein
30May01_H06_1GAL62_096	2.752	2.013	LOC421949	CD727489	similar to hypothetical protein DKFZp566A1524
11May01_K06_1GAL33_101	2.734	3.547	LDHA	CD733412	lactate dehydrogenase A
20Jul01_E22_1GAL57_054	2.734	2.457	RPL5	CD738178	ribosomal protein L5
22Dec00_K09_1GAL21_048	2.719	2.611	IL16	CD739959	interleukin 16 (lymphocyte chemoattractant factor)
3Jul01_G06_1GAL91_095	2.71	3.678	SSU72	CD738579	SSU72 RNA polymerase II CTD phosphatase homolog (S. cerevisiae)
19Jan01_P23_1GAL6_080	2.702	2.442	JMJD4	CD731949	jumonji domain containing 4
24Dec00_E04_1GAL22_055	2.695	3.057	MAN2C1	CD740160	mannosidase, alpha, class 2C, member 1
10Mar01_M20_1GAL46_034	2.635	3.365	LOC417534	CD737292	similar to chemokine ah221
pat.pk0050.a12.f	2.624	2.382	LOC420129	XM_418246.2	similar to interferon, gamma-inducible protein 30 (LOC420129)
14Feb01_K15_1GAL25_046	2.616	2.247	RBP2	CD740542	retinol binding protein 2, cellular

3Feb01_D09_1GAL27_042	2.606	2.271	ZYX	CD740609	zyxin
TGF-beta 4	2.588	3.894	LEFTY2	NM_204764	left-right determination factor 2
27Apr01_E15_1GAL52_038	2.581	2.855	LOC416564	CD734513	similar to Rho GTPase activating protein 17
29Jun01_C04_1GAL86_061	2.57	2.499	TBC1D24	CD731852	TBC1 domain family, member 24
29Jun01_F15_1GAL86_040	2.559	2.86	CD74	CD731815	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
31July01_K06_1GAL75_096	2.55	2.916	RAB8A	CD736310	RAB8A, member RAS oncogene family
28Jan01_K22_1GAL2_061	2.532	3.096	LOC425977	CD735007	similar to papillary renal cell carcinoma translocation-associated gene product
18Aug01_B16_1GAL96_052	2.509	2.276	CXCR4	CD739451	chemokine (C-X-C motif) receptor 4
11Dec00_A11_1GAL18_069	2.506	2.313	LOC419542	CD733765	similar to AT rich interactive domain 1A
22Mar01_L20_1GAL50_041	2.491	3.146	TAP1	CD733712	transporter associated with antigen processing 1
pat.pk0060.h1.f	2.487	2.102	LOC396216	NM_205279	mature cMGF (LOC396216)
pmp1c.pk005.g20	2.478	2.625	NCF1	NM_001030709	neutrophil cytosolic factor 1 (NCF1)
20Jul01_M23_1GAL57_082	2.469	3.421	LOC424360	CD738054	Regulator of G-protein signalling 1
20Jul01_F04_1GAL57_055	2.464	2.29	SLC43A2	CD738185	solute carrier family 43, member 2
11Dec00_E07_1GAL18_006	2.443	2.6	LOC416147	CD733803	similar to Zinc finger protein 147 (Tripartite motif protein 25) (Estrogen responsive finger protein) (Efp)
11May01_D05_1GAL33_078	2.44	2.961	LOC427623	CD733399	similar to beta-2 adrenergic receptor
25Mar01_L10_1GAL44_073	2.385	2.523	LRPPRC	CD736931	leucine-rich PPR-motif containing
6Jul01_H13_1GAL84_016	2.382	2.518	LOC768766	CD729262	similar to macrophage hemoglobin scavenger receptor CD163
2Feb01_D21_1GAL23_042	2.374	2.953	LOC424105	CD740291	similar to inducible T-cell co-stimulator
3Jul01_H17_1GAL91_080	2.365	3.114	LOC422551	CD738531	Ectonucleotide pyrophosphatase/phosphodiesterase 6
pat.pk0050.d12.f	2.341	2.671	IL2RG	NM_204527	interleukin 2 receptor, gamma (IL2RG)
8Dec00_K22_1GAL17_073	2.337	2.118	RCJMB04_1f23	CD733436	similar to cathepsin S preproprotein

3Jul01_F10_1GAL91_056	2.329	2.699	RHOQ	CD738550	ras homolog gene family, member Q
19May01_J14_1GAL60_034	2.311	2.6	IL7R	CD727187	interleukin 7 receptor
20Jan01_J21_1GAL8_038	2.29	2.148	LOC395976	CD732391	glutathione S-transferases CL2
3Feb01_B22_1GAL27_050	2.285	2.054	SLC43A2	CD740759	solute carrier family 43, member 2
21Jun01_B18_1GAL79_086	2.283	2.188	ZYX	CD736529	zyxin
7Mar01_C06_1GAL51_092	2.28	2.806	XPNPEP3	CD734315	X-prolyl aminopeptidase (aminopeptidase P) 3, putative
NK-lysin	2.274	2.992	LOC693257	NM_001044680	NK-lysin
22Dec00_D02_1GAL21_029	2.271	2.689	LOC426107	CD739891	similar to T cell receptor delta chain
29Jun01_D21_1GAL86_046	2.248	2.578	LOC415493	CD731758	Abhydrolase domain containing 2
3Jul01_F12_1GAL91_088	2.239	2.223	PTPN6	CD738553	protein tyrosine phosphatase, non-receptor type 6
20Apr01_O23_1GAL31_090	2.237	2.076	RAB14	CD732589	RAB14, member RAS oncogene family
22Jun01_E12_1GAL80_087	2.212	2.756	TGM4	CD737566	transglutaminase 4 (prostate)
70.d3.f	2.205	2.748	IL12	NM_213588	Interleukin 12
21Jun01_D08_1GAL79_030	2.205	2.004	MMP-13	CD736576	matrix metalloproteinase-13
26Jun01_J17_1GAL83_082	2.203	2.278	LOC415442	CD729085	similar to normal mucosa of esophagus specific 1
3Feb01_A23_1GAL27_065	2.185	2.283	LOC420796	CD740739	similar to Nuclear transcription factor, X-box binding 1
29Jun01_L14_1GAL86_042	2.185	2.632	PCOLN3	CD731927	procollagen (type III) N-endopeptidase
Lymphotactin	2.177	2.354	LOC395914	NM_205046	Lymphotactin
7May01_K23_1GAL38_089	2.156	3.547	LOC424953	CD731407	similar to eukaryotic translation initiation factor 4 gamma, 1
29Jun01_D19_1GAL86_012	2.144	2.439	FIP1L1	CD731756	FIP1 like 1 (S. cerevisiae)
11Dec00_O06_1GAL18_103	2.141	2.477	HK2	CD733943	hexokinase 2
22Dec00_D17_1GAL21_078	2.115	2.104	IGLV	CD739927	Ig light chain variable region
4Feb01_M22_1GAL28_055	2.113	3.021	LOC419816	CD726858	Hypothetical LOC419816
7Mar01_O18_1GAL51_102	2.113	2.062	SLC2A2	CD734447	solute carrier family 2 (facilitated glucose transporter), member 2
IL-8	2.081	2.582	IL8	NM_205498	Interleukin 8

20e7-long2 (pMal)	2.061	2.212	IL2RG	NM_204527	interleukin 2 receptor, gamma (IL2RG)
2Aug01_K04_1GAL71_064	2.056	2.596	DDX47	CD731547	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47
6Jan01_C10_1GAL4_061	2.045	2.018	LOC415442	CD730183	similar to normal mucosa of esophagus specific 1
11Dec00_F23_1GAL18_072	2.04	2.7	CD44	CD733748	CD44 molecule
18Jul01_I16_1GAL85_056	2.04	2.388	RAC2	CF074851	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)
12c4	2.026	2.149	GM-CSF	NM_001007078	Granulocyte-macrophage colony stimulating factor
8Dec00_G05_1GAL17_079	2.021	2.156	COL4A3BP	CD733496	collagen, type IV, alpha 3 binding protein
22Dec00_E06_1GAL21_087	2.011	2.048	HARS	CD739957	histidyl-tRNA synthetase
pat.pk0062.c8.f	2.003	2.381	LOC770026	XM_001233342	Hypothetical protein LOC770026

Appendix II. The AVIELA genes commonly down-regulated in chicken lung following P22 and IBDV-P22 infections

Clone ID	Fold changes		Gene symbol	GenBank Accession ID	Gene description
	P22	IBD-P22			
29Jun01_M05_1GAL86_083	0.0963	0.108	LOC419791	CD731941	Hypothetical LOC419791
24Dec00_G08_1GAL22_030	0.107	0.129	LOC770561	CF075140	Archain 1
6Jun01_J01_1GAL70_018	0.111	0.12	LOC423942	CD735572	similar to DMBT1 4.7 kb transcript
11May01_N08_1GAL33_036	0.149	0.196	HBB	CD733413	hemoglobin, beta
24Dec00_I01_1GAL22_008	0.15	0.222	LOC421949	CD740182	similar to hypothetical protein DKFZp566A1524
22Mar01_M13_1GAL50_018	0.169	0.229	LOC420045	CD733580	similar to keratin 17n
27Apr01_F06_1GAL52_087	0.172	0.199	LOC427380	CD734564	similar to chromosome 9 open reading frame 97; PP4189
24Dec00_E21_1GAL22_038	0.193	0.222	LOC419498	CD740178	similar to guanylin precursor
24Dec00_C01_1GAL22_012	0.204	0.207	LOC419390	CD740249	similar to enhancer of split related protein-7
15Dec00_K16_1GAL16_073	0.217	0.253	B-G	CD733178	V-region-like B-G antigen
27Mar01_B08_1GAL12_021	0.224	0.241	TSPAN8	CD730930	tetraspanin 8
24Dec00_D08_1GAL22_029	0.225	0.235	ARF6	CF075142	ADP-ribosylation factor 6
1Jun01_C07_1GAL64_013	0.246	0.264	LOC415791	CD728040	similar to carbonic anhydrase VII
30Jun01_L19_1GAL87_026	0.247	0.26	ALDOB	CD729850	aldolase B, fructose-bisphosphate
18Feb01_P24_1GAL43_102	0.256	0.374	TMEM23	CD736793	transmembrane protein 23
22Mar01_O23_1GAL50_090	0.257	0.26	REV1	CD733717	REV1 homolog (S. cerevisiae)
25July01_J24_1GAL59_097	0.268	0.299	CDH17	CD738704	cadherin 17, LI cadherin (liver-intestine)
24Dec00_C04_1GAL22_061	0.268	0.312	DNAJC16	CD740250	DnaJ (Hsp40) homolog, subfamily C, member 16
15Dec00_H06_1GAL16_096	0.286	0.298	LOC768796	CD733233	Hydrolethalus syndrome 1
24Dec00_A12_1GAL22_085	0.293	0.333	LOC424862	CD740111	similar to prostaglandin transporter
22Mar01_O17_1GAL50_090	0.295	0.339	VTN	CD733706	vitronectin
22Mar01_K02_1GAL50_032	0.297	0.245	CDKN2A	CD733653	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)

pat.pk0038.h8.f	0.308	0.252	ITGA1	NM_205069	integrin, alpha 1 (ITGA1)
21Jun01_A17_1GAL79_069	0.325	0.441	PAN3	CD736592	PAN3 polyA specific ribonuclease subunit homolog (S. cerevisiae)
19Mar01_A19_1GAL45_003	0.335	0.362	LOC771974	CD737227	similar to Cytochrome P450 4A2 precursor (CYPIVA2) (Lauric acid omega-hydroxylase) (P450-LA-omega 2) (P450 K-5) (P-450 K-2)
15Jun01_K02_1GAL65_041	0.342	0.235	UGT1A10	CD728259	UDP glucuronosyltransferase 1 family, polypeptide A10
17July01_N16_1GAL93_067	0.343	0.347	ALDH2	CD739080	aldehyde dehydrogenase 2 family (mitochondrial)
5Jun01_N07_1GAL39_020	0.343	0.441	PHPT1	CD735765	phosphohistidine phosphatase 1
5Jun01_D12_1GAL39_094	0.349	0.425	GOLPH2	CD735739	golgi phosphoprotein 2
7May01_C12_1GAL38_093	0.351	0.421	DYRK1A	CD731318	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
31July01_N19_1GAL75_019	0.352	0.459	MYH9	CD736318	myosin, heavy chain 9, non-muscle
7Jun01_B16_1GAL37_054	0.357	0.486	ATAD1	CD735411	ATPase family, AAA domain containing 1
27Apr01_F07_1GAL52_007	0.366	0.333	LOC422284	CD734565	similar to Rho-GTPase-activating protein 6 (Rho-type GTPase-activating protein RhoGAPX-1)
2Aug01_P01_1GAL71_027	0.372	0.427	SLMAP	CD731592	sarcolemma associated protein
3Jun01_P15_1GAL63_060	0.373	0.455	EPAS1	CD727877	endothelial PAS domain protein 1
29Jun01_C20_1GAL86_029	0.373	0.274	PGDS	CD731744	prostaglandin-D synthase
10Jun01_M24_1GAL66_099	0.374	0.35	LECT2	CD728934	Leukocyte cell-derived chemotaxin 2
7May01_M02_1GAL38_035	0.381	0.342	GDPD4	CD731452	glycerophosphodiester phosphodiesterase domain containing 4
19May01_A08_1GAL60_021	0.386	0.482	LOC428569	CD727174	Solute carrier family 3 (cystine, dibasic and neutral amino acid transporters, activator of cystine, dibasic and neutral amino acid transport), member 1

20Mar01_K11_1GAL32_080	0.387	0.412	LOC428771	CD733155	similar to aminopeptidase A
27Mar01_D19_1GAL12_013	0.39	0.455	LOC419988	CD730915	ABI gene family, member 3
29May01_A04_1GAL53_053	0.395	0.301	ANXA13	CD734717	annexin A13
15Aug01_J04_1GAL88_056	0.396	0.334	LOC425272	CD729989	similar to This CDS feature is included to show the translation of the corresponding V_region. Presently translation qualifiers on V_region features are illegal
24Dec00_G07_1GAL22_014	0.398	0.418	LOC418569	CD740103	similar to ubiquitin specific protease 9, X-linked
24Dec00_E19_1GAL22_006	0.401	0.419	LOC422224	CD740173	similar to growth and transformation-dependent protein
25Mar01_J21_1GAL44_049	0.407	0.458	KPNA2	CD736903	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
20Mar01_G08_1GAL32_030	0.408	0.405	RCJMB04_17m23	CD733109	similar to hypothetical protein FLJ25467
20Mar01_M24_1GAL32_098	0.412	0.373	LGALS3	CD733054	lectin, galactoside-binding, soluble, 3
20Mar01_I13_1GAL32_008	0.412	0.418	RCJMB04_2n20	CD732993	Tumor protein p53 inducible nuclear protein 1
5Jun01_C16_1GAL39_061	0.414	0.407	LOC427838	CD735718	Hypothetical LOC427838
20Mar01_C10_1GAL32_060	0.416	0.488	MOV10	CD733087	Mov10, Moloney leukemia virus 10, homolog (mouse)
20Mar01_L13_1GAL32_025	0.423	0.491	SUCLG2	CD733001	succinate-CoA ligase, GDP-forming, beta subunit
29Jun01_G03_1GAL86_045	0.426	0.436	STK25	CD731848	serine/threonine kinase 25 (STE20 homolog, yeast)
4Jul01_F15_1GAL92_040	0.429	0.369	LOC424751	CD738857	similar to myosin VIIA
22Dec00_P08_1GAL21_043	0.431	0.407	ULK1	CD740037	unc-51-like kinase 1 (C. elegans)
26Jun01_N24_1GAL83_100	0.432	0.403	SLC25A6	CD729154	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
pat.pk0032.g5.f	0.437	0.443	IRAK2	NM_001030605	interleukin-1 receptor-associated kinase

					2 (IRAK2)
17July01_O12_1GAL93_102	0.443	0.39	ADHFE1	CD739016	alcohol dehydrogenase, iron containing, 1
20Mar01_O11_1GAL32_090	0.445	0.414	TNFRSF21	CD732997	tumor necrosis factor receptor superfamily, member 21
7Mar01_N11_1GAL51_083	0.446	0.481	CEP170	CD734397	centrosomal protein 170kDa
29May01_D15_1GAL53_046	0.447	0.418	LOC429330	CF074994	similar to Mll2 protein
24Dec00_E22_1GAL22_055	0.447	0.463	PCNA	CD740179	proliferating cell nuclear antigen
12May01_L08_1GAL35_042	0.449	0.441	GPATCH3	CD734263	G patch domain containing 3
15Dec00_N04_1GAL16_068	0.453	0.3	RBP2	CD733170	retinol binding protein 2, cellular
11Aug01_A12_1GAL94_083	0.454	0.437	LGALS3	CD739216	lectin, galactoside-binding, soluble, 3
6Jul01_O22_1GAL84_075	0.458	0.46	LOC777441	CD729298	Hypothetical protein LOC777441
24Apr01_F22_1GAL41_055	0.459	0.482	LOC427199	CD733296	similar to molybdopterin synthase large subunit
pat.pk0049.h5.f	0.465	0.462	ITM2A	NM_001012571	integral membrane protein 2A (ITM2A)
30May01_N19_1GAL62_020	0.468	0.438	RW1	CD727660	RW1 protein
7Mar01_C18_1GAL51_092	0.471	0.495	PSCD4	CD734326	pleckstrin homology, Sec7 and coiled-coil domains 4
18Aug01_N12_1GAL96_098	0.474	0.436	FCGBP	CD739581	Fc fragment of IgG binding protein
22Mar01_D21_1GAL50_045	0.475	0.451	IFNAR1	CD733646	interferon (alpha, beta and omega) receptor 1
7May01_N04_1GAL38_068	0.479	0.443	SGPL1	CD731495	sphingosine-1-phosphate lyase 1
20Mar01_G13_1GAL32_014	0.496	0.47	ADA	CD733122	adenosine deaminase

Bibliography

Alexander, D.J. (2000) A review of avian influenza in different bird species. Veterinary Microbiology 74, 3-13.

Balamurugan, V. and Kataria, J.M. (2006) Economically important non-oncogenic immunosuppressive viral diseases of chicken--current status. Vet Res Commun 30(5), 541-566.

Banks, J., Speidel, E.C., McCauley, J.W. and Alexander, D.J. (2000) Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses. Archives of Virology 145(5), 1047-1058.

Banks, J., Speidel, E.S., Moore, E., Plowright, L., Piccirillo, A., Capua, I., Cordioli, P., Fioretti, A. and Alexander, D.J. (2001) Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 146(5), 963-973.

Bean, W.J., Kawaoka, Y., Wood, J.M., Pearson, J.E. and Webster, R.G. (1985) Characterization of virulent and avirulent A/chicken/Pennsylvania/83 influenza A viruses: potential role of defective interfering RNAs in nature. J Virol 54(1), 151-160.

Bosch, F.X., Orlich, M., Klenk, H.D. and Rott, R. (1979) The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology 95(1), 197-207.

Campitelli, L., Ciccozzi, M., Salemi, M., Taglia, F., Boros, S., Donatelli, I. and Rezza, G. (2006) H5N1 influenza virus evolution: a comparison of different epidemics in birds and humans (1997-2004). J Gen Virol 87(Pt 4), 955-960.

Capua, I. and Marangon, S. (2007) The use of vaccination to combat multiple introductions of Notifiable Avian Influenza viruses of the H5 and H7 subtypes between 2000 and 2006 in Italy. Vaccine 25(27), 4987-4995.

Capua, I.A.D.j. (2007) Avian influenza infections in birds - a moving target. Influenza(1), 11-18.

Cheung, C.Y., Poon, L.L., Lau, A.S., Luk, W., Lau, Y.L., Shortridge, K.F., Gordon, S., Guan, Y. and Peiris, J.S. (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 360(9348), 1831-1837.

Claas, E.C., Osterhaus, A.D., van Beek, R., De Jong, J.C., Rimmelzwaan, G.F., Senne, D.A., Krauss, S., Shortridge, K.F. and Webster, R.G. (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351(9101), 472-477.

Consortium, I.C.G.S. (2004) Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432(7018), 695-716.

Degen, W.G., Smith, J., Simmelink, B., Glass, E.J., Burt, D.W. and Schijns, V.E. (2006) Molecular immunophenotyping of lungs and spleens in naive and vaccinated chickens early after pulmonary avian influenza A (H9N2) virus infection. Vaccine 24(35-36), 6096-6109.

Deshpande, K.L., Fried, V.A., Ando, M. and Webster, R.G. (1987) Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. Proc Natl Acad Sci U S A 84(1), 36-40.

Dobbin, K., Shih, J.H. and Simon, R. (2003) Questions and answers on design of dual-label microarrays for identifying differentially expressed genes. J Natl Cancer Inst 95(18), 1362-1369.

Ennis, F.A., Wells, M.A., Butchko, G.M. and Albrecht, P. (1978) Evidence That Cytotoxic T Cells Are Part of Hosts Response to Influenza Pneumonia. Journal of Experimental Medicine 148(5), 1241-1250.

Farooq, M.D., F.R., Imran,N; Durani, Z and, Chand, N. (2003) Prevalence and Economic Losses Due to Infectious Bursal Disease in Broilers in Mirpur and Kotli Districts of Kashmir. International Journal of Poultry Science 2(4), 267-270.

Fiers, W., De Filette, M., Birkett, A., Neirynck, S. and Min Jou, W. (2004) A "universal" human influenza A vaccine. Virus Res 103(1-2), 173-176.

Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B. and Osterhaus, A.D. (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79(5), 2814-22.

Garcia, M., Crawford, J.M., Latimer, J.W., Rivera-Cruz, E. and Perdue, M.L. (1996) Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J Gen Virol 77 (Pt 7), 1493-1504.

Gomez-Puertas, P., Albo, C., Perez-Pastrana, E., Vivo, A. and Portela, A. (2000) Influenza virus matrix protein is the major driving force in virus budding. J Virol 74(24), 11538-11547.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M. and Si-Tahar, M. (2005) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 280(7), 5571-5580.

Gulati, U., Hwang, C.C., Venkatramani, L., Gulati, S., Stray, S.J., Lee, J.T., Laver, W.G., Bochkarev, A., Zlotnick, A. and Air, G.M. (2002) Antibody epitopes on the neuraminidase of a recent H3N2 influenza virus (A/Memphis/31/98). J Virol 76(23), 12274-12280.

Halvorson, D.E.S.a.D.A. (2008) Influenza, 1324 pp. Twelfth ed. Diseases of Poultry edited by Y.M. Saif. 1 vols. Blackwell Publishing.

Hatta, M., Gao, P., Halfmann, P. and Kawaoka, Y. (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293(5536), 1840-1842.

Higashihara, M., Saijo, K., Fujisaki, Y. and Matumoto, M. (1991) Immunosuppressive effect of infectious bursal disease virus strains of variable virulence for chickens. Vet Microbiol 26(3), 241-248.

Hirst, M., Astell, C.R., Griffith, M., Coughlin, S.M., Moksa, M., Zeng, T., Smailus, D.E., Holt, R.A., Jones, S., Marra, M.A., Petric, M., Krajden, M., Lawrence, D., Mak, A., Chow, R., Skowronski, D.M., Tweed, S.A., Goh, S., Brunham, R.C., Robinson, J., Bowes, V., Sojonky, K., Byrne, S.K., Li, Y., Kobasa, D., Booth, T. and Paetzel, M. (2004) Novel avian influenza H7N3 strain outbreak, British Columbia. Emerg Infect Dis 10(12), 2192-2195.

Honda, A., Mizumoto, K. and Ishihama, A. (2002) Minimum molecular architectures for transcription and replication of the influenza virus. Proc Natl Acad Sci U S A 99(20), 13166-13171.

Hong, Y.H., Lillehoj, H.S., Dalloul, R.A., Min, W., Miska, K.B., Tuo, W., Lee, S.H., Han, J.Y. and Lillehoj, E.P. (2006) Molecular cloning and characterization of chicken NK-lysin. Vet Immunol Immunopathol 110(3-4), 339-347.

Horimoto, T. and Kawaoka, Y. (2001) Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev 14(1), 129-149.

Horimoto, T. and Kawaoka, Y. (2005) Influenza: lessons from past pandemics, warnings from current incidents. Nat Rev Microbiol 3(8), 591-600.

Horimoto, T., Nakayama, K., Smeekens, S.P. and Kawaoka, Y. (1994) Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol 68(9), 6074-6078.

Horimoto, T., Rivera, E., Pearson, J., Senne, D., Krauss, S., Kawaoka, Y. and Webster, R.G. (1995) Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 213(1), 223-230.

Hosack, D.A., Dennis, G., Jr., Sherman, B.T., Lane, H.C. and Lempicki, R.A. (2003) Identifying biological themes within lists of genes with EASE. Genome Biol 4(10), R70.

Hu, Q., Pan, Z., Deen, S., Meng, S., Zhang, X., Zhang, X. and Jiao, X.A. (2007) New alleles of chicken CD8 alpha and CD3d found in Chinese native and western breeds. Vet Immunol Immunopathol 120(3-4), 223-233.

Humberd, J., Guan, Y. and Webster, R.G. (2006) Comparison of the replication of influenza A viruses in Chinese ring-necked pheasants and chukar partridges. J Virol 80(5), 2151-2161.

Ismail, N.M. and Saif, Y.M. (1991) Immunogenicity of infectious bursal disease viruses in chickens. Avian Dis 35(3), 460-469.

Isoda, N., Sakoda, Y., Kishida, N., Bai, G.R., Matsuda, K., Umemura, T. and Kida, H. (2006) Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch Virol 151(7), 1267-1279.

Ito, T., Goto, H., Yamamoto, E., Tanaka, H., Takeuchi, M., Kuwayama, M., Kawaoka, Y. and Otsuki, K. (2001) Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol 75(9), 4439-4443.

Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R.G. and Kida, H. (1995) Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 140(7), 1163-1172.

Jin, W., Riley, R.M., Wolfinger, R.D., White, K.P., Passador-Gurgel, G. and Gibson, G. (2001) The contributions of sex, genotype and age to transcriptional variance in Drosophila melanogaster. Nat Genet 29(4), 389-395.

Jones, I.M., Reay, P.A. and Philpott, K.L. (1986) Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. Embo J 5(9), 2371-2376.

Julkunen, I., Melen, K., Nyqvist, M., Pirhonen, J., Sareneva, T. and Matikainen, S. (2000) Inflammatory responses in influenza A virus infection. Vaccine 19, S32-S37.

Kaufer, I. and Weiss, E. (1980) Significance of bursa of Fabricius as target organ in infectious bursal disease of chickens. Infect Immun 27(2), 364-367.

Kaufman, J. (2000) The simple chicken major histocompatibility complex: life and death in the face of pathogens and vaccines. Philos Trans R Soc Lond B Biol Sci 355(1400), 1077-1084.

Kaufmann, A., Salentin, R., Meyer, R.G., Bussfeld, D., Pauligk, C., Fesq, H., Hofmann, P., Nain, M., Gemsa, D. and Sprenger, H. (2001) Defense against influenza A virus infection: essential role of the chemokine system. Immunobiology 204(5), 603-613.

Kawaoka, Y., Naeve, C.W. and Webster, R.G. (1984) Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology 139(2), 303-316.

Kawaoka, Y., Nestorowicz, A., Alexander, D.J. and Webster, R.G. (1987) Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. Virology 158(1), 218-227.

Kawaoka, Y. and Webster, R.G. (1985) Evolution of the A/Chicken/Pennsylvania/83 (H5N2) influenza virus. Virology 146(1), 130-137.

Kawaoka, Y. and Webster, R.G. (1988) Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A 85(2), 324-328.

Kawaoka, Y. and Webster, R.G. (1989) Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin. J Virol 63(8), 3296-3300.

Keeler, C.L., Jr., Bliss, T.W., Lavric, M. and Maughan, M.N. (2007) A functional genomics approach to the study of avian innate immunity. Cytogenet Genome Res 117(1-4), 139-145.

Kim, C.H., Hyun S. Lillehoj, Travis W. Bliss, Calvin L. Keeler Jr., Yeong Ho Hong, Dong Woon Park, Mat Yamage, Wongi Min, Erik P. Lillehoj. (2008) Construction and application of an avian intestinal intraepithelial lymphocyte cDNA microarray (AVIELA) for gene expression profiling during Eimeria maxima infection. Veterinary Immunology and Immunoparhology. Aug 15, 124(3-4):351-354

Kim, I.J., Gagic, M. and Sharma, J.M. (1999) Recovery of antibody-producing ability and lymphocyte repopulation of bursal follicles in chickens exposed to infectious bursal disease virus. Avian Dis 43(3), 401-413.

Knipe, D.K.P.M.H., Diane E Griffin, Robert A Lamb, Malcolm A Martin. (2007) Fields Virology.

Kobasa, D., Takada, A., Shinya, K., Hatta, M., Halfmann, P., Theriault, S., Suzuki, H., Nishimura, H., Mitamura, K., Sugaya, N., Usui, T., Murata, T., Maeda, Y., Watanabe, S., Suresh, M., Suzuki, T., Suzuki, Y., Feldmann, H. and Kawaoka, Y. (2004) Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 431(7009), 703-707.

Kuiken, T., Holmes, E.C., McCauley, J., Rimmelzwaan, G.F., Williams, C.S. and Grenfell, B.T. (2006) Host species barriers to influenza virus infections. Science 312(5772), 394-397.

Kwon, J.S., Lee, H.J., Lee, D.H., Lee, Y.J., Mo, I.P., Nahm, S.S., Kim, M.J., Lee, J.B., Park, S.Y., Choi, I.S. and Song, C.S. (2008) Immune responses and pathogenesis in immunocompromised chickens in response to infection with the H9N2 low pathogenic avian influenza virus. Virus Res 133(2), 187-194.

La Gruta, N.L., Kedzierska, K., Stambas, J. and Doherty, P.C. (2007) A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol 85(2), 85-92.

Landolt, G.A. and Olsen, C.W. (2007) Up to new tricks - a review of cross-species transmission of influenza A viruses. Anim Health Res Rev 8(1), 1-21.

Lazarowitz, S.G. and Choppin, P.W. (1975) Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 68(2), 440-454.

Lee, C.W., Lee, Y.J., Swayne, D., Senne, D., Linares, D.J. and Suarez, D. (2007) Assessing potential pathogenicity of avian influenza virus: current and experimental system. Avian Dis 51(1 Suppl), 260-263.

Lee, C.W., Swayne, D.E., Linares, J.A., Senne, D.A. and Suarez, D.L. (2005) H5N2 avian influenza outbreak in Texas in 2004: the first highly pathogenic strain in the United States in 20 years? J Virol 79(17), 11412-11421.

Li, K.S., Guan, Y., Wang, J., Smith, G.J., Xu, K.M., Duan, L., Rahardjo, A.P., Puthavathana, P., Buranathai, C., Nguyen, T.D., Estoepangestie, A.T., Chaisingh, A., Auewarakul, P., Long, H.T., Hanh, N.T., Webby, R.J., Poon, L.L., Chen, H., Shortridge, K.F., Yuen, K.Y., Webster, R.G. and Peiris, J.S. (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430(6996), 209-213.

Lutticken, D. (1997) Viral diseases of the immune system and strategies to control infectious bursal disease by vaccination. Acta Vet Hung 45(3), 239-249.

MacDonald, M.R.V.V., S.M; Guo, X; Xia, J; Moon, D.A.; Magor, K.E. (2007) Genomics of antiviral defenses in the duck, a natural host of influenza and hepatitis B viruses. Cytogenet Genome Res(117), 195-206.

Martin, K. and Helenius, A. (1991) Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell 67(1), 117-130.

Matrosovich, M., Zhou, N., Kawaoka, Y. and Webster, R. (1999) The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 73(2), 1146-1155.

McFerran, J.B., McNulty, M.S., McKillop, E.R., Connor, T.J., McCracken, R.M., Collins, D.S. and Allan, G.M. (1980) Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. Avian Pathol 9(3), 395-404.

Morgan, A. (2006) Avian influenza: an agricultural perspective. J Infect Dis 194 Suppl 2, S139-146.

Mozdzanowska, K., Maiese, K. and Gerhard, W. (2000) Th cell-deficient mice control influenza virus infection more. effectively than Th- and B cell-deficient mice: Evidence for a Th-independent contribution by B cells to virus clearance. Journal of Immunology 164(5), 2635-2643.

Muller, H., Islam, M.R. and Raue, R. (2003) Research on infectious bursal disease--the past, the present and the future. Vet Microbiol 97(1-2), 153-165.

Munster, V.J., Wallensten, A., Baas, C., Rimmelzwaan, G.F., Schutten, M., Olsen, B., Osterhaus, A.D. and Fouchier, R.A. (2005) Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis 11(10), 1545-1551.

Neumann, G., Hughes, M.T. and Kawaoka, Y. (2000) Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. Embo J 19(24), 6751-6758.

O'Neill, R.E., Jaskunas, R., Blobel, G., Palese, P. and Moroianu, J. (1995) Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. J Biol Chem 270(39), 22701-22704.

OIE. (2006) Avian Influenza. International Animal Health Code.

Palese, P., Shaw, M. (2007) Othomyxoviridae: The viruses and their replication. 5th ed. Fields Virology, edited by D.M.H. Knipe, Peter M., 1. 2 vols. Lippincott Williams & Wilkins.

Pasick, J., Handel, K., Robinson, J., Copps, J., Ridd, D., Hills, K., Kehler, H., Cottam-Birt, C., Neufeld, J., Berhane, Y. and Czub, S. (2005) Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol 86(Pt 3), 727-731.

Perdue, M.L. and Swayne, D.E. (2005) Public health risk from avian influenza viruses. Avian Dis 49(3), 317-327.

Perkins, L.E. and Swayne, D.E. (2001) Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet Pathol 38(2), 149-164.

Perkins, L.E. and Swayne, D.E. (2003) Comparative susceptibility of selected avian and mammalian species to a Hong Kong-origin H5N1 high-pathogenicity avian influenza virus. Avian Dis 47(3 Suppl), 956-967.

Peters, M.A., Lin, T.L. and Wu, C.C. (2004) Infectious bursal disease virus polyprotein expression arrests growth and mitogenic stimulation of B lymphocytes. Arch Virol 149(12), 2413-2426.

Pinto, L.H., Holsinger, L.J. and Lamb, R.A. (1992) Influenza virus M2 protein has ion channel activity. Cell 69(3), 517-528.

Plotch, S.J. and Krug, R.M. (1977) Influenza virion transcriptase: synthesis in vitro of large, polyadenylic acid-containing complementary RNA. J Virol 21(1), 24-34.

Poch, O., Sauvaget, I., Delarue, M. and Tordo, N. (1989) Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. Embo J 8(12), 3867-3874.

Poole, E., Elton, D., Medcalf, L. and Digard, P. (2004) Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. Virology 321(1), 120-133.

Rautenschlein, S., Kraemer, C., Montiel, E., Vanmarcke, J. and Haase, C. (2007) Bilateral effects of vaccination against infectious bursal disease and Newcastle disease in specific-pathogen-free layers and commercial broiler chickens. Avian Dis 51(1), 14-20.

Rautenschlein, S., Yeh, H.Y. and Sharma, J.M. (2003) Comparative immunopathogenesis of mild, intermediate, and virulent strains of classic infectious bursal disease virus. Avian Dis 47(1), 66-78.

Reed, L.J., and H. Muench. (1938) A simple method for estimating 50 percent endpoints. Am J Hyg 37, 493.

Riegert, P., Andersen, R., Bumstead, N., Dohring, C., DominguezSteglich, M., Engberg, J., Salomonsen, J., Schmid, M., Schwager, J., Skjodt, K. and Kaufman, J. (1996) The chicken beta(2)-microglobulin gene is located on a non-major histocompatibility complex microchromosome: A small, G+C-rich gene with X and Y boxes in the promoter. Proceedings of the National Academy of Sciences of the United States of America 93(3), 1243-1248.

Rimmelzwaan, G.F., van Riel, D., Baars, M., Bestebroer, T.M., van Amerongen, G., Fouchier, R.A., Osterhaus, A.D. and Kuiken, T. (2006) Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol 168(1), 176-183; quiz 364.

Rohm, C., Horimoto, T., Kawaoka, Y., Suss, J. and Webster, R.G. (1995) Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? Virology 209(2), 664-670.

Rosenberger, J.K. and Gelb, J., Jr. (1978) Response to several avian respiratory viruses as affected by infectious bursal disease virus. Avian Dis 22(1), 95-105.

Rosenberger, J.K., Cloud SS. (1989) The effects of age, route of exposure, and coinfection with infectious bursal disease virus on the pathogenicityand transmissibility of chicken anemia agent (CAA). . Avian Dis 33(4), 753-759.

Rott, R., Orlich, M. and Scholtissek, C. (1976) Attenuation of pathogenicity of fowl plague virus by recombination with other influenza A viruses nonpathogenic for fowl: nonexculsive dependence of pathogenicity on hemagglutinin and neuraminidase of the virus. J Virol 19(1), 54-60.

Rott, R., Orlich, M. and Scholtissek, C. (1979) Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J Gen Virol 44(2), 471-477.

Ruysschaert, J.M., Goormaghtigh, E., Homble, F., Andersson, M., Liepinsh, E. and Otting, G. (1998) Lipid membrane binding of NK-lysin. FEBS Lett 425(2), 341-344.

Saif, Y.M. (1991) Immunosuppression induced by infectious bursal disease virus. Vet Immunol Immunopathol 30(1), 45-50.

Sanz-Ezquerro, J.J., Fernandez Santaren, J., Sierra, T., Aragon, T., Ortega, J., Ortin, J., Smith, G.L. and Nieto, A. (1998) The PA influenza virus polymerase subunit is a phosphorylated protein. J Gen Virol 79 (Pt 3), 471-478.

Scholtissek, C., Rohde, W., Harms, E. and Rott, R. (1977a) Correlation between base sequence homology of RNA segment 4 and antigenicity of the hemagglutinin of influenza viruses. Virology 79(2), 330-336.

Scholtissek, C., Rott, R., Orlich, M., Harms, E. and Rohde, W. (1977b) Correlation of pathogenicity and gene constellation of an influenza A virus (fowl plague). I. Exchange of a single gene. Virology 81(1), 74-80.

Sharma, J.M., Kim, I.J., Rautenschlein, S. and Yeh, H.Y. (2000) Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. Dev Comp Immunol 24(2-3), 223-235.

Sims, L.D., Ellis, T.M., Liu, K.K., Dyrting, K., Wong, H., Peiris, M., Guan, Y. and Shortridge, K.F. (2003) Avian influenza in Hong Kong 1997-2002. Avian Dis 47(3 Suppl), 832-838.

Sioson, A.A., Mane, S.P., Li, P., Sha, W., Heath, L.S., Bohnert, H.J. and Grene, R. (2006) The statistics of identifying differentially expressed genes in Expresso and TM4: a comparison. BMC Bioinformatics 7, 215.

Snyder, D.B., Lana, D.P., Savage, P.K., Yancey, F.S., Mengel, S.A. and Marquardt, W.W. (1988) Differentiation of infectious bursal disease viruses directly from infected tissues with neutralizing monoclonal antibodies: evidence of a major antigenic shift in recent field isolates. Avian Dis 32(3), 535-539.

Sorrell, E.M., Ramirez-Nieto, G.C., Gomez-Osorio, I.G. and Perez, D.R. (2007) Genesis of pandemic influenza. Cytogenet Genome Res 117(1-4), 394-402.

Squires, B., Macken, C., Garcia-Sastre, A., Godbole, S., Noronha, J., Hunt, V., Chang, R., Larsen, C.N., Klem, E., Biersack, K. and Scheuermann, R.H. (2008) BioHealthBase: informatics support in the elucidation of influenza virus host pathogen interactions and virulence. Nucleic Acids Res 36(Database issue), D497-503.

Steinhauer, D.A. and Skehel, J.J. (2002) Genetics of influenza viruses. Annu Rev Genet 36, 305-332.

Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.D. and Garten, W. (1992) Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. Embo J 11(7), 2407-2414.

Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S.A., Bloom, B.R., Krensky, A.M. and Modlin, R.L. (1998) An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 282(5386), 121-125.

Suarez, D.L. (2000) Evolution of avian influenza viruses. Vet Microbiol 74(1-2), 15-27.

Suarez, D.L., Perdue, M.L., Cox, N., Rowe, T., Bender, C., Huang, J. and Swayne, D.E. (1998) Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 72(8), 6678-6688.

Suarez, D.L., Senne, D.A., Banks, J., Brown, I.H., Essen, S.C., Lee, C.W., Manvell, R.J., Mathieu-Benson, C., Moreno, V., Pedersen, J.C., Panigrahy, B., Rojas, H., Spackman, E. and Alexander, D.J. (2004) Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg Infect Dis 10(4), 693-699.

Subbarao, K. and Joseph, T. (2007) Scientific barriers to developing vaccines against avian influenza viruses. Nature Reviews Immunology 7(4), 267-278.

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K. and Cox, N. (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279(5349), 393-396.

Suzuki, Y. (2006) Natural selection on the influenza virus genome. Mol Biol Evol 23(10), 1902-1911.

Swayne, D.E. (1997) Pathobiology of H5N2 Mexican avian influenza virus infections of chickens. Vet Pathol 34(6), 557-567.

Swayne, D.E. (2007) Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. Avian Dis 51(1 Suppl), 242-249.

Swayne, D.E. and Suarez, D.L. (2000) Highly pathogenic avian influenza. Rev Sci Tech 19(2), 463-482.

Szretter, K.J., Gangappa, S., Lu, X., Smith, C., Shieh, W.J., Zaki, S.R., Sambhara, S., Tumpey, T.M. and Katz, J.M. (2007) Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. J Virol 81(6), 2736-2744.

Tecle, T., White, Mitchell .R. and Hartshorn, Kevan L. (2005) Innate Immunity to Influenza A Virus Infection. Current Respiratory Medicine Reviews 1, 127-145.

Treanor, J.J., Tierney, E.L., Zebedee, S.L., Lamb, R.A. and Murphy, B.R. (1990) Passively Transferred Monoclonal-Antibody to the M2 Protein Inhibits Influenza-a Virus-Replication in Mice. Journal of Virology 64(3), 1375-1377.

Tucker, S.P. and Compans, R.W. (1993) Virus infection of polarized epithelial cells. Adv Virus Res 42, 187-247.

van der Goot, J.A., de Jong, M.C., Koch, G. and Van Boven, M. (2003) Comparison of the transmission characteristics of low and high pathogenicity avian influenza A virus (H5N2). Epidemiol Infect 131(2), 1003-1013.

Wallis, J.W., Aerts, J., Groenen, M.A., Crooijmans, R.P., Layman, D., Graves, T.A., Scheer, D.E., Kremitzki, C., Fedele, M.J., Mudd, N.K., Cardenas, M., Higginbotham, J., Carter, J., McGrane, R., Gaige, T., Mead, K., Walker, J., Albracht, D., Davito, J., Yang, S.P., Leong, S., Chinwalla, A., Sekhon, M., Wylie, K., Dodgson, J., Romanov, M.N., Cheng, H., de Jong, P.J., Osoegawa, K., Nefedov, M., Zhang, H., McPherson, J.D., Krzywinski, M., Schein, J., Hillier, L., Mardis, E.R., Wilson, R.K. and Warren, W.C. (2004) A physical map of the chicken genome. Nature 432(7018), 761-764.

Wareign, M.D., Lyon, A.B., Lu, B., Gerard, C, and Sarawar, S. (2004) Chemokine expression during the development and resolution of a pulmonary leucocyte response to influenza A virus infection in mice. Journal of LeuKocyte Biology 76(October), 886-895.

Wasilenko, J.L., Lee, C.W., Sarmento, L., Spackman, E., Kapczynski, D.R., Suarez, D.L. and Pantin-Jackwood, M.J. (2008) NP, PB1, and PB2 viral genes contribute to altered replication of H5N1 avian influenza viruses in chickens. J Virol 82(9), 4544-4553.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56(1), 152-179.

Webster, R.G., Reay, P.A. and Laver, W.G. (1988) Protection against lethal influenza with neuraminidase. Virology 164(1), 230-237.

Webster, R.G., Shortridge, K.F. and Kawaoka, Y. (1997) Influenza: interspecies transmission and emergence of new pandemics. FEMS Immunol Med Microbiol 18(4), 275-279.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J. and Murti, K.G. (1978) Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84(2), 268-278.

WHO. WHO manual on Animal Influenza Diagnosis and Surveillance, pp. 105.

Withers, D.R., Young, J.R. and Davison, T.F. (2005) Infectious bursal disease virus-induced immunosuppression in the chick is associated with the presence of undifferentiated follicles in the recovering bursa. Viral Immunol 18(1), 127-137.

Wijburg, O.L., DiNatale, S., Vadolas, J., van Rooijen, N. and Strugnell, R.A. (1997) Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection. J Virol 71(12), 9450-9457.

Wood, G.W., Mccauley, J.W., Bashiruddin, J.B. and Alexander, D.J. (1993) Deduced Amino-Acid-Sequences at the Hemagglutinin Cleavage Site of Avian Influenza-a Viruses of H5 and H7 Subtypes. Archives of Virology 130(1-2), 209-217.

Wood, J.M., Webster, R. G., and Nettles, V. F. (1984) Host Range of A/Chicken/Pennsylvania/83 (H5N2). Avian Diseases 29(1), 198-207.

Xu, X., Subbarao, Cox, N.J. and Guo, Y. (1999) Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 261(1), 15-19.

Zekarias, B., Ter Huurne, A.A., Landman, W.J., Rebel, J.M., Pol, J.M. and Gruys, E. (2002) Immunological basis of differences in disease resistance in the chicken. Vet Res 33(2), 109-125.