MULTI-SPECIES USE OF AN AVIAN MICROARRAY

by

Lorna Dougherty

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Animal Science with Distinction.

Spring 2009

MULTI-SPECIES USE OF AN AVIAN MICROARRAY

by

Lorna Dougherty

Approved: _____
Calvin Keeler, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Marlene Emara, Ph.D.
Committee member from the Department of Animal and Food Sciences

Approved: _____
Jung-Youn Lee, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____
Ismat Shah, Ph.D.
Chair of the University Committee on Student and Faculty Honors

ACKNOWLEDGMENTS

I would like to thank Dr. Gelb, and his entire lab for providing spleen samples, and providing me with select results of their experiment that aided in my analysis. I would also like to thank the graduate students in my lab, specifically Ida Chung, and Michele Maughan for their continued support and instruction. Finally, I would like to thank the USDA for providing me with a grant to support my research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Ducks represent a natural host and reservoir for avian influenza virus and significant differences are observed in the biological response of ducks to strains of avian influenza recovered from other avian species. The objective of this project was to study the immune response of ducks at the transcriptional level to three species-adapted strains of avian influenza. Our goal was to determine if differences in the innate immune response to species-adapted strains of avian influenza might suggest a contribution to these observed biological differences. Microarray analysis revealed that a common set of genes exhibited significant expression in response to chicken-, duck- and turkey- derived strains of avian influenza, while many other genes exhibited a significant response to only one of the species-adapted strains. Overall, similar expression values were seen in cytokines and chemokines, and genes involved in the TLR and NFκβ activation pathways in response to the species-adapted strains of avian influenza. Several genes important in the immune response of ducks to avian influenza that exhibited different expression values when infected with species-adapted strains were identified that may correlate with the differences seen in the biological response of ducks to the different strains of avian influenza.

Chapter 1

INTRODUCTION

1.1 Impact of Avian Influenza on the Poultry Industry and Human Health

Avian influenza is an important disease due to transmission of the virus among species, and the high mortality rates associated with highly pathogenic strains of the virus. Highly pathogenic avian influenza (HPAI) outbreaks in the commercial poultry industry are associated with serious economic consequences due to bird death, depopulation costs, and national and international trade restrictions (30). In the last twenty years, the number of outbreaks of HPAI has increased, as have the number of birds involved. In the period of 1959-1992 there were eleven outbreaks in which one resulted in more than 500,000 poultry dying or being slaughtered, while in the 13 outbreaks that occurred from 1993-2006, mortality in eight of the outbreaks far exceeded 500,000 birds (7). Since 1959 there have been HPAI outbreaks in South Africa, England, Canada, Australia, Germany, Ireland, Pennsylvania, Mexico, Pakistan, Italy, and Hong Kong (51). Despite control efforts, outbreaks still occur today. During a routine surveillance in 2008, highly pathogenic influenza A virus was detected in live chickens in Lokoss, Benin. Considering this was India's third outbreak of H5 avian flu since 2006, government officials ordered the culling of more than two million chickens and ducks (3). Recent outbreaks have also occurred in China, Egypt, Japan, Indonesia, Russia, Thailand, Turkey, and Vietnam (2). Low-pathogenic avian influenza persists in the north-eastern United States live-bird markets today, and

periodic outbreaks of circulating market strains of virus occur among commercial poultry farms (30).

In relation to human health, the total number of confirmed human cases of avian influenza since 2003 is 385, with 243 of them being fatal infections (2). It has been documented that multiple avian influenza virus subtypes have been transmitted directly from domestic poultry to humans (23). Infections can arise through reassortment between an avian influenza virus and the circulating human influenza virus. This mechanism is responsible for two of the three influenza pandemics in the 20th century, and has caused a range of disease symptoms, varying in severity from asymptomatic to fatal (23). Most human infections of HPAI are characterized by severe pneumonia and rapid progression to acute respiratory distress syndrome, and multi-organ failure (6). Additional effects include lymphopenia, thrombocytopenia, high nasopharyngeal viral loads, elevated chemokines and cytokines, and eventual death (15). When isolating a specific strain of HPAI from humans, it was discovered that the virus genome was wholly avian in origin (23), indicating the importance of avian influenza in the human health industry.

1.2 Avian Influenza

Strains of avian influenza are classified as either low pathogenic (LPAI) or highly pathogenic (HPAI), based on their ability to cause disease and mortality (16). Strains are further subtyped by the form of glycoproteins present on the virus, specifically hemagglutinin (H), and neuraminidase (N). Aquatic birds are the source of all influenza viruses in other species, with every subtype combination found in the aquatic bird population (53). While disease rarely presents symptoms in the aquatic bird reservoir, disease often presents varying levels of morbidity and mortality when

the virus enters another host (16). Influenza A viruses are found globally, with viruses transmitting across long distances due to trade of live animals, and the movement of animals within production systems. The virus often spreads via contaminated feed, water, or equipment, along wild bird migration routes, and through the movement of people (16).

Clinical signs associated with influenza A virus infection in avian species vary with the strain of the virus. Whether LPAI or HPAI, the primary targets of virus replication include the lungs, lymphoid tissues, and visceral organs containing epithelial cells such as the kidneys and pancreas (51). LPAI infections may be asymptomatic or may cause a variety of clinical signs ranging from mild to severe signs of disease affecting respiratory, urogenital, and/or enteric systems (51). Clinical signs reported in LPAI infected chickens include lethargy, decreased feed and water consumption, decreased egg production, decreased fertility and hatchability of eggs, misshapen eggs, and increased mortality (48). Conjunctivitis and diarrhea have also been reported, however, respiratory effects are the primary symptom of LPAI infection and include cyanosis and facial edema (8; 48). Concurrent infections with other pathogens, environmental stress, and the host species and age also influence the severity of disease (51). Unlike the high mortality rates associated with HPAI, the reported mortality rates during LPAI outbreaks typically range from 0.25% to 25% (25; 31; 12).

Highly pathogenic influenza produces a systemic infection accompanied by central nervous system involvement, with death occurring within one week (53). In fact, mortality rates associated with HPAI can be as high as 100% (7). Typical signs of highly pathogenic viruses include decreased egg production, respiratory distress, rales,

excessive lacrimation, sinusitis, cyanosis of unfeathered skin, especially of the combs and wattles, edema of the head and face, ruffled feathers, diarrhea, nervous disorders, depression, and decreased feed and water intake (53). These signs often are not observed because bird death occurs quickly. While many birds are found dead with few or no preceding signs (49), initial signs reported from documented outbreaks include tremors and incoordination followed by depression and anorexia (31), mild respiratory signs, severe diarrhea and depression, and reduced egg production (18), hemmorhage and inflammation of the trachea, and severe respiratory signs (42).

Viral shedding in LPAI and HPAI has been studied by examining the amount of virus particles present in the feces and respiratory secretions. LPAI viruses have been detected in respiratory secretions as early as one day after infection, while virus was not found in the feces until day two (42). Such viral shedding may occur without accompaniment of observed clinical signs since LPAI infections can be asymptomatic. Studies involving HPAI conclude that viral shedding occurs slightly earlier than, or around the same time as the first clinical signs, which occur one to two days after inoculation (42).

HPAI viruses have also been found in meat and eggs after inoculation. While HPAI virus may be present in the meat before the onset of clinical signs, presence in the eggs does not occur until after the appearance of clinical signs (42). LPAI viruses, on the other hand, are not observed in poultry products. Since LPAI viruses remain localized in the respiratory and gastrointestinal tract, LPAI virus is not observed in meat or eggs (47, 55).

1.3 Avian Influenza and Wild Birds

Wild birds were first connected to the ecology of influenza in 1972 when influenza was isolated from free-living ducks (46). Since then, numerous surveillance studies have revealed that all fifteen hemagglutinin and nine neuraminidase subtypes of influenza can be found in wild birds (46).

Naturally occurring infections of avian influenza have been reported in free-living birds representing more than ninety species in twelve avian orders, with most occurring in the Anseriformes order (ducks, geese, and swans) and the Charadriiforms order (gulls, terns, and shorebirds) (43). With viruses reported in forty-seven of the one hundred fifty-eight species of ducks and geese worldwide, ducks have accounted for most of the avian influenza isolations (33), with the most isolation from mallard ducks (43). In accordance with migratory behaviors, avian influenza prevalence in ducks peaks in the late summer and early fall when there is an increase in concentration of susceptible hatching-year birds. The prevalence of avian influenza in North America is greatest in waterfowl staging areas in Canada and the northern United States. During migration, prevalence rapidly decreases, and in wintering areas, avian influenza prevalence can drop to as low as 1-2% (44).

Transmission and maintenance of avian influenza in wild bird populations is dependent on fecal/ oral transmission. In wild ducks, influenza viruses replicate preferentially in the cells lining the intestinal tract, cause no signs of disease, and are excreted in high concentrations in the feces (53). Concentrations can be as high as 10^7 infectious particles per gram, and the virus may survive for more than forty-four days (7). As described earlier, in both LPAI and HPAI, virus can be found in the feces and respiratory secretions of infected birds as early as two days post infection, although the actual amount shed and first appearance of virus shed varies with the strain (42).

Ducks, and other bird species become infected when they ingest feces-contaminated water. While the greatest threat of spread of avian infuenza virus is by fecal/oral transmission, the primary source of secondary spread in domestic poultry is through movement of fomites by man (7). Fomites are inanimate objects capable of transmitting pathogens (39), and can include cages, clothing, shoes and other objects that come in contact with infected birds. Fomites can be transported through movements of caretakers, staff, trucks and drivers moving birds or delivering food, artificial inseminators (7), and equipment.

An example of the transmission of avian influenza from a wild bird population to domestic poultry is illustrated in an H5N1 outbreak in 2004 in Thailand. In addition to causing twelve human deaths and seventeen human infections, the outbreak resulted in the death or slaughter of sixty-million domestic fowl, and the disruption of poultry production and trade (40). In late 2003, poultry farms in the central and northern regions of Thailand experienced large-scale mortality, spurring a surveillance program for poultry in January 2004 (50). From January to May 2004, HPAI virus was detected in 188 villages in 42 of 76 provinces throughout Thailand. Field studies in 2004 determined that ducks were silent carriers of this HPAI virus. Importantly, Thailand has commercial hybrid broilers and layers, but backyard poultry are raised for food in most villages. It was found that greater than 50% of infected flocks were from backyard-raised origin (50). Thai health officials recognized that the spread of the H5N1 influenza virus to domestic chickens was correlated with the distribution of free-grazing ducks, and forbade the practice of raising ducks in open fields and transporting grazing ducks (40). One year after these changes were implemented, only one case of human H5N1 infection was reported. The source of

infection was found to be 3,000 to 5,000 free-range ducks that were illegally grazing in rice fields in the area. Although no direct contact between these ducks and domestic poultry were noted, within two weeks of the ducks arriving, chickens in the area began dying. One person who had direct contact with these chickens died from H5N1 infection, but it remains unknown if the chickens contracted the infection from the free-grazing ducks (40).

1.4 Influenza A Virus Structure

Influenza A viruses are members of the *Orthomyxoviridae* family. They are spherical in shape, and are 80 to 120 nm in diameter. The virus envelope is composed of a host-derived lipid bilayer that contains the virus-encoded glycoproteins hemagglutinin (HA), and neuraminidase (NA). Medial to the bilayer is an inner shell of matrix protein, and at the center of the particles is the viral genome, which is composed of eight unique segments of negative-sense single-stranded RNA. Multiple nucleoprotein molecules encase the viral genome. To be infectious, a single virus particle must contain each of the eight unique RNA segments, which collectively encode ten recognized gene products; PB1, PB2, and PA polymerases, hemagglutinin, neuraminidase, nucleoprotein, M1 and M2 proteins, and nonstructural NS1 and NS2 proteins (53).

1.4.1 Hemagglutinin and Neuraminidase

Hemagglutinin and neuraminidase are integral membrane proteins that serve as the major surface antigens of the influenza virus virion. HA is responsible for the binding of virions to host cell receptors, and the fusion of the virion envelope to the host cell. NA, however, facilitates virus spread by freeing virus particles from host

cell receptors, allowing progeny virions to escape from their originating cell (53). HA and NA are both subject to high rates of antigenic drift (45), with sixteen known subtypes of HA and nine known subtypes of NA. Antigenic drift occurs as a result of mutations due to the limited proof-reading ability of RNA polymerase in the replication of the influenza genome. New strains accumulate random point mutations that result in amino acid substitutions, and allow new variants to avoid humoral immunity (45).

Antigenic shift can also bring about new influenza virus strains. The segmented nature of the influenza genome allows for reassortment of virus segments (45). Concurrent infection of a cell by two viruses may allow reassortment of the RNA segments and this results in a virus with novel surface and internal proteins. Reassortment is thought to occur between human and animal reservoirs of influenza (45). As a result, agricultural practices that allow close proximity between humans, ducks, poultry, and pigs (45) serve as a major risk for the creation of pandemic influenza strains.

While all sixteen hemagglutinin subtypes may be classified as LPAI, only the H5 and H7 subtypes may be classified as HPAI (16). The H5 and H7 subtypes have shown an ability to mutate from the low pathogenic virus to a highly pathogenic form, leading to high mortality (21).

1.4.2 Nucleoprotein

The viral nucleoprotein is produced and transported into the infected cell nucleus where it serves a structural role by binding and encapsulating the viral RNA. Nucleoprotein also plays an important role in switching viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis. Nucleoprotein is also a major

target of the host cytotoxic T-cell immune response. While highly abundant, the pattern of nucleoprotein phosphorylation is host cell dependent, and may be related to viral host range restriction (53).

1.4.3 AI Infectivity

In order for influenza A virus to be infectious, the HA precursor, HA0, requires post-translational cleavage by host proteases (7). The HA0 precursor proteins of LPAI viruses have a single arginine at the cleavage site. As a result, these viruses are limited to cleavage by extracellular trypsin-like host proteases, and so are restricted to replication sites in the respiratory and intestinal tract (7). HPAI viruses, however, possess multiple basic amino acids at the HA0 cleavage site, as a result of insertion or substitution, and are cleavable by ubiquitous intracellular proteases allowing the HPAI virus to replicate throughout the bird, damaging vital organs and tissues resulting in disease and death (7). While it appears that HPAI viruses arrive from mutation of LPAI viruses after they are introduced into poultry, the factors that bring about such mutation are unknown. However, the low fidelity of replicase in the virus replication cycle is the most likely source for the variation and mutation in influenza viruses (35). Replicase is a polymerase enzyme that serves as a catalyst for the self-replication of single stranded RNA (5). RNA viruses contain error-prone, self-encoded replicases that may lack proofreading functions (35), which lead to variation in the virus.

1.5 Virus Replication

The genome of influenza virus is negative-stranded RNA (vRNA). vRNAs are templates for cRNA (full-length copies of the vRNA that can be used as a template

for vRNA synthesis) and mRNA synthesis, but unlike other negative-sense, single-stranded RNA viruses, the transcription and replication site for the influenza virus genome is in the nucleus of infected cells (13). Furthermore, three polymerase subunits (PB2, PB1, and PA) and NP are required for viral transcription and replication (13).

To begin replication, an influenza virus particle binds to cells via receptor-binding sites of HA. Following binding, the attached virion is endocytosed by the cell, resulting in the fusion of the viral and vesicular membranes, releasing the contents of the virion into the cytoplasm of the cell. The nucleocapsid of the parent virus enters the cell nucleus and begins primary transcription of the mRNA to be used for translation of viral proteins (53).

Nucleoprotein (NP) is encoded by segment five of the virus genome (13), and is one of the essential components for virus replication. The amino terminus of NP protein contains an RNA-binding domain, and encapsulates the viral RNA in a sequence nonspecific manner (13). In addition to this structural role, NP plays an important role in switching viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis (53). NP is also a major target of the host cytotoxic T-cell immune response. While highly abundant, the pattern of nucleoprotein phosphorylation is host cell dependent, and may be related to viral host range restrictions (53).

While translation of host mRNA is blocked, viral proteins are abundantly translated, including HA and NA, which integrate into the cell membrane. Nucleocapsids also migrate out of the nucleus to assemble into progeny viral particles in the cytoplasm, which will bud from the cell membrane (53). The final step in virus

maturation is the cleavage of HA into HA1 and HA2 by host proteases. The location where this cleavage is performed is dependent on the pathogenicity of the virus. LPAI viruses are cleaved extracellularly, while HPAI are cleaved intracellulary . This difference accounts for the difference in virulence seen between LPAI and HPAI as described in the "AI Infectivity" section. It can be noted, however, that cleaved HA is unstable at low pH. Since avian influenza is transmitted primarily by the fecal-oral route, cleavage most likely occurs after excreted virions enter their new host and pass through the stomach (53).

1.6 Humoral Immune Response to Avian Influenza

The humoral immune response stimulated by avian influenza in poultry includes antibody production in both the systemic system and mucosa. The systemic antibody response in chickens and turkeys is the production of IgM and IgY starting as early as five days post-infection (46). The surface proteins, HA and NA, are the only antigens capable of inducing neutralizing antibody formation. Since antibodies raised to one strain of the virus are unable to neutralize a different strain, vaccination must be specific to each strain. Antibodies against the HA protein are the principle determinant for protection in the host against disease, so vaccination of poultry is targeted toward the HA subtype. Secretory antibody in the mucosal immune system, specifically IgA, has also been shown to be produced in the duck and chicken. This response is thought to play a role in the recovery of infected birds, and provide protection from future infections, especially LPAI, which is primarily a mucosal infection. (46).

1.7 Microarrays

A microarray is a tool used for analyzing gene expression that consists of a glass slide containing samples of many genes arranged in a regular pattern. Sequences from thousands of different genes are fixed to the glass slide, and can consist of DNA, cDNA, or oligonucleotides (4). A sample containing fluorescently labeled nucleic acid molecules is then added to the slide, and the labeled molecules act as "mobile probes" (4) as they base pair with their complements fixed to the slide. After hybridization, a laser scanner is used to excite the fluorescent tags, and the ratio of the fluorescence of the spot to the background is used to determine the expression of the specific genes.

The microarray has become a powerful tool for the study of immune system function. In avian species, several low-density and high-density cDNA based microarrays have been developed (10). Such arrays have been used to study gene expression in specific avian systems and tissues such as the liver, immune system, metabolic/somatic system, and the neuroendocrine/ reproductive system (10). We have previously demonstrated the utility of the microarray in determining gene expression in avian species from macrophages, as well as specific tissues. This tool can be used to compare and contrast the avian immune response to different pathogens at the transcriptional level.

1.8 Avian Innate Immunity Microarray

A 4,949 element avian innate immunity microarray (AIIM) has been created and used to examine the avian innate immune response (24). The array was constructed from EST libraries of stimulated avian (chicken) macrophages and supplemented by genes of interest from several specific innate immune pathways. The

elements are spotted in triplicate on the slide, resulting in 14,877 total spots per slide. Each subarray contains several blank spots as negative controls. Elements are fixed to the slide using UV cross-linking. The array contains 13 interleukin, 7 chemokine, and 5 cytokine elements. The AIIM also contains elements for the Toll-like receptor pathway, the avian interferon/viral response pathway, and genes involved in oxidative burst (24).

1.9 Objectives

In past experiments we have shown the utility of the AIIM to monitor the transcriptional response of immune cells in turkeys and ducks as well as chickens (24). We believe this approach will prove to be especially useful in studying avian influenza, where some strains of the virus are non-pathogenic for ducks, but highly pathogenic for turkeys and chickens (22). Considering that the outbreaks of both high and low pathogenic avian influenza in domestic poultry appear to be the result of introduction initially from feral birds (8), analysis of the transcriptional response of ducks and chickens to avian influenza isolates originating from different avian species will allow inspection of the various pathogenicity in heterologous hosts. To our knowledge, there is limited data on the transcriptional response of ducks to avian influenza. The objective of my study was to examine the hypothesis that ducks will respond differently at the transcriptional level to low pathogenic avian influenza viruses originally derived from chickens, ducks, and turkeys.

Chapter 2

MATERIALS AND METHODS

2.1 Infection of Pekin ducks with chicken-, duck-, and turkey-derived strains of LPAI

Infection of Pekin ducks with different strains of avian influenza took place within a larger experiment performed by Dr. Gelb's lab at the University of Delaware to analyze the responses of SPF white leghorns, commercial broad breasted white turkeys, and commercial Pekin ducks to twelve strains of LPAI. The viruses used in analyzing the transcriptional response of ducks to LPAI are listed in Table 2.1. The duck- and turkey-derived viruses, A/Pintail/MN/423/99 H7N3 and A/Turkey/VA/67/02 H7N2 respectively, were obtained from the Southeastern Poultry Research Laboratory, ARS, USDA. The chicken-derived virus, A/Chicken/MD/MinMah/04 H7N2, was offered from Dr. Gelb at the University of Delaware. The viruses were cultivated in 9- to 11-day old embryonated chicken eggs to prepare seed stock. The stocks were then titrated in 9- to 11-day old SPF chicken embryos inoculated via the chorioallantoic sac. The turkey- and chicken-derived viruses were the subtype H7N2, while the duck-derived virus was subtype H7N3.

Table 2.1 Summary of LPAI Infected Pekin Duck Experiment

Group	Number of Pekin Ducks per Cage	Challenge Virus	Subtype	Species of Virus Origin
Control	15	-	-	-
Treatment 1	12	CK/MD/MinMah/04	H7N2	Chicken
Treatment 2	12	TK/VA/67/02	H7N2	Turkey
Treatment 3	15	Pintail/MN/423/99	H7N3	Duck

Pekin ducks were divided into treatment groups, including one control group, containing approximately 12 birds per group and housed in biosafety level three (BSL-3) biocontainment units in the Allen Laboratory. Ducks were infected at two-weeks of age with 10^6 50% embryo infective dose (EID_{50}) in 0.1 ml via the intrachoanal route.

Ducks were observed daily for clinical signs. Birds were scored as follows; no clinical signs were zero (0); mild depression was one (1); moderate to severe respiratory signs including depression, decreased feed intake and neurological signs were two (2); and dead birds were scored as three (3). Birds unable to access food and water due to severity of disease were euthanized using AVMA-approved procedures.

Oral/pharyngeal (O/P) and cloacal swabs were obtained on days 2, 4, 7, 10, and 14 post-inoculation to evaluate virus shed. On day three post-inoculation, three birds from each treatment group were selected for histopathology and immunohistochemistry (IHC) to evaluate tissue tropism. Tissues were placed in formalin and included heart, lung, pancreas/duodenum, kidney, liver, ileum, jejunum, ceca, bursa, thymus, spleen, breast muscle, thigh muscle, brain, nasal cavity, adrenal gland, cecal tonsil, trachea, and reproductive organs. One-hundred to 150 mg of spleen tissue were also collected from each bird, and stored in 5-10 volumes of RNAlater at -80^{o}C.

2.2 Real-Time RT-PCR

Virus amounts were determined from O/P and cloacal swabs. Viral RNA was extracted using Mag-MAX ™ -96 AI/ND Viral RNA Isolation Kit (Ambion, Inc.,

Austin, TX) with the automated King Fisher 96 (Thermo Fisher Scientific, Waltham, MA) in a 96-well format. Each plate contained the respective test virus dilutions (10^{-1} to 10^{-6}) in order to establish a standard curve, which was applied to the values obtained for the samples. These values were used to determine an estimated viral titer in the sample. Using the Ambion AgPath-ID™ (Ambion, Inc., Austin, TX) and the USDA Matrix primers and probe (41), quantitative real-time RT-PCR was performed in the Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA) using the 7500 Fast System Sequence Detection System (SDS) Software Version 1.4.0. The following cycle parameters were used: 45°C for 10 minutes; 95°C for 10 minutes; 45 cycles at 94°C for 1 second and 60°C for 30 seconds.

2.2 AIIM slide preparation

Spotted slides were stored under vacuum. In order to prevent material from binding to areas of the slides where there were no spots, prior to hybridization slides were heated on a 100°C heat block for 3 minutes, soaked in a blocking solution (5xSSC, 0.1%SDS, 1%BSA) and heated to 50°C for 30 minutes. Slides were then rinsed with water three times, and dried by centrifuge at 1200 RPM for 1 minute.

2.3 RNA isolation from duck spleen samples

Total cellular RNA was isolated from 100mg of spleen tissue using the RNeasy Midi RNA Purification Kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocols. Stored spleen tissue was removed from RNAlater and homogenized in 4mL of Buffer RLT using a mortar and pestle. The lysate was centrifuged for 10 minutes at 3,000xg. Next, one volume of 70% ethanol was added to the homogenized lysate and applied to an RNeasy midi column and centrifuged for 5

minutes at 3000xg. Four mL of Buffer RW1 was added to the RNeasy column and centrifuged for 5 minutes at 3,000xg to wash the column. Next, 2.5mL of Buffer RPE was added to the RNeasy column and centrifuged for 2 minutes at 3,000xg to wash the column. Another 2.5mL of Buffer RPE was added to the column, and centrifuged for 5 minutes at 3,000xg to dry the silica gel membrane in the column. Two elutions were performed to isolate total RNA in which 150uL of RNase-free water was added to the silica-gel membrane, stood for 1 minute, then centrifuged for 3 minutes at 3,000xg in each elution. RNA purity was determined spectrophotometrically, and RNA quality was assessed using the Agilent RNA 6000 Nano Assay Protocol (Agilent Technologies) in the Agilent 2100 Bioanalyzer. RNA Integrity Numbers (RINs) were obtained from the bioanlayzer for each sample to confirm sample quality. RIN values can range from 1-10, where 1 is the lowest quality RNA, and 10 is the highest quality (14).

2.4 RNA Amplification

1 ug of total cellular RNA from each sample was amplified into amino allyl modified RNA (aRNA) using the Ambion Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion Inc., Austin, TX) using two rounds of amplification. Total cellular spleen RNA underwent reverse transcription with an oligo(dT) primer bearing a T7 promoter and reverse transcriptase to synthesize virtually full-length single strands of cDNA. The cDNA then underwent second strand synthesis to create double stranded cDNA, and was purified using 250uL of cDNA binding buffer, which binds to synthesized cDNA. The solution was passed through a cDNA filter and washed with 500uL of wash buffer to eliminate unicorporated nucleotides. The cDNA was then eluted twice with 9uL of nuclease-free water heated to 55°C. The resulting

purified, double-stranded cDNA was then used as a template for in vitro transcription (IVT) with T7 RNA polymerase. During the IVT reaction, which occurred for 14 hours at 37°C, antisense RNA (aRNA) copies of each mRNA in the sample were generated. aRNA was purified by adding 350uL of aRNA binding buffer, passing the solution through an aRNA filter cartridge, then washing the column with 650uL of wash buffer, and eluting the aRNA with 100uL of nuclease-free water heated to 50°C. In order to generate enough aRNA for use in the microarray, 2ug of each aRNA sample was used in a second round of amplification. A similar method as described above was used to generate double-stranded cDNA. This cDNA was purified and placed in an IVT reaction where the modified nucleotide 5-(3-aminoallyl)-UTP (aaUTP), was incorporated into the aRNA. aaUTP contains a reactive primary amino group on the C5 position of uracil that can be chemically coupled to N-hydroxysuccinimidyl ester. aRNA was then purified as described above.

2.5 Labeling and Hybridization

10ug of aRNA was fluorescently labeled with Alexa Fluor 555 using a mixture of 9uL of coupling buffer, and Alexa Fluor 555 resuspended in 11uL of DMSO incubated at room temperature for 3 hours in the dark. Labeled aRNA was purified to remove excess dye, then analyzed spectrophotometrically to determine the concentration and labeling efficiencies of aRNA (number of dye molecules per 100 bases) using the dye:base ratio calculator (http://probes.invitrogen.com). Labeled aRNA was then combined with hybridization solution to bring the volume of each sample to 50uL. The mixture was incubated at 100°C for 1 min, then pipetted onto blocked AIIM slides covered with a NuncM Series LifterSlip (Nunc Brand; Rochester,

NY). Each sample hybridized to a blocked AIIM slide overnight in a five-slide hyb-chamber in a 42^{o}C water bath.

2.6 Washing and Scanning the AIIM Slides

After hybridization, slides were put through a washing regimen to remove unbound nucleotides that would cloud the background of the slide and make analysis difficult. Slides were first rinsed in 0.5xSSC and 0.01%SDS solution at room temperature. Next, slides were washed in 0.2xSSC, 0.2%SDS at 42^{o}C for 15 minutes in a nutator to keep a continuous, gentle, mix occurring. After the second wash, slides were washed three times for one minute each in 0.2xSSC solution at room temperature. During these washes slides were agitated using forceps to encourage removal of unbound material. Finally, slides were rinsed three times in water at room temperature. In order to dry the slides in preparation for scanning, slides were centrifuged at 1200RPM for 5 minutes, then placed in corning tubes surrounded by aluminum foil to eliminate photobleaching. Nitrogen gas was then added to the tubes for fluorescent dye preservation. Microarray slides were scanned using a one-dye scan in an Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA).

2.7 Slide Analysis

Spot and background intensities were acquired using GenePix Pro 4.1 Software (Molecular Devices, Sunnyvale, CA). Abnormal spots, including dust and air bubbles, were flagged as absent and removed from further analysis. Spot intensity was determined using a local background subtraction method. A GenePix Results file was also generated in Microsoft Excel 2007 (Microsoft; Seattle, WA). The percentage of unflagged spots was calculated for each slide to determine if the results were suitable

for further evaluation. All slides with at least 72.8-90.6 percent of spots unflagged were further analyzed using GeneSpring.

Data from the analyzed slides were imported to GeneSpring v7.0 (Silicon Genetics, Redwood, City, CA). Each slide was compared to the control slide to determine relative spot intensities, and the degree of up-regulation or down-regulation for each gene. A gene list was created from those elements that appeared in two of the three locations in each slide, in all three experimental conditions. This gene list was exported to Ingenuity Pathway Analysis (IPA) for further analysis.

Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA) was used to identify the canonical pathways containing the greatest expression of genes, and to compare the up-regulation or down-regulation of genes involved in virus and immunity-related pathways. Canonical pathways are well-characterized metabolic and cell signaling pathways present in IPA that have been created based on information from journal articles, review articles, text books, and KEGG Ligand (https://analysis.ingenuity.com).

Chapter 3

RESULTS

3.1 Cross Species Use of the AIIM

Microarray results from the hybridization of chicken, duck, and turkey spleen aRNA to elements on the AIIM revealed that lowering the hybridization temperature from 50^{o}C, which is standard for use on chicken samples, allowed elements to hybridize to turkey- and duck-derived labeled aRNA. Cy3 labeled aRNA from control spleens of ducks and turkeys were hybridized in separate experiments at 25^{o}C, 32^{o}C, 37^{o}C, 42^{o}C, and 50^{o}C, while chicken spleen control samples were hybridized at 37^{o}C only. Slides were scanned with an ArrayWoRx scanner (Applied Precision, Issaquah, WA) using Cy3 filter at an exposure time of 2.00 seconds. Spot and background intensities were acquired using SoftWoRx tracker (Aplied Precision) and those elements exhibiting a spot intensity greater than the sum of the background median intensity plus two times the background standard deviation from two of the three element spots on the array were considered to have produced a positive signal for hybridization. In these experiments, the greatest hybridization success was seen at 42^{o}C for turkey spleen aRNA with 71% of spots with two good replicate spots out of three on the microarray slide, and 25^{o}C for duck spleen aRNA, with approximately 70% of spots with two good replicate spots out of three on the microarray slide. However, when duck aRNA was hybridized at 42^{o}C in later experiments, greater than 90% of spots contained two good replicate spots.

When turkey control spleen aRNA was hybridized to the AIIM slide at 42°C, approximately 71% of spots had two good replicate spots out of three on the microarray slide. When duck control spleen aRNA was hybridized at 42°C , 95.7% of spots had two good replicate spots out of three on the microarray slide. Past experiments performed by our lab had shown that chicken spleen aRNA had greater than 98% of spots display two good replicate spots out of three on the microarray slide when hybridized at 50°C . Based on these results, 42°C was chosen as the temperature to continue further experiments with.

GeneSpring analysis of the turkey, duck, and chicken control spleen aRNA hybridized to AIIM slides revealed that the AIIM has great utility for analyzing gene expression of genes important in the immune response in all three avian species. Table 3.1 displays the number of cytokine and chemokine elements detected in chicken, turkey, and duck spleen tissue using the AIIM, and Table 3.2 depicts the genes in the TLR pathway detected in chicken, turkey, and duck spleen tissue. In both tables, chicken spleen aRNA used in this analysis was hybridized to an AIIM slide overnight at 50°C in which 69.59% of elements had two good replicate spots out of three. Duck spleen aRNA used in the analysis was hybridized at 25°C in which 69.47% of elements had two good replicate spots, and turkey spleen aRNA was hybridized at 37°C in which 74.97% of elements had two good replicate spots. 87% of turkey and duck cytokine and chemokine elements were detected using the AIIM, while 60% were detected in all three species. In the TLR pathway, 76.2% of the elements on the AIIM were detected in turkey spleen, 92.9% of the elements were detected in duck spleen, and 61.9% were detected in all three species.

Following identification of genes detected on the AIIM slides for each species, genes from the TLR pathway, cytokines and chemokines, and housekeeping genes such as GAPDH, Beta-Actin, Gas41, Ppia, ribosomal proteins, Arbp, and SDHA were examined to determine homology among chickens, ducks, and turkeys. On average, it was found that specific genes within the lists above had 91% homology across the species.

3.2 Biological results of Pekin ducks infected with chicken-, turkey-, and duck-derived low pathogenic avian influenza viruses.

In monitoring the response of Pekin ducks to infection with chicken-, turkey-, and duck-derived strains of LPAI, no clinical signs of infection were observed in the Pekin ducks infected with any of the three avian influenza virus isolates. Birds were monitored for mild and moderate to severe clinical signs including mild depression, and depression, anorexia, neurological signs and death respectively. As can be seen in table 3.3, however, no clinical signs were observed between days 2 and 14 after infection.

Additionally, as can be seen in Table 3.4, minimal gross lesions were observed in the nasal cavity, trachea, and pulmonary and renal systems of Pekin ducks infected with chicken-, duck-, and turkey- derived LPAI strains. Three days after infection with LPAI, three birds from each treatment group were sampled for detection of gross lesions. While gross lesions were observed in each of these organ systems in at least one strain of virus, there were no statistically significant differences in gross lesions among the viruses.

Table 3.1 Cytokine and Chemokine elements present on the AIIM detected in chicken, duck, and turkey control spleen aRNA. A picture of the species indicates the gene was detected in at least one replicate of three on the AIIM slide hybridized to the spleen aRNA of the respective species.

Gene	Chicken	Turkey	Duck
chCCLi7			
TNFSF5			
TNFSF5			
IL8			
IL4			
IL13			
IL12beta			
IL10			
IL6			
chCCL20			
chCXCLi1			
IL18			
K203			
ah221			
ah294			
IL16			
IL1-beta			
PDGF-B			
LPSc_A07			
IL18			
Clone 391 cytokine			
iNOS			
macrophage inflammatory protein 1-beta			
K60 protein			
iNOS			
CXCL3			
IL1-beta			
CCL7			
CXCL3			
CCL16			

Table 3.2 Elements in the TLR pathway present on the AIIM detected in chicken, duck, and turkey control spleen aRNA. A picture of the species indicates the gene was detected in at least one replicate of three on the AIIM slide hybridized to the spleen aRNA of the respective species.

Gene	Chicken	Turkey	Duck
MHC class 1 antigen			
Jun protein			
NF-kB1			
Lipopolysaccharide binding protein			
Toll/interleukin-1 receptor 8			
Mitogen-activated protein kinase 8			
TLR10			
Helix-loop-helix kinase			
MAP3K7			
TLR5 precursor			
TLR3			
TRAF1			
TOLLIP			
TLR7			
TLR5			
TLR4			
TAB1			
PKR			
MKK3			
MEKK1			
MD2			
IL12beta			
IL10			
ICSBP			
COX2			
IL6			
Myeloid differentiation response gene			
IL1-beta			
Heat shock protein 90			
iNOS			
T1L			
Jun protein			
iNOS			
IL1-beta			
IRF-2			
IRAK-2			
NF-kB p50 subunit			
IL1-beta cDNA clone			
C-fos proto-oncogene			
TRAF6			
TLR2			
MHC class 1 glycoprotein			

Table 3.3 Clinical Signs* Observed in Pekin Ducks Infected with Chicken-, Duck-, and Turkey-derived Strains of LPAI

Challenge Virus	Day 2	Day 4	Day 7	Day 10	Day 14
Chicken/MD/MinMah/04	0	0	0	0	0
DuckPintail/MN/423/99	0	0	0	0	0
Turkey/VA/67/02	0	0	0	0	0

* No signs = 0; Mild to moderate respiratory signs (mild depression in ducks) = 1; Moderate to severe (i.e. depressed, not eating, neurological signs) = 2; Dead = 3

Table 3.4 Gross Lesions ** Observed in Pekin Ducks at Day 3 Post-infection with Chicken-, Duck-, and Turkey-derived Strains of LPAI

Challenge Virus	Nasal Cavity	Pulmonary	Trachea	Renal
Chicken/MD/MinMah/04	2/3	1/3	1/3	1/3
DuckPintail/MN/423/99	2/3	0/3	0/3	0/3
Turkey/VA/67/02	1/3	1/3	0/3	0/3

** Number of birds with significant lesions / Total number of birds sampled

Differences were observed in viral recovery and persistence of the different LPAI strains in ducks when comparing viruses isolated from oral/pharyngeal and cloacal swabs. As can be seen in Table 3.5, the DuckPintail/MN/423/99 virus had the highest recovery in the oral/pharyngeal swabs throughout the experiment, although this increase was not statistically significant. In contrast, significant differences among the virus strains were observed in persistence and recovery when examining cloacal swabs. The virus titre was calculated using quantitative real-time PCR. There was both greater recovery and longer persistence of the DuckPintail/MN/423/99 virus when compared to the chicken- and turkey- derived viruses. As can be seen in Table 3.6, the log titre for chicken-derived virus ranged from 0 to 1.7, while turkey-derived virus ranged from 0 to 1.9 over days 2 through 14 after infection. The virus titre for the duck-derived virus, however, ranged from 1.4 to 5.2 over the course of the experiment. This greater viral recovery and persistence was the most biologically significant result observed.

Table 3.5 Virus Isolation by qRT-PCR* of Oral/Pharyngeal Swabs

Challenge Virus	Day 2	Day 4	Day 7	Day 10	Day 14
Chicken/MD/MinMah/04	3.7	1.8	3.6	2.0	0
DuckPintail/MN/423/99	4.9	4.8	4.3	3.3	0.3
Turkey/VA/67/02	4.2	2.5	3.0	0.4	0.1

* Log of titre

Table 3.6 Virus Isolation by qRT-PCR* of Cloacal Swabs

Challenge Virus	Day 2	Day 4	Day 7	Day 10	Day 14
Chicken/MD/MinMah/04	1.0	0	1.7	0	0
DuckPintail/MN/423/99	3.7	5.2	1.0	3.4	1.4
Turkey/VA/67/02	0.7	0	1.9	0	0

* Log of titre

3.3 Infecting ducks with LPAI alters gene expression.

Flourescently labeled spleen aRNA isolated from ducks infected with chicken-, duck-, or turkey-derived strains of LPAI were hybridized to AIIM slides at 42^{o}C. Slides were scanned and analyzed to determine the elements on the AIIM that appeared in two of the three replicates on each slide of all three virus-origin microarray slides. Microarray analysis revealed that infecting ducks with LPAI alters gene expression. When comparing genes that exhibited a two-fold response after infection with chicken-, turkey-, or duck- derived LPAI it was found that 61 genes exhibited at least a two fold response to all three virus strains. As can be seen in Figure 3.1, many genes exhibited at least a two-fold response to more than one of the virus strains, while there were also genes that exhibited altered expression uniquely to each strain of LPAI.

The 61 genes exhibiting at least a two-fold response to all three genes included several interferon-, and lipopolysaccharide- stimulated genes, IL12, and outer membrane proteins. The names of the 61 genes as they appear in GeneSpring, along with their expression levels in response to the chicken-, duck-, and turkey- derived virus strains are in Table 3.7. Similar fold changes were seen in 55 of the specific genes (approximately 90%), while six of the genes exhibited unique expression in response to a specific virus strain. IFNb_P10 was down-regulated in response to chicken-derived LPAI, but induced in response to duck- and turkey-derived LPAI strains. IFNk_M07 exhibited a 11.46 fold change in response to turkey-derived LPAI strain, but only a 2.588 and 2.067 fold change in response to chicken- and duck-derived LPAI strains respectively. LPSe_G08 exhibited a 9.03 fold change in response to turkey-derived LPAI strain, but only a 2.176 and 3.082 fold change in response to

chicken- and duck-derived LPAI strains respectively. LPSh_C21 displayed a 66.3 fold change in response to chicken-derived LPAI, but only a 2.28 and 3.53 fold change in response to duck-, and turkey-derived LPAI strains respectively. LPSj_O22 exhibited a 12.64 fold change in response to chicken-derived virus, but only a 2.319 and 4.488 fold change in response to duck-, and turkey-derived LPAI strains respectively. Finally pmp1c.pk005.n11 demonstrated a 0.0768 fold change (a down-regulation of 13.021) in response to turkey-derived LPAI, but only a 0.196 (down regulation of 5.102) and a 0.105 (down-regulation of 9.524) in response to chicken- and duck-derived LPAI strains respectively.

In comparing the elements that responded to only one of the LPAI strains, all virus strains caused changes in expression in control genes, interferon-, and lipopolysaccharide- stimulated genes, and surface proteins. However, a few unique genes responded only to one of the virus strains. Groups of elements on the AIIM that responded only to the chicken-derived strain of LPAI included 11 control genes, 36 interferon-stimulated genes, 45 lipopolysaccharide-stimulated genes, CD18, and 7 surface proteins. Elements that responded only to the duck-derived strain of the virus included 26 control genes, 29 interferon-stimulated genes, 45 lipopolysaccharide-stimulated genes, and 18 surface proteins. Additionally, Toll-Like Receptor 1 (TLR1) displayed a 2.247 downward fold change, while SOCS5 (suppressor of cytokine signaling) exhibited a 2.36 downward fold change in response to duck-derived virus. Elements that responded only to the turkey-derived virus included 98 control genes, 178 interferon-stimulated genes, 226 lipopolysaccharide-stimulated genes, and 107 surface proteins.

Additionally, in response to the turkey-derived virus, TLR7 exhibited a 3.316 fold change increase in expression.

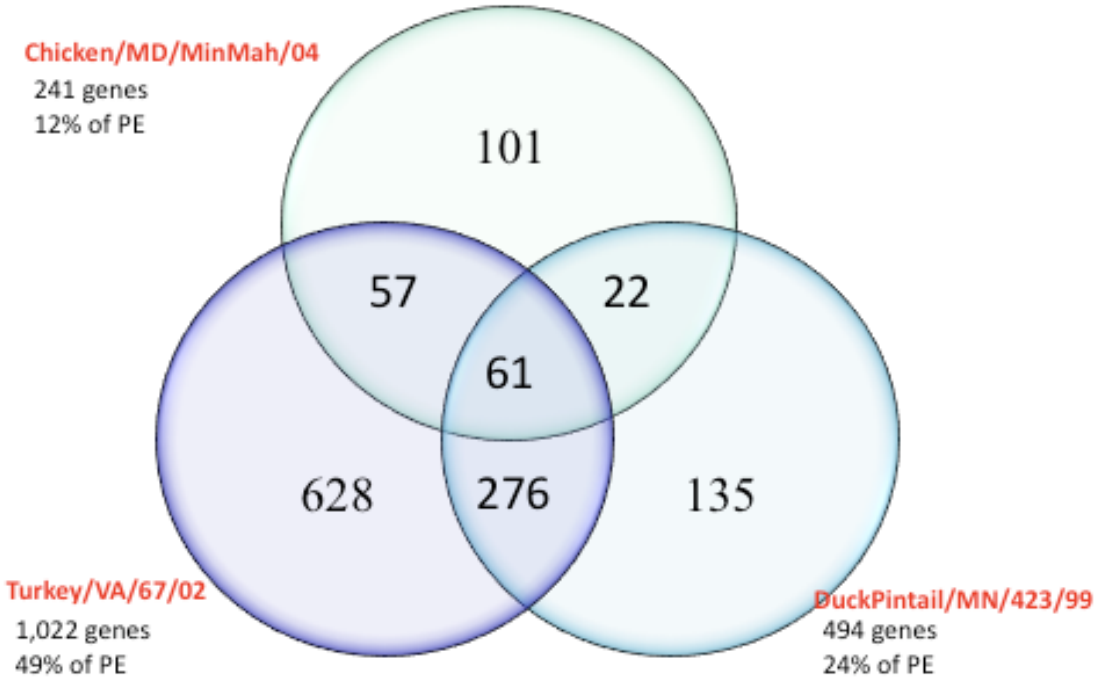

Figure 3.1 Genes displaying a two-fold change in expression in response to infection with strains of LPAI derived from chickens, ducks, and turkeys. PE is all genes that were detected in two of three replicates on each AIIM slide in each of the experimental condition slides.

Table 3.7 Expression levels of the 61 genes exhibiting at least a two-fold change in expression in response to infection with chicken-, duck-, or turkey-derived LPAI strains. Gene names are those that appear in GeneSpring. The list includes control genes ("control"), interferon-stimulated genes ("IFN"), lipopolysaccharide-stimulated genes ("LPS"), interleukins ("IL12"), placental growth factor ("pgf"), a linker protein ("pgl"), and outer membrane proteins ("pmp"). Elements that experienced a fold change greater than 1.00 had increased expression when compared to the expression observed in control spleen, while those with less than a 1.00 fold change had decreased expression when compared to the expression observed in control spleen.

	Name in GeneSpring	Fold change in response to chicken-derived LPAI	Fold change in response to duck- derived LPAI	Fold change in response to turkey-derived LPAI
Control genes	Control_C10	0.484	0.449	0.221
	Control_P03	0.495	0.237	0.192
	Controlb_D13	2.119	2.165	2.459
	Controld_B11	0.34	0.167	0.171
	Controld_D16	0.364	0.405	0.162
	Controle_B09	0.463	0.441	0.491
	Controle_H24	0.169	0.131	0.0866
Interferon-stimulated genes	IFN_G21	0.414	0.279	0.104
	IFNb_C17	2.228	3.673	5.751
	IFNb_D05	0.444	0.403	0.285
	IFNb_F19	7.116	7.097	7.716
	IFNb_J20	0.448	0.476	0.491
	IFNb_P10	0.489	2.181	2.116
	IFNc_A12	0.424	0.313	0.145
	IFNc_D07	3.001	2.014	2.18
	IFNd_I21	2.655	2.225	2.293
	IFNe_L18	0.271	0.188	0.109
	IFNe_L20	5.581	7.261	10.77
	IFNg_C08	0.212	0.298	0.122
	IFNh_N10	0.192	0.44	0.293
	IFNk_J19	0.443	0.427	0.387
	IFNk_M07	2.588	2.067	11.46
	IFNm_A18	2.303	3.072	5.551
	IFNm_E02	0.411	0.414	0.377
	IFNm_J23	0.396	0.453	0.197
Lipopolysaccharide--stimulated genes	LPS_I03	3.007	41.46	2.07
	LPS_P16	2.562	3.385	4.795
	LPSb_A24	0.485	0.33	0.364
	LPSc_M06	0.409	0.26	0.206
	LPSc_P18	2.751	2.229	3.706
	LPSe_G08	2.176	3.082	9.033
	LPSf_B18	0.47	0.373	0.194
	LPSf_I05	0.461	0.31	0.0641
	LPSg_D08	0.389	0.449	0.241
	LPSg_G07	0.345	0.481	0.246
	LPSg_K02	0.392	0.48	0.256
	LPSg_K06	0.5	0.416	0.37
	LPSg_L22	0.38	0.449	0.477
	LPSh_C21	66.33	2.28	3.52
	LPSh_H08	6.983	2.483	4.295
	LPSh_I04	0.242	0.334	0.0779
	LPSi_A24	0.301	0.482	0.452
	LPSi_E07	0.306	0.405	0.159
	LPSi_L05	3.253	7.512	4.477
	LPSj_O22	12.64	2.319	4.488
	LPSk_B11	2.992	2.083	2.831
	LPSl_M21	0.351	0.331	0.447
	LPSl_O09	0.409	0.449	0.159
	LPSl_P06	0.404	0.28	0.0356
	LPSm_B10	0.433	0.336	0.328
	LPSm_G24	0.383	0.264	0.187
	LPSm_K14	0.495	0.4	0.232
	MM.IL12beta	2.028	2.187	2.735
	pgf1n.pk003.i17	0.427	0.233	0.161
	pgl1n.pk014.k6	0.356	0.232	0.146
Outer membrane proteins	pmp1c.pk005.n11	0.196	0.105	0.0768
	pmp1c.pk005.o06	0.336	0.493	0.412
	pmp1c.pk006.h06	0.363	0.342	0.439
	pmp1c.pk007.d10	0.488	0.356	0.336
	pmp1c.pk007.n16	0.37	0.365	0.437
	pmp1c.pk007.o04	0.472	0.206	0.143

The regulation of a subset of chemokines and cytokines, proteins that regulate the nature of immune responses, present in IPA was analyzed for each virus strain to determine variability in the innate immune response of ducks to each strain. As can be seen in Figure 3.2, there was a similar, weak (less than two-fold response to infection) cytokine and chemokine response to all strains of LPAI. Most genes exhibited less than a two-fold response to infection, with gene expression values close to those observed in the control spleen sample. These patterns are consistent with the lack of differences observed in the biological results of the experiment.

The most robust response was seen in infection of Pekin ducks with the turkey-derived virus. IL2, IL8, IL12, CD40, and CCL4 all experienced at least a two-fold induction in expression. The duck-derived virus, on the other hand, elicited the weakest chemokine and cytokine response with only IL12 experiencing at least a two-fold up-regulation. The only gene with at least a two-fold down-regulation of expression occurred in CCL7 in response to the chicken-derived virus. As can be seen in Figure 3.2, IL1B, CCL5, and IL12 each experienced two-fold up-regulation in response to the chicken-derived virus.

In Figure 3.2 we observe that IL2 had the most significant differences in response to the different LPAI strains. IL2 was significantly induced in response to both the turkey- and chicken- derived virus, with the greatest expression seen in response to the turkey-derived virus, while no significant change in expression was observed in response to the duck-derived virus. This result, however, has yet to be confirmed by RT-PCR analysis.

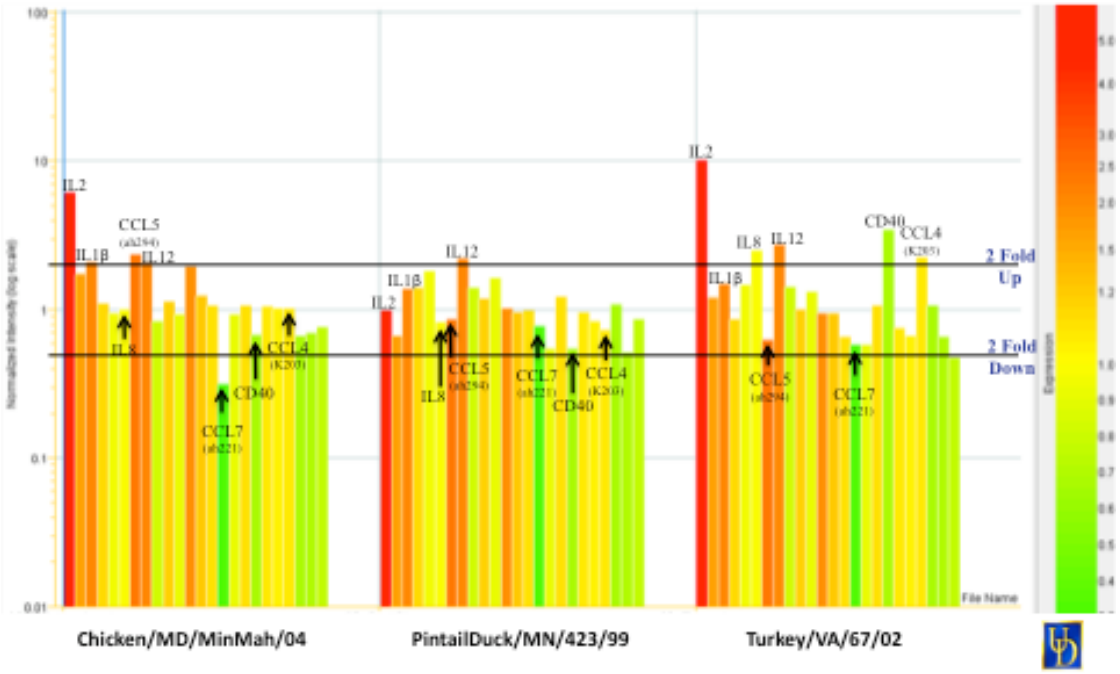

Figure 3.2 Expression of Cytokines and Chemokines in Ducks infected with Chicken-, Duck-, and Turkey- derived strains of LPAI. Elements in red were up-regulated, while elements in green were down-regulated. Fold changes in gene expression were calculated by comparing the expression seen in control duck spleen to the expression seen in duck spleen excised from ducks on day 3 post-infection with chicken- duck-, or turkey-derived LPAI.

3.4 Canonical Pathway Analysis

Canonical pathways that had the greatest number of genes with expression values when compared to the total number of genes in the pathway were identified for each virus strain. Each species-derived strain shared the same five canonical pathways that had the greatest ratio of the number of expressed genes to the total number of genes in the pathway observed as identified by IPA. These five pathways included integrin signaling, glucocorticoid signaling, Fcy receptor (which recognize immunoglobulins) mediated phagocytosis in macrophages and monocytes, ephrin receptor signaling (involved in cell to cell communication during development), and PTEN (phosphatase and tensin homolog, which is a tumor suppressor gene) signaling. Expression levels of genes present in canonical pathways involving the innate immune response were then compared to determine if genes present in the pathways exhibited different responses to each of the virus strains. Findings from the TLR pathway and NF-κβ signaling pathway are presented below.

The expression of genes involved in the five canonical pathways that had the greatest ratio of the number of expressed genes to the total number of genes in the pathway were largely similar among viral strains, although a few differences of importance to immune response were noted. In the PTEN signaling pathway and Glucocorticoid receptor signaling pathway, there was a 1.037 fold change up in BCL2 in response to chicken-derived virus, while there was a 1.014 and 1.241 fold change down in response to duck- and turkey-derived viruses respectively. Also, there was a 1.038 fold change up in BCL2L1 (BCL2-like 1) in response to chicken-derived virus, while there was a 1.239 and 2.762 fold change down in response to duck- and turkey-derived viruses respectively. Also within the PTEN signaling pathway, Caspase 3 was

induced in response to all three virus strains, but experienced only a fold change of 1.552 in response to chicken-derived LPAI, while it experienced a 9.128 fold change in response to duck-derived virus, and an 8.602 fold change in response to turkey-derived virus.

Overall, the TLR pathway exhibited similar gene expression, with only a few differences in response to the different species-derived virus strains. The TLR pathway shown in Figure 3.3 was condensed to show only the TLR3 and TLR7 receptors since they are the receptors involved in the response of ducks to avian influenza. TLR3 and TLR7 were both up-regulated in response to each virus strain. TLR3 experienced the greatest up-regulation in response to the chicken-derived virus (4.357 fold change) , while TLR7 experienced the greatest up-regulation in response to the turkey-derived virus (3.316 fold change) . Continuing along the pathway, RAK was similarly down-regulated in response to all three virus isolates with a fold change of -1.479 in response to chicken-derived LPAI, -2.053 in response to turkey-derived LPAI, and -1.832 to duck-derived LPAI. TOLLIP, however, was differentially expressed as it was down-regulated (-1.057 fold change) in response to the chicken-derived virus, but up-regulated in response to the duck-, and turkey-derived virus with fold changes of 2.118 and 3.123 respectively. TAK1 was down-regulated in response to all three virus strains , and MEKK1 was induced in all strains. Map kinase MKK3/MKK6 was another gene that was down-regulated in response to the chicken-derived virus, while it was induced in response to the duck-, and turkey-derived viruses. IKKα was down-regulated in all three virus strains, but exhibited a very weak response in each as indicated by the light green color in Figure 3.3. Finally, c-Jun was significantly down-regulated in each virus strain, with the greatest down-regulation

observed in duck- (-9.524 fold change) and turkey-derived (-13.021 fold change) viruses. The fold change of c-Jun was only -5.102 in response to chicken-derived virus.

The genes present in the NF-κβ activation by virus pathway also exhibited similar expression, with a few exceptions, to infection with the different strains of LPAI. NF-kB is known to regulate expression of anti-viral cytokines and to promote the induction of pro-apoptotic factors, which, in the case of influenza viruses, may result in enhanced virus propagation (54). Genes in which differential expression was observed in response to the different virus strains included BCL2 and BCL-XL (BCL2L1), which were described above, cIAP, and Casepase 3 and 6. BCL2 and BCL-XL each were up-regulated in response to the chicken-derived virus, but down-regulated in response to the duck-, and turkey-derived virus strains. The gene cIAP was down-regulated in response to the chicken-, and turkey-derived virus, but induced in response to the duck-derived virus. Casepase 3 and 6 were both induced in response to the chicken-derived virus, with the greatest induction seen in response to the duck-, and turkey-derived virus. The expression of Casepase 3 is described above. Casepase 6 had a 1.474 fold change in response to chicken-derived LPAI, a 9.586 fold change in response to duck-derived virus, and a 10.750 fold change in response to turkey-derived virus.

Other canonical pathways involving the innate immune system were viewed to identify other genes that displayed a different response to the three virus isolates that could help explain the subtle biological difference seen *in vivo*. The genes identified include MIP1β (CCL4), EIF2AK2, IL2 and MX1. MIP1β experienced a 1.040 fold change up in response to chicken-derived virus, a -1.050 fold change in

response to duck-derived virus, and a -1.330 fold change in response to turkey-derived virus. EIF2AK2 experienced a -1.550 fold change in response to chicken-derived virus, and a 1.572 and 1.945 fold change up in response to duck- and turkey-derived strains of LPAI respectively. IL2 experienced a negative fold change in response to the chicken- and duck-derived viruses, but an induction in response to the turkey-derived virus. MX1 had a fold change of -1.828 in response to the chicken-derived virus, and a positive 2.107 and 2.651 fold change in response to duck- and turkey-derived strains of LPAI respectively.

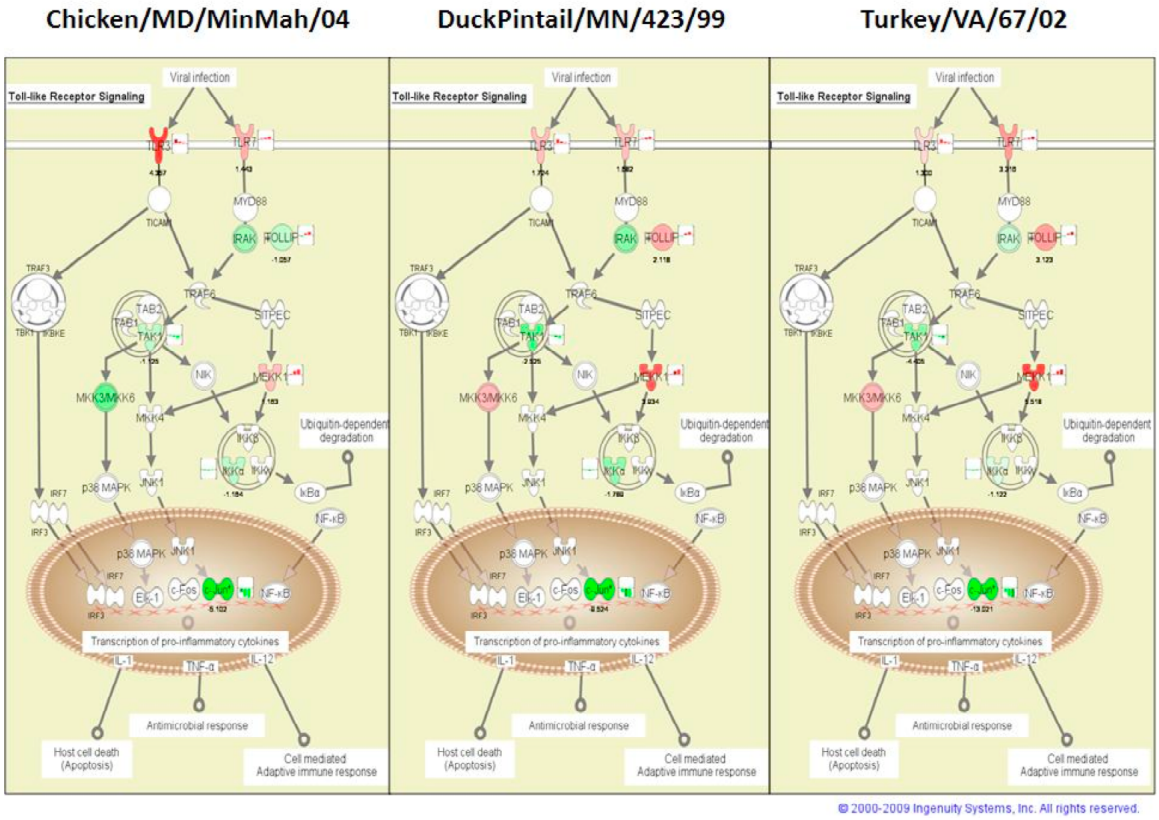

Figure 3.3 Expression of Genes involved in the TLR Pathway in Response to Chicken-, Duck-, and Turkey-Derived Strains of LPAI. Genes colored in red were up-regulated, while genes colored in green were down-regulated. The intensity of the color is indicative of the expression level. The bar chart next to the genes indicate the expression level of the genes in all three experimental slides with chicken-derived virus represented by the left column, duck-derived virus in the middle column, and turkey-derived virus in the right column.

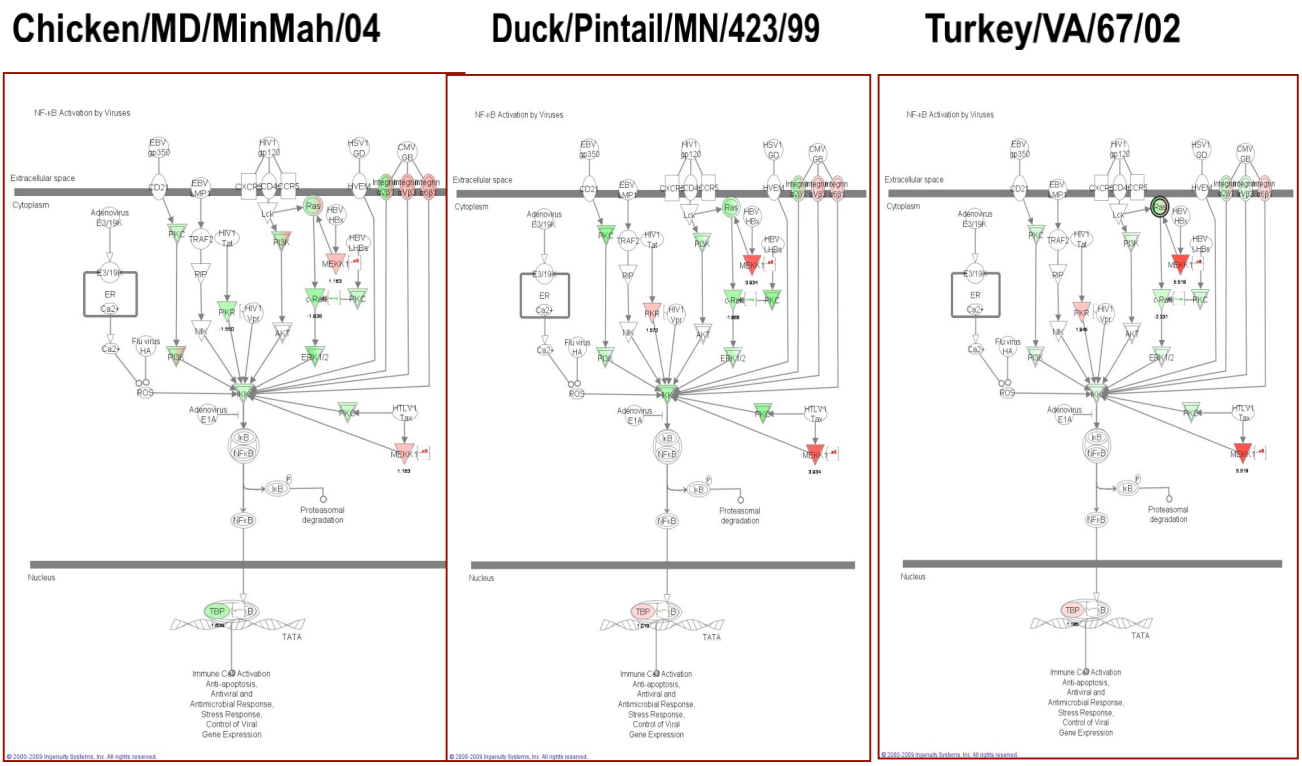

Figure 3.4 Expression of Genes involved in the NF-κβ Activation by Virus Pathway in Response to Chicken-, Duck-, and Turkey-Derived Strains of LPAI. Genes colored in red were up-regulated, while genes colored in green were down-regulated. The intensity of the color is indicative of the expression level. The bar chart next to the genes indicate the expression level of the genes in all three experimental slides with chicken-derived virus represented by the left column, duck-derived virus in the middle column, and turkey-derived virus in the right column.

Chapter 4

DISCUSSION

4.1 Cross-Species use of the AIIM

Based on microarray analysis, the AIIM can be used for analysis of gene expression in chickens, ducks, and turkeys. Results showed that lowering the hybridization temperature increased the number of elements that were detected in the turkey and duck spleen samples on AIIM slides. Lowering the temperature of hybridization decreased the stringency, allowing cross hybridization to occur. Considering that 71% and 95.7% of AIIM elements were detected in turkey and duck samples hybridized at 42^{o}C to AIIM slides respectively, and to minimize the loss of stringency by lowering the hybridization temperature too much, which would allow background materials to hybridize to the slide, 42^{o}C was chosen as the temperature to continue further experiments with. Furthermore, the utility of the AIIM in monitoring gene expression in chickens as well as turkeys and ducks was shown in the great number of genes detected in both turkeys and ducks from the TLR pathway (76.2% of elements from the TLR pathway were detected in the turkey control sample, and 92.9% were detected in the duck sample), and chemokine and cytokine gene lists (87% of elements in the cytokine and chemokine list were detected in duck and turkey control samples). When examining the homology of specific genes within those lists among species, the genes had an average homology of 91% indicating conservation of innate immune genes. Thus the chicken cDNA used to form the spots on the AIIM is

capable of hybridizing to duck and turkey gene homologs. Based on their homology, I would expect a great number of the chicken elements on the AIIM to hybridize to the equivalent duck and turkey genes.

4.2 Biological results reaffirm the asymptomatic nature of LPAI infection in ducks

No differences were observed in the clinical signs and gross lesions in response to infection of ducks with LPAI H7 viruses of chicken-, duck-, and turkey-origin. Due to the low pathogenicity of the three strains, few clinical symptoms and gross lesions would be expected in ducks, which serve as a natural reservoir of LPAI, and infection of whom is often asymptomatic.

Biological results also indicated that virus replication and persistence in ducks was greater in the cloacal swabs than the oral/pharyngeal swabs. The reason for this is two-fold. First, in wild ducks it is known that LPAI is preferentially replicated in cells lining the intestinal tract, and therefore excreted in high amounts in the feces. Secondly, due to the asymptomatic nature of LPAI infection in ducks, few respiratory signs are observed in infected birds. Therefore, we would expect to obtain greater amounts of virus from cloacal swabs rather than oral/pharyngeal swabs. The biological results also revealed that greater virus replication and persistence was seen in response to the duck-origin virus over the other strains. The reason for this is unclear, but perhaps the duck-origin virus possesses a natural advantage for replication in ducks since it originated in that species.

4.3 Transcriptional response to LPAI infection

4.3.1 Genes experiencing a two-fold response to LPAI infection

GeneSpring analysis revealed that 61 genes present on the AIIM experienced a two-fold change in response to the three virus strains, while other genes uniquely responded to the different strains. Overall this shows that infecting ducks with LPAI alters gene expression. The 61 genes exhibiting at least a two-fold response to all three genes included several interferon-, and lipopolysaccharide-stimulated genes, as well as outer membrane proteins. The expression level in response to each of the virus strains was similar across virus strains for 90% of these genes, indicating that a similar pattern of gene expression was elicited by the chicken-, duck-, and turkey-derived strains of LPAI.

There were also unique genes that responded only to turkey- or duck-derived virus that could help to explain duck response to the LPAI strains. First, there was a negative 2.36 fold change of a suppressor of cytokine signaling in response to duck-derived LPAI. Since cytokines are an integral part of amounting an immune response to avian influenza, decreasing the expression of a suppressor of cytokine signaling would cause an increase in cytokine signaling, and thus may lead to an increase in the immune response. Such a response would explain why no clinical signs were observed in ducks expose to LPAI. A positive 3.316 fold change was seen in Toll-like receptor 7 (TLR7) in response to infection with turkey-derived LPAI. Activation of TLR7 leads to an up-regulation of proinflammatory cytokines and IFNα, which could contribute to the antiviral defense of ducks (29).

4.3.2 Cytokine and chemokine response to LPAI strains

Cytokines and chemokines are secreted proteins that possess growth, differentiation, and activation functions that regulate the nature of immune responses (11). The production of certain cytokines determines whether the individual mounts a cytotoxic, humoral, cell-mediated, or allergic response (11). In addition to initiating the immune response, cytokines and chemokines generate symptoms associated with infections and inflammatory disorders such as fever, lethargy, anorexia, and vascular leakage (11). The chicken-, duck-, and turkey-derived strains of LPAI all elicited a similar pattern of cytokine and chemokine response. Consistent with the lack of clinical signs and gross lesions observed in the ducks, most cytokines and chemokines experienced less than a two-fold response to LPAI infection.

4.3.3 Potential genes of interest based on Canonical Pathways

Examination of canonical pathways in IPA revealed differential expression of several genes within pathways important in innate immunity that may help to explain the subtle biological differences seen in response to the different strains of LPAI as well as lead to a better understanding of the response of ducks to LPAI. Such genes warrant further investigation with RT-PCR to determine quantitative differences in expression in response to each strain. Genes of particular interest due to their role in immune response, and in that they responded differently to the chicken-, duck-, and turkey-derived LPAI strains include BCL2, MIP1β (CCL4), EIF2AK2 (PKR), Caspase 3/6, IL2, and MX1.

As seen in Figure 3.4, there was a 1.037 fold change up in BCL2 in response to chicken-derived virus, while there was a 1.014 and 1.241 fold change down in response to duck- and turkey-derived viruses respectively. The dominant role

of BCL2 is to encode a protein that prolongs the survival of hematopoetic cells in the absence of required growth factors or in the presence of stimuli inducing cellular death (17). In chickens, BCL2 has its highest expression levels in the thymus, with lower levels occurring in the spleen, kidney, heart, ovary, nervous system, bone marrow and bursa (17). The up-regulation of this anti-apoptotic gene could partially explain the minimal gross lesions and clinical signs observed in ducks to response to LPAI.

Macrophage inflammatory protein-1 beta (MIP1β) or CCL4 is a member of the chemokine superfamily that has been shown to attract the migration of chicken heterophils (27). Expression of MIP1β has been linked to respiratory rales, coughing, and nasal discharge in response to *Mycoplasma gallisepticum* in chickens as a result of accumulation of lymphoid cells (27). MIP1β experienced a 1.040 fold change up in response to chicken-derived virus, and a 1.050 and 1.330 fold change down in response to duck- and turkey-derived LPAI respectively. The less than two-fold response of MIP1β in Pekin ducks in response to all three virus isolates, as well as the slight down-regulation in duck-derived virus could play a role in the asymptomatic response of duck to LPAI, and the insignificant amounts of virus isolated from oral/pharyngeal swabs.

Eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2 or PKR) is a gene involved in several cellular roles including apoptosis, growth, transformation, proliferation, and antiviral response (Ingenuity Pathway Analysis). Molecular functions include nucleotide binding, double-stranded RNA (dsRNA) binding, protein kinase activity, and protein and ATP binding to name a few (Ingenuity Pathway Analysis). Following activation by dsRNA during viral infection, the kinase catalyzes a reaction that stops the initiation of protein synthesis (1).

EIF2AK2 experienced a -1.550 fold change in response to chicken-derived virus, and a 1.572 and 1.945 fold change up in response to duck- and turkey-derived strains of LPAI respectively. Increased expression in ducks infected with duck-origin virus leading to the question of if ducks have an advantage in immune response to dsRNA virus intermediates by induction of pro-inflammatory cytokines.

Casepase 3 and 6 are genes that are both involved in apoptosis and according to IPA analysis were induced in response to the three virus strains, but especially in response to duck- and turkey-derived virus strains. Caspase 3 experienced a fold change of 1.552 in response to chicken-derived LPAI, while it experienced a 9.128 fold change in response to duck-derived virus, and an 8.602 fold change in response to turkey-derived virus. Casepase 6 had a 1.474 fold change in response to chicken-derived LPAI, a 9.586 fold change in response to duck-derived virus, and a 10.750 fold change in response to turkey-derived virus. Casepase 3 and 6 encode proteins that play a central role in the execution-phase of apoptosis, and serve as modulators of apoptosis (Ingenuity Pathway Analysis). Further examination of these genes may help to determine the role of apoptosis in duck responses to AIV. This, in turn may lead to an explanation of the longer viral shedding and persistence seen in ducks infected with duck-origin virus.

Interleukin 2 (IL2) encodes cytokines that are important for the proliferation of T and B lymphocytes. IL2 is involved in biological processes such as anti-apoptosis, cell-cell signaling, natural killer cell activation, T-cell proliferation, and negative regulation of inflammatory response (Ingenuity Pathway Analysis). IL2 has also been shown to induce in vitro proliferation of duck splenocytes and strengthen duck immune responses induced by injection of an inactivated oil emulsion

vaccine against avian influenza virus (56). IPA analysis revealed that IL2 experienced reduced expression in response to the chicken- and duck-derived viruses, while experiencing induction as a result of the turkey-virus strain. More research needs to be done to determine the role IL2 plays in the antiviral immune response to LPAI. Perhaps, though, the down-regulation of IL2 leads to impaired immune cell proliferation, and thus leads to higher virus isolation and persistence in the duck intestinal tract.

MX1, which codes for the Mx protein, has been implicated in resistance to influenza A viruses in mice (9). However, the antiviral contribution of Mx protein in avians is not well defined. In one study, transfected mouse and chick cells expressing cloned duck Mx protein did not exhibit increased resistance to influenza virus despite the fact that, similar to mammals, avian Mx1 was strongly induced in response to virus and dsRNA (9). However, in another study, Mx genes from different species of chicken were demonstrated to confer antiviral responses to influenza virus (26). Antiviral activity was specifically linked to an amino acid substitution of Ser to Asn at position 631 (26). According to our analysis, MX1 had a fold change of -1.828 in response to the chicken-derived virus, and a positive 2.107 and 2.651 fold change in response to duck- and turkey-derived strains of LPAI respectively. The induction in turkey- and duck-derived strains is consistent with previous experiments in which Mx1 was induced in response to avian influenza infection. Further studies on the antiviral properties of Mx in different avian species will still need to be done to obtain a fuller understanding of the role Mx plays in avians infected with avian influenza. Considering that ducks are natural reservoirs for avian influenza, often experiencing asymptomatic infection, natural selection may not

have favored an adaptation in Mx for antiviral properties as it may have in mammals, which experience clinical signs during infection. This could help to explain the differences in antiviral properties seen in Mx between mammals and avians.

In conclusion, the AIIM is a tool that allows us to monitor duck immune gene transcription in addition to chicken and turkey. We observe no differences in clinical signs and gross lesion in the response of ducks to LPAI H7 viruses of chicken-, duck-, and turkey- origin, however, the duck-origin LPAI H7 appears to have an advantage in replication and persistence in ducks. Analysis of immune gene transcription/expression in the spleen reveals few substantive differences when the three viruses are compared. However, when analyzed in the context of immune pathway analysis, there are some intriguing and subtle expression differences that might correlate with the differences seen *in vivo*.

REFERENCES

1. Eukaryotic translation initiation factor 2-alpha kinase 2. 2009. 2009.

2. H5N1 avian influenza: Timeline of major events. World Health Organization. 2009.

3. H5N1 avian influenza risk assessment report - August 2008. Ministry of Health: Communicable diseases division . 2008.

4. Microarrays: Chipping away at the mysteries of science and medicine. 2007. 2007.

5. Anonymous RNA replicase. 2009. 2004.

6. Abdel-Ghafar, A. Update on Avian Influenza A (H5N1) virus infection in humans. N. Engl. J. Med. 358:261-273. 2008.

7. Alexander, D. J. An overview of the epidemiology of avian influenza. Vaccine 25:5637-5644. 2007.

8. Alexander, D. J. A review of avian influenza in different bird species. Vet. Microbiol. 74:3-13. 2000.

9. Bazzigher, L., Schwarz, A., and Staehel, P. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. Virology 195:100-112. 1993.

10. Bliss, T. W., Dohms, J. E., Emara, M. G., and Keeler, C. L. Gene expression profiling of avian macrophage activation. Vet. Immunol. Immunopathol. 105:289-299. 2005.

11. Borish, L. C., and Steinke, J. W. Cytokines and chemokines. J Allergy Clin Immunol 11:S460-S475. 2003.

12. Bowes, V. A., Ritchie, S. J., Byrne, S., Sojonky, K., Bidulka, J. J., and Robinson, J. H. Virus characterization, clinical presentation and pathology associated with H7N3 avian influenza in British Columbia broiler breeder chickens in 2004. Avian Dis. 48:928-934. 2004.

13. Cheung, T. K. W., and Poon, L. L. M. Biology of influenza A virus. Ann. N. Y. Acad. Sci. 1102:1-25. 2007.

14. Copois, V., Bibeau, F., Bascoul-Mollevi, C., Salvetat, N., Chalbos, P., Bareil, C., Candeil, L., Fraslon, C., Conseiller, E., Granci, V., Maziere, P., Kramar, A., Ychou, M., Pau, B., Martineau, P., Molina, F., and Del Rio, M. Impact of RNA degradation on gene expression profiles: Assessment of different methods to reliably determine RNA quality. J. Biotechnol. 127:549-559. 2007.

15. de Jong, M. D., and Hien, T. T. Avian influenza A (H5N1). J. Clin. Virol. 35:2-13. 2006.

16. Ducatez, M. G., Webster, R. G., and Webby, R. J. Animal influenza epidemiology. Vaccin 26:D67-D69. 2008.

17. Eguchi, Y., Ewert, D. L., and Tsujimoto, Y. Isolation and characterization of the chicken bcl-2 gene: expression in a variety of tissues including lymphoid and neuronal organs in adult and embryo. Nucleic Acids Res. 20:4187-4192. 1992.

18. Elbers, A. R. W., Fabri, T. H. F., de Vries, T. S., de Wit, J. J., Pijpers, A., and Koch, G. The highly pathogenic avian influenza A (H7N7) virus epidemic in the Netherlands in 2003- lessons learned from the first five outbreaks. Avian Dis. 48:691-705. 2004.

19. Garamszegi, L. Z., and Moller, A. P. Prevalence of avian influenza and host ecology. Proc. R. Soc. B 274:2003-2012. 2007.

20. Hackett, S. J., Kimball, R. T., Reddy, S., Bowie, R. C. K., Braun, E. L., Braun, M. J., Chojnowski, J. L., Cox, W. A., Han, K., Harshman, J., Huddleston, C. J., Marks, B. D., Miglia, K. J., Moore, W. S., Sheldon, F. H., Steadman, D. W., Witt, C. C., and Yuri, T. A Phylogenomic Study of Birds Reveals Their Evolutionary History. Science 320:1763-1768. 2008.

21. Hall, C. Impact of Avian Influenza on U.S. Poultry Trade Relations - 2002; H5 or H7 Low Pathogenic Avian Influenza. Ann. N. Y. Acad. Sci. 1026:47-53. 2004.

22. Horimoto, T., and Kawaoka, Y. Pandemic threat posed by avian influenza A viruses. Clin. Microbiol. Rev. 14. 2001.

23. Katz, J. M., Veguilla, V., Belser, J. A., Maines, T. R., Van Hoeven, N., Pappas, C., Hancock, K., and Tumpey, T. M. The public health impact of avian influenza viruses. Poultry Sci. 88:872-879. 2009.

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

25. Kinde, H., Read, D. H., Daft, B. M., Hammarlund, M., Moore, J., Uzal, F., and Mukai, J. W., P. The occurrence of avian influenza A subtype H6N2 in commercial layer flocks in southern California (2000-02): Clinopathologic findings. Avian Dis. 47:1214-1218. 2003.

26. Ko, J. H., Jin, H. K., Asano, A., Takada, A., Ninomiya, A., Kida, H., Hokiyama, H., Ohara, M., Tsuzuki, M., Nishibori, M., Mizutani, M., and Watanabe, T. Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res. 12:595-601. 2000.

27. Lam, K. M., and DaMassa, A. J. Mycoplasma gallisepticum-induced release of macrophage inflammatory protein-1 beta from chicken monocytes-macrophages. J. Comp. Path. 122:35-42. 2000.

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

29. MacDonald, M. R. W., Xia, J., Smith, A. L., and Magor, K. E. The duck toll like receptor 7: Genomic organization, expression, and function. Mol. Immunol. 45:2055-2061. 2008.

30. McQuiston, J. H., Garber, L. P., Porter-Spalding, B. A., Hahn, J. W., Pierson, F. W., Wainwright, S. H., Senne, D. A., Brignole, T. J., Akey, B. L., and Holt, T. J. Evaluation of risk factors for the spread of low pathogenicity H7N2 avian influenza virus among commercial poultry farms. JAVMA 226:767-772. 2005.

31. Mutinelli, F., Capua, I., Terregino, C., and Cattoli, G. Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenicity avian influenza epidemics in Italy during 1999 and 2000. Avian Dis. 47:844-848. 2003.

32. Nagata, K., Kawaguchi, A., and Naito, T. Host factors for replication and transcription of the influenza virus genome. Rev. Med. Virol. 18:247-260. 2008.

33. Olsen, B., Munster, V. J., Wallensten, A., Waldenstrom, J., Osterhaus, A., and Fouchier, R. Global patterns of influenza A virus in wild birds. Science 312:384-388. 2006.

34. Peiris, J. S., deJong, M. D., and Guan, Y. Avian Influenza Virus (H5N1): a Threat to Human Health. Clin. Microbiol. Rev. 20:243-267. 2007.

35. Pita, J. S., de Miranda, J. R., Schneider, W. L., and Roossinck, M. J. Environment determines fidelity for an RNA virus replicase. J. Virol. 81:9072-9077. 2007.

36. Sanderlin, S. Pandemic influenza preparedness phase II. World Health Organization . 2008.

37. Sarmento, L., Afonso, C. L., Estevez, C., Wasilenko, J., and Pantin-Jackwood, M. Differential host gene expression in cells infected with highly pathogenic H5N1 avian influenza viruses. Vet. Immunol. Immunopathol. 125:291-302. 2008.

38. Savill, N. J., St. Rose, S. G., and Woolhouse, M. E. J. Detection of mortality clusters associated with highly pathogenic avian influenza in poultry: a theoretical analysis. J. R. Soc. 5:1409-1419. 2008.

39. Sinclair, R. Factors affecting fomite survival and the future of fomites. The Center for Advancing Microbial Risk Assessment .

40. Songserm, T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D. J., Sturm-Ramirez, K. M., and Webster, R. G. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerg. Infect. Dis. 12:575-581. 2006.

41. Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T., and Suarez, D. L. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256-3260. 2002.

42. Spickler, A. R., Trampel, D. W., and Roth, J. A. The onset of virus shedding and clinical signs in chickens infected with high-pathogenicity and low-pathogenicity avian influenza viruses. Avian Pathol. 37:555-577. 2008.

43. Stallknecht, D. E., and Brown, J. D. Wild birds and the epidemiology of avian influenza. J. Wildl. Dis. 43:S15-S20. 2007.

44. Stallknecht, D. E., and Shane, S. M. Host range of avian influenza-virus in free-living birds. Vet. Res. Commun. 12:125-141. 1988.

45. Stephenson, I., and Zambon, M. The epidemiology of influenza. Occup. Med. 52:241-247. 2002.

46. Suarez, D. L., and Schultz-Cherry, S. Immunology of avian influenza virus: a review. Dev. Comp. Immunol. 24:269-283. 2000.

47. Swayne, D. E., and Beck, B. R. Experimental study to determine if low-pathogenicity and high pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. Avian Dis. 49:81-85. 2005.

48. Swayne, D. E., and Pantin-Jackwood, M. Pathogenicity of avian influenza viruses in poultry. Dev. Biol. (Basel). 124:61-67. 2006.

49. Swayne, D. E., and Slemons, R. D. Using mean infectious dose of high- and low-pathogenicity avian influenza viruses originating from wild duck and poultry as one measure of infectivity and adaptation to poultry. Avian Dis. 52:455-460. 2008.

50. Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., Parakamawongsa, T., Premashthira, S., Amonsin, A., Gilbert, M., Nielen, M., and Stegeman, A. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg. Infect. Dis. 11:1664-1671. 2005.

51. Tollis, M., and Ditrani, L. Recent developments in avian influenza research: epidemiology and immunoprophylaxis. Vet. J. 164:202-215. 2002.

52. Webby, R. J., and Webster, R. G. Emergence of influenza A viruses. Phil. Trans. R. Soc. Lond. B 356:1817-1828. 2001.

53. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., and Kawaoka, Y. Evolution and Ecology of Influenza A Viruses. Microbiol. Rev. 56:152-179. 1992.

54. Wurzer, W. J., Ehrhardt, C., Pleschka, S., Berberich-Siebelt, F., Wolff, T., Walczak, H., Planz, O., and Ludwig, S. NF-kappa-B-dependent Induction of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) and Fas/FasL Is Crucial for Efficient Influenza Virus Propagation. J. Biol. Chem. 279:30931-30937. 2004.

55. Zepeda, C., and Salman, M. D. Assessing the probability of the presence of low pathogenicity avian influenza virus in exported chicken meat. Avian Dis. 51:344-351. 2007.

56. Zhou, J., Wang, J., Chen, J., Wu, J., Gong, H., Teng, Q., Guo, J., and Shen, H. Cloning, in vitro expression and bioactivity of duck interleukin-2. Mol. Immunol. 42:589-598. 2005.