ABSTRACT

Title of Thesis: ADAPTATION OF A/MALLARD/POTSDAM/178-4/83 (H2N2) IN JAPANESE QUAIL LEADS TO REPLICATION AND TRANSMISSION IN CHICKENS

Erin Maureen Sorrell, Master of Science, 2005

Thesis Directed By: Assistant Professor Daniel R. Perez
Department of Veterinary Medicine
VA-MD Regional College of Veterinary Medicine and The University of Maryland, College Park

Influenza is a single stranded, negative-sense RNA virus with a segmented genome that can infect avian and mammalian species. Influenza viruses from the avian reservoir do not seem to replicate efficiently in humans upon direct transmission. Therefore, an intermediate host is involved in generating mutations to create a more transmissible or an avian-human reassortant virus.

Quail have been highlighted as a potential reservoir and intermediate host of avian influenza. To assess the potential of quail as an intermediate host, A/Mallard/Postdam/178-4/83, H2N2 was tested to determine if through adaptation in quail a mallard strain can replicate and transmit in quail, as well as other avian species. After six serial passages of lung homogenate a virus arose, which replicated and transmitted directly to contact quail. When chickens were infected with this

quail-adapted virus replication and transmission were observed, while no replication was noted in the chickens infected with wild type H2N2 virus.

ADAPTATION OF A/MALLARD/POTSDAM/178-4/83 (H2N2) IN JAPANESE QUAIL LEADS TO REPLICATION AND TRANSMISSION IN CHICKENS

By

Erin Maureen Sorrell

Thesis 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
Master of Science
2005

Advisory Committee:
Assistant Professor Daniel Perez Chair/ Advisor
Associate Professor Jeffrey DeStefano
Professor Mary Ann Ottinger
Professor Siba Samal
Assistant Professor Xiaoping Zhu

ACKNOWLEDGMENTS

My parents not only raised me with love, they taught me to be an independent person. Each in their own way showed me how to be strong, intelligent and to reach for your goals. I am here, writing this acknowledgement because of them.

I worked with many intelligent, perceptive scientists in my lab and I want to thank each and every one, Dr. Haichen Song, Dr. Hongquan Wan, Sharon Azogue, Dr. Gloria Ramirez Nieto, Dr. Xiaoyuan Huang, Dr. Annabelle Pascua and Dr. Jaber Hossain. I would especially like to thank my advisor, Dr. Daniel Perez, who gave me ideas and let me build on them. He pushed me forward in my thinking and introduced me to a world of research and opportunities I had not known. I would also like to thank Dr. Haichen Song who has become my mentor. She is a terrific scientist and teacher and I thank her for all the time and instruction she has given me.

Thank you to my committee members, Dr. Siba Samal, Dr. Xiaoping Zhu, Dr. Mary Ann Ottinger and Dr. Jeffrey DeStefano whose guidance and intellect have made my research stronger. I would also like to thank the department of Veterinary Medicine. The faculty, students and staff provide a wonderful working environment.

My friends and family have been there with me through it all. They have read books on influenza, learned about the CDC and WHO and are always ready to listen to me ramble on about my newest experiment or finding. My sisters, Colleen and Megan, have been the encouragement and support necessary for a life of graduate work. They are both intelligent women who are living examples of how to be respected and valued for what you can bring to the table. Patrick, you have always believed in me. Thank you for making life fun, I know laughter will always be a part of life with you.

How does one say thank you to a group who has given up their life for science? To all the quail, chickens and ducks that worked with me, I am ever in debt to you. Your sacrifice not only gave me my M.S. but will save many more avian lives in the future. A big thank you to Emma Lavoie and Michael Quinn for all their help with setting eggs and hatching all the quail used for this study and many others in our lab.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

Antb	antibiotic / antimycotic
^{o}C	degrees Celsius
cDNA	compliment DNA
cRNA	compliment RNA
CDC	Centers for Disease Control
EID_{50}	egg infectious dose 50
HA Assay	hemagglutinin assay
HEF	hemagglutinin-esterase fusion
HPAI	high pathogenic avian influenza
LPAI	low pathogenic avian influenza
MDCK	Mandin Darby Canine Kidney
ml	milliter
mRNA	messenger RNA
nt	nucleotide
PBS	phosphate-buffered saline
RT-PCR	reverse transcriptase-polymerase chain reaction
µl	microliter
vRNP	viral ribonucleoprotein
vRNA	viral RNA
WHO	World Health Organization
WT	wild type

One-Letter Abbreviations for Amino Acids

G	Glycine
A	Alanine
V	Valine
L	Leucine
I	Isoleucine
M	Methonine
F	Phenylanine
W	Tryptophan
P	Proline
S	Serine
T	Threonine
C	Cysteine
Y	Tyrosine
N	Asparagine
Q	Glutamine
K	Lysine
R	Arginine
H	Histidine
D	Aspartic Acid
E	Glutamic Acid

Influenza Genes and Their Function

Gene	Segment	Size (nt)	Function
PB2	1	2341	Transcriptase: cap binding
PB1	2	2341	Transcriptase: elongation
PA	3	2233	Transcriptase: vRNA replication
HA	4	1778	Hemagglutinin: Host cell attachment
NP	5	1565	Nucleoprotein: RNA binding, part of transcriptase complex, nuclear / cytoplasmic transport of vRNA
NA	6	1413	Neuraminidase: viral release
M	7	1027	Matrix: M1 major component of virion M2 integral membrane protein-ion channel
NS	8	890	Nonstructural: NS1 nucleus; effects on cellular RNA transport, splicing, translation. Anti-interferon protein NS2 also known as NEP (nuclear export protein)

CHAPTER 1, INTRODUCTION

1.1 General Introduction

Molecular alterations in the genome of avian influenza viruses from wild birds are thought to be required before these strains can infect humans (Beare and Webster, 1991; Hinshaw et al., 1983). Intermediate hosts that are susceptible to influenza viruses from aquatic birds can generate novel strains. These strains have the potential to cross the species barrier a second time. Therefore, identifying potential intermediate hosts and the molecular changes associated to adaptation of influenza viruses is vital if we want to prepare for and potentially prevent the emergence of influenza strains with pandemic potential. This research has evaluated the role that quail could play as an intermediate host in which an influenza virus can change its phenotypic characteristics and increase its host range. This study shows that quail could be such a host.

Japanese Quail (*Coturnix coturnix*), among other terrestrial birds, are extensively farmed around the world. Until a few years ago, quail were underrated and ignored as a potentially important reservoir of avian influenza viruses. In 1997 with the direct transmission of avian H5N1 influenza to humans, extensive analysis of avian influenza samples was conducted. Analysis highlighted a quail H6N1 influenza virus whose internal genes were identical to the 6 internal genes of the human H5N1 virus.

Further investigation and molecular characterization of influenza virus strains isolated from quail in Hong Kong and other parts of Southeast China showed that many of these viruses were phylogenetically related to those that gained the capacity to cross to humans, particularly H9 and H5 subtypes (Chin et al., 2002; Guan et al., 2000; Hoffmann et al., 2000). As a result, quail were banned from live bird poultry markets in Hong Kong in 2002 under the suspicion that they were a contributing factor in the perpetuation of avian influenza viruses in the markets.

However, quail remain a part of live bird markets around the world including Europe and North America. Infections in quail with avian influenza viruses is almost unequivocally established in the respiratory tract and transmitted by aerosol, two features in common to influenza viruses that have become endemic in humans and swine. Based on these observations we have hypothesized that quail provide an environment where influenza viruses from the natural reservoir, wild aquatic birds, could change their phenotypic characteristics and increase their host range.

1.2 Research Objectives and Outline

The main goal of this research is a study of adaptation with influenza virus, A/Mallard/Potsdam/178-4/83 (H2N2), in Japanese quail and its subsequent ability to transmit to other land-based birds.

The research objectives of my thesis are to:

I. To determine whether A/Mallard/Potsdam/178-4/83 (H2N2) replicates but can not transmit in Japanese Quail.

II. Adapt A/Mallard/Potsdam/178-4/83 in quail by serially passing the virus in lung homogenate from the previous passage. Confirm transmission through direct contact with sentinel cage mates.

III. Determine if adaptation of a mallard virus in quail creates a virus better adapted to other land-based birds as well as being able to infect the original host.

IV. Determine the changes necessary, at the molecular level, for adaptation and transmission of A/Mallard/Potsdam/178-4/83 (H2N2) in quail.

The results of my research are:

I. A/Mallard/Potsdam/178-4/83 (H2N2) established an infection in quail directly inoculated with the virus and shed until day 3 post-infection. No transmission to sentinel cage mates was observed.

II. After six passages of lung homogenate a virus arose that was adapted to quail. Transmission to contact quail occurred on day 3 post-infection and all contact quail became infected shedding just as much virus as the infected quail.

III. Quail-adapted H2N2 virus established an infection and transmitted to White Leghorn chickens while in contrast the original, wild type, A/Mallard/Postdam/178-4/83 was unable to establish an infection even in chickens directly inoculated with virus. Quail-adapted H2N2 was able to establish an infection in mallard ducks.

IV. Changes occurred at both surface and internal genes, including the HA, PB2, PB1 and NP genes.

CHAPTER 2, LITERATURE REVIEW

2.1 Influenza Virus

2.1.1 History and Pandemics

Influenza viruses can cause serious outbreaks of upper-respiratory infections in humans and other species. The history of influenza dates back to 412 BC when Hippocrates described an outbreak of flu-like illness that wiped out the Athenian army (Hoehling, 1961). Today influenza viruses are subdivided into types A, B, and C. Influenza A is the most contagious and caused the most human illnesses even leading to a number of pandemics claiming millions of lives.

The first recorded European pandemic was 1173-74 although the first generally accepted pandemic occurred between 1510 and1580 (Hoehling, 1961). Since then there have been 31 documented pandemics although it is impossible to clearly identify the early history of flu with any certainty. Historical data gathered from 1500 through 1800 collected by Webster and Hirsch outlined a pattern for influenza epidemics. There were three major observations. Epidemics occurred frequently however at irregular intervals and occasionally the disease disappeared for periods of time, epidemics varied in severity usually causing mortality in the elderly and some epidemics, particularly those that occurred in 1781 and 1830 appeared to spread across Russia from Asia.

There appears to have been two pandemics in the 19th century, occurring in 1847 and 1889. The 1889 pandemic marked the beginning of modern epidemiology. Influenza pandemics occurred every 18-34 years during 1510-1889 and between 1889-1977 they occurred every 9-39 years. Pandemics this century have occurred in 1918, 1957 and 1968. After 1889, influenza became and remained an important cause of mortality and morbidity. It has been suggested from the 1889 pandemic a major change occurred in epidemiology and for the next 25 years epidemics occurred at irregular intervals and were associated with increased mortality. The baseline rate never returned to pre-1889 levels (Stuart-Harris et al., 1976).

In modern human history the most serious pandemic occurred in 1918. It was referred to as the "Spanish Flu" although the pandemic is thought to have originated in China (Cox and Subbarao, 2000). The virus then became known as the "Spanish flu" because the country was the first to report an epidemic. All armies of Europe were hit hard by the outbreak, 80% of the U.S Army's war deaths were due to influenza. After the initial infection influenza spread over America at alarming speed with over 100,000 cases reported nationally in the first month.

Almost every country was affected by this pandemic. The second and third waves of the pandemic were associated with a large number of deaths, claiming roughly 50% mortality in the 20 to 30 age group (Stuart-Harris et al., 1976). In 1919 during the third wave the virus began to run its course and disappeared almost as

rapidly as it began. It is estimated that the pandemic that was of a H1N1 subtype, killed more than 40 million people worldwide (Cox and Subbarao, 2000).

Up until the 1950s the H1N1 virus was still endemic all over the world. In 1957 there was another influenza pandemic, the "Asian Flu". The virus was a H2N2 subtype, which originated from China. The 1957 pandemic was a reassortant of the 1918 H1N1 and an avian H2N2 strain. The hemagglutinin (HA), neuraminidase (NA) and polymerase (PB1) genes of the H2N2 were of avian origin and the remaining 5 genes from the human H1N1 (Kawaoka, Krauss, and Webster, 1989; Scholtissek et al., 1978). The emergence of the H2N2 led to the disappearance of the H1N1 virus. There were two waves to the 1957 pandemic with extreme mortality occurring in each (Cox and Subbarao, 2000).

The third influenza pandemic of the 20th century occurred in 1968. This pandemic was caused by a H3N2 virus, later known as the "Hong Kong Flu", it was a result of the HA and PB1 of an H3 avian strain and the remaining genes from the '57 H2N2 (Kawaoka, Krauss, and Webster, 1989; Scholtissek et al., 1978). In 1977 the re-emergence of the H1N1 influenza virus caused a mild pandemic. The virus arose in China and spread throughout the world mainly infecting those born after 1957. Since then mild influenza epidemics caused by the H1N1 and H3N2 viruses have been reported.

In May1997 H5N1 emerged as an avian influenza strain that during the course of seven months infected 18 people and of those, killed 6 (Subbarao et al., 1998; Yuen et al., 1998). To battle this avian to human transmission the Hong Kong government ordered a mass culling of poultry in chicken farms and markets. Recent outbreaks of H5N1 viruses in Southeast Asia are affecting its victims at alarming speed; almost a 70% mortality rate is associated with this virus (www.CDC.gov). The incapability of this strain to transmit human to human is still a concern for virologist and influenza surveillance, once it has mutated to become capable of transmitting from human to human this strain has all the makings of a pandemic.

Avian influenza is a disease that ranges from a mild, possibly asymptomatic infection to an acute, fatal disease of chickens, turkeys, guinea fowl, quail, and other avian species, especially migratory water fowl. Fowl plague was described in Italy in 1878, but it was not until 1955 that the fowl plague virus was identified as an influenza virus (Schaeffer, 1955). Once domestic birds are infected, avian influenza outbreaks are nearly impossible to control and often lead to major economic impacts including the loss of millions of chickens through culling.

2.1.2 Discovery of Influenza Viruses

In the 1880s and 1890s it became possible to culture bacteria and using the method organisms such as *Vibrio cholerae* were discovered and shown to cause epidemic diseases such as cholera. Therefore, scientists looked for a characteristic

bacterium that caused influenza. The epidemic of 1889 presented an opportunity to identify the causative agent if it were bacterium. In the early 1900s the concept of pathogenic filterable viruses was established by a method of isolation through the filtration of bacteria and then reproducing the disease by inoculating plants or animals.

It was in 1933 that Christopher Andrewes demonstrated that a filterable virus was present that could be transmitted serially from ferret to ferret causing an immune response protecting them from re-infection (Smith and Stuart-Harris, 1933). Francis and Magill independently isolated a new type of influenza virus, using ferrets and mice, which had no antigenic relation to the previous isolates (Francis 1940). The new virus was referred to as type B, while the previously isolated virus was type A.

Influenza viruses were successfully grown in embryonated chicken eggs (Smith 1935). The use of egg isolation led to the discovery of the third type of influenza virus in humans, type C. It is a mild, nondescript common cold that occurs sporadically and not in recognizable epidemics. Type C is significantly different at the biological level from influenza types A and B.

2.1.3 Types of Influenza: A, B, C

The structures of A and B viruses are very similar. The main difference between types A and B is the membrane channel lining the lipid envelope. In influenza A it is the M2 protein and in influenza B it is the NB protein. The

membrane channel for influenza C is the CM2 protein. A and B viruses also differ in their genome size with 14,639 nucleotides for type B and 13,588 nucleotides for A. The difference in genome size is not due to the size of the proteins, rather it is because of larger noncoding sequences in type B viruses. The major characteristic identifying type C versus types A and B is that influenza C has only one surface glycoprotein and contains only seven RNA segments. The surface glycoprotein in C, referred to as the hemagglutinin-esterase fusion (HEF) protein contains the fusion and receptor properties of A and B's HA gene as well as the receptor destroying activity of NA. This enzymatic activity is not a neuraminidase but an esterase activity. The HEF protein has the same morphology as A and B's HA.

2.1.4 Propagation of Influenza

Influenza viruses can be grown in embryonated chicken eggs or in primary tissue culture systems (Smith, 1935; Burnet, 1936). Egg inoculation is still used today and is the system of choice for vaccine production and the acquisition of a large volume of virus stock used for laboratory studies. Isolation of human viruses through tissue culture systems from either primary monkey kidney or Mandin Darby Canine Kidney (MDCK) cells is well established. Most human and avian influenza viruses can be grown in eggs and detected through the agglutination of erythrocytes (Hirst 1941). The surface glycoproteins of the virus bind sialic acid receptors on erythrocytes causing the agglutination. Influenza will replicate, cause cytopathology and produce plaques in many primary tissue cultures. However few cell systems

other than primary kidney cells are suitable for plaque formation unless trypsin is added to cleave the HA, activating the virus.

2.1.5 Morphology and Nature of the Genome

Influenza particles are highly pleiomorphic. The morphological characteristics of influenza viruses are a genetic trait but spherical morphology depends on passage in eggs or tissue culture (Kilbourne, 1963). Most influenza viruses isolated from humans and other species after a single passage in culture show great shape and size variation in contrast to viruses from infected eggs or tissue culture fluids, which reveal irregularly shaped, spherical particles. The majority of viruses are found to be spherical, 80-120 nm in diameter but many forms occur, including filamentous particles up to 2000 nm long. Different strains of virus tend to have different filamentous forms, a property which maps to the matrix protein, which lines the inside of the viral envelope (Hay, 1974; Klenk, 1974).

2.2 Transmission of Influenza

2.2.1 Species and Subtype Variation

Representatives from each of the known subtypes of influenza A have been isolated in aquatic birds, the virus' natural reservoir. Influenza viruses have been isolated worldwide from domestic and wild avian species. The largest number of

viruses have been gathered from feral water birds including ducks, geese, terns and gulls as well as from a large group of domestic terrestrial birds like turkeys, chickens, quail and pheasants (Easterday, 1975). Influenza B viruses are restricted to humans while type C viruses have been isolated from humans and pigs.

The clinical symptoms associated with influenza infections in avian species range considerably with each strain of the virus. Infections with most strains cause asymptomatic infections though a few can cause disease including systemic infections with CNS involvement and mortality within a week of infection. The strains, which cause this pathogenic infection in the birds, are of the H5 and H7 subtypes and are referred to as highly pathogenic avian influenza strains (HPAI). The major concern with mallard viruses is the transmission of these strains to domestic birds, especially those housed outdoors or in free range environments where the fecal material from wild, migratory birds are accessible to the chickens, turkeys or quail in the outdoor pens. These outbreaks not only jeopardize the health of the particular flock infected but also those of surrounding flocks and those who work with the birds. Economic losses as well as political uneasiness also play roles in each outbreak here in the U.S. and across the globe.

In ducks the majority of viruses replicate in the cells lining the intestinal tract while there is some replication in lung epithelial cells (Webster et al., 1978). The virus gains access by passage in the digestive tract, despite the low pH of the gizzard, and are shed in high concentrations in the feces. The mechanism by which influenza

infections can persist and spread in ducks from year to year is not well understood. Influenza has been shown to infect juvenile birds when they congregate prior to migration and those viruses can then persist in water surrounding those areas. Some ducks may shed virus, asymptomatically, up to 30 days therefore requiring few passages to maintain the virus in population. Current and available information supports the continuous circulation of influenza in migrating waterfowl (Webster et al., 1992). The avirulent nature of avian influenza in its reservoir is the result of adaptation over many centuries, creating an environment that allows the perpetuation and evolution of this virus. Avian influenza from ducks has been linked to outbreaks in mammals including seals, whales, pigs, horses, ferrets, cats (domesticated and wild), some breeds of dogs, as well as in other poultry species like chicken and quail (Guo et al., 1992; Hinshaw et al., 1986; Scholtissek et al., 1983; Webby, Hoffmann, and Webster, 2004; Webster et al., 1981).

Studies on the ecology of the virus show that all mammalian influenza viruses derive from the avian reservoir (Reid et al., 1999; Reid et al., 2002; Reid et al., 2000). Phylogenetic analysis of the NP protein show avian influenza has evolved into five host-specific lineages. They are an ancient equine lineage (not isolated in the last 25 years), a recent equine lineage, a lineage in gulls, one in swine and one in humans. It appears the ancestor of the swine and human viruses is one of avian origin, one that derived all eight of its genes from avian sources (Reid et al., 2004; Webster et al., 1992). Studies with the NP and other gene segments indicate separate sublineages of influenza in Eurasia and the Americas, demonstrating birds with a longitudinal

migration path play a role in the transmission of these viruses (Liu et al., 2004; Makarova et al., 1999; Schafer et al., 1993). Influenza virus evolution in avian reservoirs has reached stasis. There is no evidence of net evolution in the past 60 + years. Nucleotide changes continue to occur at similar rates to mammalian viruses but these changes no longer lead to amino acid changes, thus referred to as synonymous changes. This level of conservation suggests that avian viruses are approaching an adaptive optimum. The source of pandemic viruses exist phenotypically unchanged in the aquatic bird, implicating that silent nucleotide changes dominate over deleterious or neutral amino acid changes (Reid et al., 2004).

Influenza A viruses in humans follow a single branch lineage, indicating no co-circulating strains of the same subtype so that the virus evolves by clonal reconstitution following the previous outbreak's extinction (Buonagurio et al., 1985). Influenza B and C have multiple lineage evolution, allowing the presence of co circulating strains within human population (Yamashita et al., 1988). Therefore, B and C type influenza are approaching equilibrium with their human hosts but the type A influenza viruses are being prevented by perturbations arising from the reassortment of human virus genomes with those from avian viruses.

2.2.2 Antigenic Shift & Drift

Influenza is a difficult disease to prevent and contain because it is able to disguise itself as a new agent, never seen before because it can mutate quickly and

regularly produces new strains through antigenic drift and shift. Antigenic drift is the appearance of a virus with a slightly changed antigenic structure resulting from the accumulation of point mutations of the viral surface proteins. The theory of antigenic drift assumes new antigen sequences arise by natural selection of mutants in influenza viruses. The rate of the point mutations depends on the evolutionary rate and is highest in human viruses (Reid et al., 2004). These point mutations prevent antibodies made after previous infections from binding to the surface HA properly in order to destroy the virus. Through these mutations influenza virus has enabled itself to replicate without being destroyed by host defense mechanisms.

Antigenic shift is a major change in the surface of a virus when completely new proteins are acquired through viral reassortment. Reassortment occurs in one cell, in a susceptible host with two different strains, either two avian or an avian and human. This new reassortant strain is unrelated to any previous strain circulating. The human pandemics of 1957 and 1968 resulted from antigenic shift when avian strains co circulating with the human strain reassorted creating a new virus for the human population (Schafer et al., 1993). These major antigenic shifts have common characteristics; appearing suddenly and antigentically distinct from the current human influenza viruses circulating. There is ample confirmation for genetic reassortment between human and avian viruses *in vivo* (Wilson et al., 1980) as well as reassortment in humans (Cox, Bai, and Kendal, 1983). The 1957 pandemic strain contained avian HA, NA and PB1 genes combined with the circulating human H1N1 while the 1968 H3N2 contained HA and PB1 avian genes (Schafer et al., 1993). Antigenic shift is

capable of causing a pandemic at any time because there is no history of infection for the subtype in the population and thus there is no immunity.

The direct transmission of avian H5N1 and H9N2 viruses to humans in southeastern China and Hong Kong in 1997, 1999 and 2004-2005 have renewed interest in the role of avian influenza virus zoonosis (Guan et al., 2000; Perez et al., 2003a; Perez et al., 2003b). These infections raise the possibility that a pandemic influenza virus could arise from the direct transmission of an avian influenza virus to humans. The one barrier holding the current H5N1 from causing real trouble in Asia and worldwide is the lack of successful human-to-human transmission. If these avian viruses are able to infect humans and establish an infection there is always a chance for reassortment between this avian virus and a co-circulating human influenza virus, thus creating a new virus that can easily transmit to humans.

2.3 Influenza Life Cycle

2.3.1 Myxoviruses

Influenza viruses are single stranded, negative-sense RNA viruses with a segmented genome. They belong to the family *Orthomyxoviridae*, which includes four genera, the influenza A, B, C viruses and *Thogotavirus*. Type B and C viruses

are restricted to humans while type A viruses infect avian species and mammals such as swine and humans. *Thogotavirus* include tick-transmitted orthomyxoviruses.

Influenza A has eight segments. They are independently encapsidated by the viral nucleoprotein (NP) and each segment is associated with a polymerase complex composed of the PA, PB1 and PB2 genes. The polymerase complex binds to the 3' and 5' ends of the vRNA and holds the ends together in a circular structure. The subviral particle consisting of the viral RNA, NP and polymerase complex is referred to as the ribonucleoprotein (RNP) particle. The NP protein is the major structural protein that interacts with the RNA segments to form the RNP. The NP binds to PB1, M1 and PB2 directly (Portela and Digard, 2002). The RNP particles are located inside a layer of M1 protein that lines the viral lipid membrane. The lipid membrane is derived from the plasma membrane of the infected host cell. It becomes part of the viral particle during the budding process. Embedded in the viral membrane are three proteins; two spike glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA), and a membrane-channel protein, M2 (Zebedee and Lamb, 1988). The HA is a trimer of identical subunits which provides the receptor-binding site and elicits neutralizing antibodies (Wharton et al., 1989). Cleavage of the HA into HA-1 and HA-2 is essential for fusion and viral infectivity. The NA is a tetramer of identical subunits that contains the receptor destroying activity needed for release of the newly formed virus from the surface of the infected cell (Colman et al., 1989). M2 forms a membrane channel allowing acidification of the virus interior while it passes through the endosome after attachment and entry. This acidification is believed necessary for

the release of RNP into the cytoplasm for viral replication and host cell infection. The two non-structural proteins, NS1 and NS2 are found in the infected cell while NS2 is also located in the virion (Pinto, Holsinger, and Lamb, 1992; Sugrue and Hay, 1991).

2.3.2 Virus Entry

Influenza viruses bind to sialic acid residues present on cell surface glycoproteins or glycolipids via the receptor-binding site in the distal tip of the HA molecule. Specificities for sialic acid linkage differs depending on specific residues in the HA receptor-binding pocket. The preference is between sialic acid linked to galactose by α2, 6 or α2, 3-linkages (Matrosovich and Klenk, 2003).

Entry of the virus into the host cell is through receptor-mediated endocytosis, historically referred to as viropexis (Figure 1). The virus enters the cell in a clathrin coat formed by invagination of specialized coated-pit domains of the host plasma membrane. After internalization the clathrin coat is removed and vesicles fuse with endosomes, beginning with a primary endosome and increasing in acidity to end with a late endosome. The acidification occurs through H^+-ATPases. The viral RNPs pass into the cytosol by crossing the membrane of the virion and endosome (Skehel and Wiley, 2000; Stegmann, 2000).

The HA mediates fusion of the viral membrane with the endosomal membrane based on the pH of the transition of HA to its low pH form. When a virion particle has been endocytosed the low pH activated ion channel of the virion-associated M2 protein allows the flow of ions from the endosome to the virion interior disrupting protein-protein interactions and freeing the RNPs from the M1 protein (Colman and Lawrence, 2003). A possible additional role for the M2 protein in ion channel activity is the preparation of HA for the fusion process (Bron et al., 1993). Low pathogenic viruses (LP) containing HA with a single positive charge at the cleavage site are cleaved by specific enzymes such as *tryptase Clarab* present in lungs, whereas HA containing multiple basic residues at the cleavage site are cleaved by proteases such as furin and subtilism-type enzymes, present throughout the body (Horimoto and Kawaoka, 1995; Kido et al., 1999). Therefore, highly pathogenic viruses (HP) can infect other tissues and spread throughout the host causing a systemic infection. Virus particles containing uncleaved HA can bind to and enter host cells but do not undergo fusion and are therefore non-infectious (Colman and Lawrence, 2003). Once the M2 ion channel acidifies the internal virion core releasing the vRNP from the M1 the vRNP moves to the cytoplasm. This M1-free vRNP is then imported into the nucleus through a nuclear pore using nuclear transport signals of NP. The M1 protein, which is dissociated from the RNP enters via active transport, its size, 70kD, is larger than the maximum 40kD allowed for passive diffusion (Neumann, Hughes, and Kawaoka, 2000).

Transcription of the RNPs to mRNA occurs in the nucleus. Transcription and replication are both catalyzed by the viral RNA-dependent RNA polymerase complex. The PB1 subunit of the polymerase complex has a major role in the catalytic activity of the viral polymerase. It contains the conserved motifs characteristic of RNA-dependent RNA polymerases and RNA-dependent DNA polymerases (Biswas and Nayak, 1994; Muller et al., 1994). The PB2 is essential for transcription (Kobayashi, Toyoda, and Ishihama, 1996). It is responsible for the recognition and binding of host mRNAs although the PB1 binds to the terminal sequences of vRNA and cRNA and performs capped RNA endonuclease activities (Li, Rao, and Krug, 2001; Ulmanen, Broni, and Krug, 1981). PA is associated with replication activity of the polymerase (Mahy et al., 1983; Perales et al., 2000). PA is associated with N-terminus of PB1 (Perez and Donis, 2001). Optimal viral RNA transcription and replication are dependent on the formation of the heterotrimer (Honda et al., 1990; Huang, Palese, and Krystal, 1990).

Figure 1. Influenza Viral Attachment and Entry

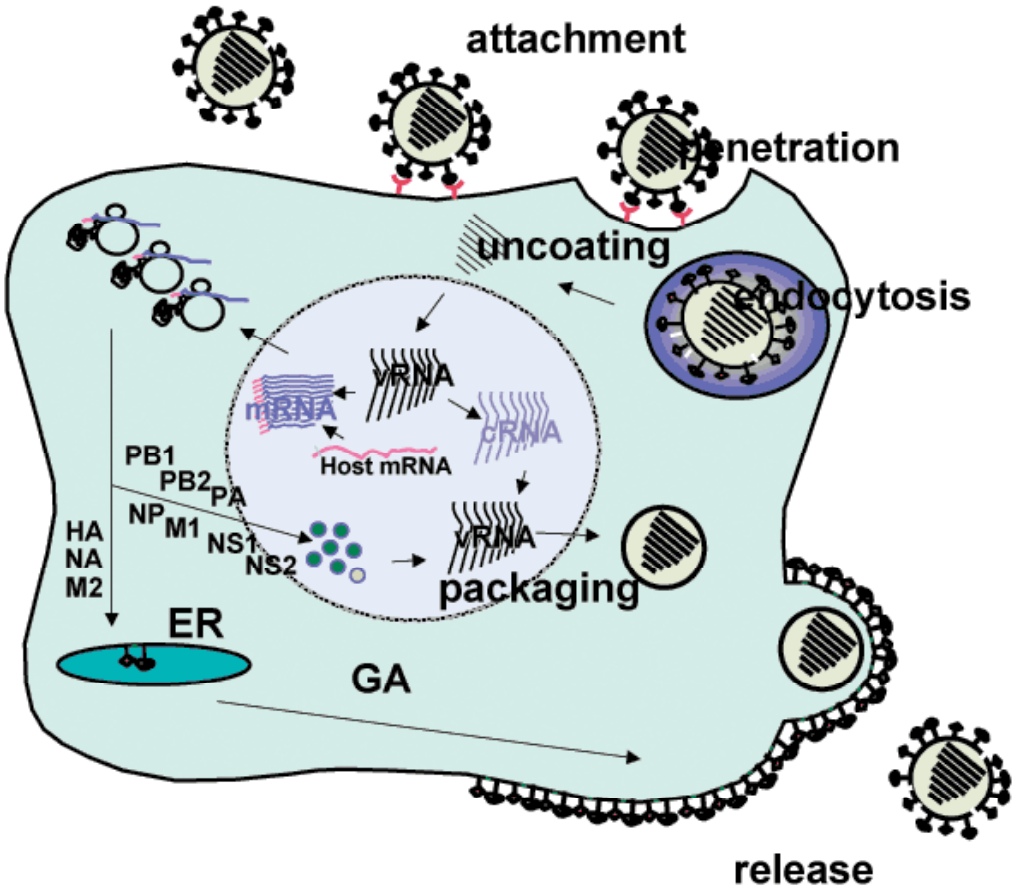


Figure from http://www.agnr.umd.edu/avianflu

2.3.3 Viral mRNA Synthesis

Viral mRNA synthesis occurs in the nucleus and requires initiation by host-cell primers, in particular capped (m^7GpppNm-containing) RNA fragments which are derived from host-cell RNA polymerase II transcripts. These host-cell primers are generated in the nucleus through the action of a viral cap-dependent endonuclease. A 5'-methylated cap structure is required for priming. Cap binding is performed by PB2 followed by capped RNA cleavage by PB1. The endonuclease activity in PB1 cleaves mRNAs 10-13 nucleotides from their 5' end, preferentially after a purine residue (Braam, Ulmanen, and Krug, 1983). Cap binding and cleavage are dependent on the interaction of the polymerase complex with vRNA. PB2 binds to methylated cap-1 structures at the 5' terminal of the transcribed cellular mRNA and they are subsequently cleaved by the P complex, producing 10 to 13-mers used for priming viral mRNA transcription. Transcription is initiated by incorporating a G residue onto the 3' end of the fragments and viral mRNA chains are then elongated until the polyadenylation signal, 5 to 7 uridine (U) residues, located 15 to 22 nucleotides before the 5' ends of the vRNAs. Polyadenlyation occurs through reiterative copying of the uridine sequence (Perez and Donis, 2001; Poon et al., 1999; Zheng et al., 1999).

During transcription, the polymerase complex transcribes the negative sense vRNAs into positive-sense mRNAs that are capped and polyadenylated. Viral RNP complexes are formed for each of the eight segments. Termination occurs from a

stuttering of the stretch of U residues, adding a poly(A) tail to the 3' ends of the viral mRNAs (Poon et al., 1999; Zheng et al., 1999). In infected cells the vRNAs are both transcribed into mRNAs and replicated.

The conserved 12 and 13 nucleotides at the 3' and 5' ends respectively are shown below. The U_6 poly(A) signal near the 5' end of the vRNA is separated by 2 or 3 segment specific residues from the conserved 13 nucleotides of the 5' end. The cap structure and the 10-13 heterologous nucleotides at the 5' end of the mRNA are derived from the host cell pre-mRNAs. The template RNA (cRNA) contains at its 5' terminus a pppA and it is a complete copy of the vRNA segment.

5'AGCAAAAGCAGG---------AAAAAAN$_{2-3}$CCUUGUUUCUACU 3' cRNA (template)

Replication

3' UCGUUUUCGUCC---------UUUUUUN$_{2-3}$GGAACAAAGAUGA 5' vRNA

Transcription

5' Cap-N$_{10-13}$AGCAAAAGCAGG---AAAAAAAAAA$_n$ 3' mRNA

5' pppAGCGAAAGCAGG---------CCUUGUUUCUACU 3' template RNA

2.3.4 vRNA Replication

Replication of vRNA occurs in two steps; the synthesis of template RNAs, the full-length copies of the vRNA and the copying of template RNAs into vRNAs. During replication a full-length copy of the vRNA, referred to as complimentary RNA (cRNA) is synthesized and serves as the template for synthesizing progeny viral RNA. Replication is primer-independent. The mechanism that triggers the switch

from primer-dependent mRNA synthesis to primer-independent cRNA synthesis is not well understood. The production of cRNA is shown to require soluble NP protein. A possible role for NP is that it binds to the developing RNA transcript, preventing the uridine stretch near the 5' end of the vRNA from slipping backwards, therefore preventing polyadenylation. NP could also play a structural role, binding to the cRNA and together with the viral polymerase complex prevent the degradation of newly synthesized cRNA molecules by cellular nucleases (Honda et al., 1988; Huang, Palese, and Krystal, 1990).

At the second step of replication, full-length anti-genomic cRNA serves as the template for progeny vRNA synthesis. vRNA synthesis, similar to cRNA, is primer-independent, requires NP, and results in the full-length viral transcripts. The switch from the synthesis of viral mRNAs to that of template RNAs requires that the capped RNA-primed initiation change to unprimed initiation. Because the switch calls for protein synthesis, one or more newly synthesized virus-specific proteins are assumed to be necessary for the unprimed synthesis and / or anti-termination at the poly(A) site. Thus, at least two polymerase complexes can be postulated to exist in infected cells. One, which is also present in the virion, uses capped primer fragments to initiate mRNA synthesis, requiring PB2 protein, while the second type initiates synthesis of either template RNA or vRNA chains without a primer, requiring NP (Area et al., 2004).

The production of viral proteins is regulated to the time they are required during replication and in relation to the amounts of structural components necessary for virion assembly. Synthesis of the different viral proteins is proportional to the amount of corresponding mRNAs. There are two phases of gene expression, early and late. The early phase begins after primary transcription and corresponds to a stage of vRNA replication and amplification of mRNA production. During the early phase synthesis of specific vRNAs, viral mRNAs and viral proteins are coupled. After primary transcription, the first event to occur is template RNA synthesis. The rate of template RNA synthesis peaks early and declines sharply. Specific template RNAs are then transcribed into vRNAs. Preferential synthesis of the NP and NS1 proteins occur in the early phase, the consequence of selective replication of the vRNA templates from which their mRNAs are transcribed (Shapiro, Gurney, and Krug, 1987; Skehel, 1972). The production of NP and NS1 in early phase highlights their role in the regulation of transcription and replication of vRNAs.

During the late phase, which focuses mainly on the production of virion structural components, the vRNAs are synthesized in equal amounts, necessary for the progeny genomes. Synthesis of all viral mRNAs peak at the beginning of the late phase but synthesis of the vRNAs remains high during the late phase. Therefore vRNA and viral mRNA are no longer coupled during this second phase. Viral protein production also remains high during the late phase, thus ending viral mRNA synthesis. Previously synthesized viral mRNAs direct the viral protein production with a change in production preference. Transcription is directed towards the HA and

M1 proteins while NS1 production decreases. The synthesis of all three virus-specific RNAs occurs in the nucleus. Template RNAs, synthesized at early phase remain in the nucleus to direct vRNA synthesis throughout infection while the vRNAs are transported to the cytoplasm at late phase. M1 protein is thought to be synthesized late because this protein stops transcription of vRNA into viral mRNA and mediates transport of the vRNA, in the form of nucleocapsids, to the cytoplasm (Marin et al., 1991). Regulation of mRNA splicing is important in controlling the ratio of full-length and spliced mRNAs for NS and M genes to produce NS1, NS2, M1 and M2. NS splicing into NS1 is regulated by cis-acting sequences in the NS1 mRNA while the production of M2 ion channel protein is controlled by viral and cellular proteins.

2.3.5 Transport and Assembly

Virus integral membrane proteins HA, NA and M2 are synthesized on membrane-bound ribosomes and are translocated across the endoplasmic reticulum (ER) in a signal recognition particle (SRP)-dependent manner. Involvement with the ER includes cleaving the N-terminal sequence of the HA by signal peptidase, glycosylation of N-linked carbohydrate chains to NA and HA and trimming of mannose-rich oligosaccharides. After correct folding and assembly of the proteins they are transported out of the ER to the Golgi apparatus where further processing of oligosaccharides occur. The M2 modifies the acidity of the *trans* Golgi network to protect the HA from possible inactivation. The integral membrane proteins are transported to the plasma membrane where virion assembly and budding occurs.

Both HA and NA have been shown to interact with lipid rafts (Hobman, 1993; Nayak and Barman, 2002). It is generally believed that viral glycoproteins determine the site of virus assembly and budding. This belief stemming from the fact that viral glycoproteins accumulate at the site of virus budding even when expressed alone (Hobman, 1993). Viral glycoproteins may not be the only determinant for selecting the site of virus budding, other viral components such as M1 and vRNP as well as host components may be involved in determining the budding site (Nayak and Hui, 2004). The RNP-M1 core acquires a membrane derived from the host cell plasma membrane, an area modified to contain almost no host membrane proteins. The PB1, PB2, PA and NP genes of the transcription / replication complex each contain karyophilic signals that direct the genes from their site of synthesis in the cytoplasm to the nucleus where they assemble into functional complexes and nucleocapsids. The polymerase complex associates with the termini of viral RNAs in RNPs. The M1 present in the nucleus joins the vRNP and facilitates export from the nucleus and prevents re-entry. The NS2 protein, also called the nuclear export protein (NEP), mediates export of the vRNPs by acting as an adaptor between the vRNP-M1 complex and nucleoporins of the nuclear export machinery. Nucleocapsids are exported out of the nucleus and progeny virus particles are packaged. The action of viral neuraminidase is necessary for the release of the effective virions.

Budding requires three additional steps; the selection of an assembly site where viral components are transported and assembled to initiate the budding process, growth of the bud and completion of the bud with release of the virus particle (Nayak

and Hui, 2004). Each step in the budding process is complex and not completely understood but it is well accepted that it requires interaction and involvement of both host and viral components. Bud formation and release are the last steps in viral replication and production of new infectious virions.

Pinching off of the virus buds require fusion of the opposing viral and cellular membranes. This leads to fission and separation of the bud from the cell (Nayak and Hui, 2004). M1 proteins have been shown to be the key component of bud formation and pinching off (Nayak and Barman, 2002; Nayak and Hui, 2004). Although M1 proteins alone can initiate and release virus buds for an influenza virus to be infectious, all eight RNA segments are required. It is not known if this mechanism is selective or random. There is evidence for both. If the mechanism were random, there would be common structural elements in all vRNPs, causing them to be incorporated randomly. Support for this model is the observation that influenza A virions can have more than eight vRNPs (Bancroft and Parslow, 2002). If the mechanism is random, the probability of packaging eight segments into a virion requires that only 12 segments need to be randomly packaged to have 10% of virions containing one copy of each of the 8 segments (Enami et al., 1991). If the mechanism is specific, the structural feature in each vRNA segment enables them to be selectively incorporated into virions. Evidence for this model comes from studies that show the various vRNAs are equimolar within viral particles although their concentrations in infected cells may differ (Smith and Hay, 1982). Viruses must be released into the surrounding environment in order to infect other cells. Bud

formation and closure causing pinching off of the virion may not be sufficient to release the virus into the external environment because the released virion may still be attached to the infected host cell by sialic acid. The enzymatic activity of the NA removes sialic acid, the receptor for influenza, from the membrane glycolipids and glycoproteins from both the virus particles and infected cells, preventing self aggregation of virus particles and reattachment to the virus-infected cell (Liu et al., 1995).

The site and nature of budding can be an important factor in viral pathogenesis. Influenza viruses that bud from the apical surface, typically from polarized epithelial cells and into the lumen of the lung are restricted to the lungs and do not cause viremia. However some influenza viruses with the polybasic amino acid cleavage site of HA (highly pathogenic influenza) are not restricted to the lungs and thus produce viremia infecting other organs (pantropism) causing sever mortality to the infected host (Liu et al., 1995; Mori et al., 1995; Subbarao and Katz, 2000).

2.4 Host Range of Influenza

2.4.1 Molecular Determinants of Host-cell Restriction

Host range is a polygenic trait (Ito and Kawaoka, 2000). The hemagglutinin (HA) subtype plays a major role in the host range and pathogenicity of each virus subtype and strain. Cleavability of the HA is an important determinant of virulence but may not be for host range. The mechanism by which certain HA subtypes infect

and cause illness in mammals while other subtypes are unable is not well understood. The receptor-specificity of influenza HA depends on the host species they evolved from, the infections are dependent on interactions between the HA and cell oligosaccharides that have sialic acid residues. Avian viruses preferentially bind to sialyloligosaccharides terminated by SAα2, 3Gal, where human viruses prefer SAα2, 6Gal conformation (Rogers and Paulson, 1983). The binding preference appears to be associated with the amino acid located at position 226 in the HA, Leu-226 in humans and Gln-226 in avian viruses. It is generally accepted that to change host range influenza viruses need to override the selective binding. A possible route is by going through an intermediate host carrying both receptors, thus creating a new virus through reassortment. Swine have been suggested to have a role due to the fact that they posses SAα2, 3Gal and SAα2, 6Gal (Ito et al., 1998; Scholtissek, Hinshaw, and Olsen, 1998). There have been only a limited number of avian and human influenza viruses that have established stable lineages in pigs (Makarova et al., 2003). Recent evidence introduces certain terrestrial poultry, such as chickens and quail, who provide an environment similar to pigs (Gambaryan, Webster, and Matrosovich, 2002; Guan et al., 1999). Such instances in 1997 with H5N1 and in 1999 with H9N2, where avian viruses circulating in poultry in Hong Kong were transmitted to humans, are scenarios that seem to be increasing in familiarity (Lin et al., 2000; Peiris et al., 1999; Shortridge, 1999) . There is no solid evidence for neuraminidase (NA) action in host-range discrimination but changes to the NA gene can alter virulence properties or the ability of the virus to form plaques in tissue culture (Schulman and Palese, 1977; Sugiura and Ueda, 1980).

2.4.2 Interspecies Transmission

Influenza A viruses cross species barriers relatively easily and often. The establishment of a stable lineage and the spread thereof is however a rare event. For the introduction of a foreign gene into a new genetic constellation by reassortment, an adaptation must take place. This adaptation must occur for optimal function of the new gene within the framework of the available viral proteins. Although aquatic birds are the natural reservoir for influenza A viruses, the viruses from these birds replicate poorly in mammals including humans (Hinshaw et al., 1980a; Hinshaw et al., 1980b; Webster et al., 1978). Therefore, viruses from aquatic species must undergo some change before they can cross the species barrier. Influenza viruses can reassort because of their segmented genome. Human influenza strains are thought to be able to acquire genes from avian influenza viruses through this reassortment or through an adaptation in an intermediate (Schafer et al., 1993).

Swine may be the mixing vessel for the reassortment of human and avian influenza viruses. Pigs have receptors in both the SAa2, 6Gal and SAa2, 3Gal conformation and are susceptible to influenza viruses from both avian and human sources (Kida et al., 1994). Therefore the pig can reassort an avian and human virus producing a new virus to which the human population is immune naive. In areas of Asia where backyard pigs co-mingle with chickens and even migratory ducks the potential for reassortment is present. The infrequent transmission of avian influenza viruses to pigs may be the limiting factor in their role as intermediate hosts. Studies

have shown evidence that H3N2 human influenza virus variants can persist in pigs after leaving the human population. However, in nature, a limited number of human and avian viruses have established stable lineages in swine and only occasional transmission of influenza from pigs to humans, resulting in respiratory disease, has been noted (Brown, 2000; Brown, Gajdusek, and Morris, 1966; Makarova et al., 2003; Shortridge et al., 1977).

In 1997 in Hong Kong, H5N1 viruses, circulating in poultry were transmitted to humans which caused 18 illnesses and led to 6 deaths (Shortridge, 1999). Genetic characterization of the isolates from humans and poultry in Hong Kong live bird markets indicated that avian H9N2 and H6N1 viruses were co-circulating with the avian H5N1 virus. Both the H6N1 and H9N2 viruses are endemic in quail in Hong Kong. The H9N2 virus was first isolated in quail in 1988 and transmitted to humans and swine by 1999 (Chin et al., 2002; Guan et al., 2000; Guan et al., 1999; Hoffmann et al., 2000; Perez et al., 2003a). In particular, A/Quail/HK/G1/97 (H9N2) is thought to have been involved in the generation of the highly pathogenic H5N1 human virus of 1997. The six internal genes of this quail H9N2 as well as the human cases of H9N2 infections were similar to the internal genes of the H5N1 viruses co-circulating in poultry in 1997 and which caused fatal human infections. The H5N1 viruses were reassortants that derived their internal genes from a Qa/HK/G1/97-like virus (Lin et al., 2000; Makarova et al., 2003). The antigenic differences between human and swine H9N2 isolates show that swine viruses were not intermediates in avian to human transmission indicating land-based poultry are a potential source of influenza

viruses that can transmit to humans (Makarova et al., 2003; Shortridge et al., 1998; Webby and Webster, 2001). These avian H9N2 viruses have receptor specificity similar to that of human H3N2 viruses providing evidence of a stable avian lineage with human virus-like receptor specificity and the ability to infect different poultry species including quail, chicken and pheasant. This clearly shows that a strict receptor-specificity, typical of influenza viruses in wild aquatic birds may not be an absolute for replication in other avian species (Matrosovich et al., 2000). Phylogenetic analysis of the H9N2 influenza viruses isolated in domestic ducks in southern China during the 2000-2001 season indicate that the H9N2 virus lineage has transmitted back to the ducks, creating double and sometimes triple reassortants with viruses resident in the ducks (Li et al., 2003). This creates a two-way transmission between terrestrial and aquatic birds generating multiple genotypes of H9N2 viruses containing internal genes of aquatic avian origin and more importantly creating a virus with pandemic potential.

The human outbreak of H5N1 in Hong Kong in 1997 was proof that avian viruses can transmit directly to humans. Accordingly there has been an increase in surveillance and characterization of influenza A viruses from quail in the region. Outbreaks in quail have been few, the first reported in Italy between 1966-1968 (Nardelli et al., 1970). Thirteen flocks of quail were found to be infected with an influenza A virus that caused respiratory distress and was lethal to quail younger than 3 months (Makarova et al., 2003). Influenza viruses of many subtypes have been isolated in North America, Europe and Asia from quail including H5N2, H7N2,

H7N3, H9N2 and H10N8 (Guan et al., 1999; Guo et al., 2000; Saito, Kawaoka, and Webster, 1993; Suarez et al., 1999).

2.4.3 Quail as Potential Intermediate Hosts

Quail have been proposed to act as an intermediate host in the process of transmitting avian viruses to humans. The 1997 human outbreak of H5N1 supports a convincing role that quail play in taking a mallard virus and adapting it, allowing replication and transmission within quail and throughout species, particularly land based birds. Quail have been shown to be a reservoir for influenza viruses, creating an environment for replication and transmission to other species. The actual method of how that transmission occurs is not well understood. Quail infected with the highly pathogenic A/Turkey/Ontario/7732/66 (H5N9) show no clinical symptoms but can transmit the virus to chickens causing morbidity and mortality (Slemons and Easterday, 1972; Tashiro, Reinacher, and Rott, 1987). Quail are also more susceptible to infection with goose H5N1 than chickens. The virus replicates in the respiratory tract of the quail and is transmitted by aerosol. The virus has a longer incubation in the quail than the chickens, displaying signs of disease and death later, therefore increasing the likelihood of transmission. Viruses like H5N1 A/Goose/Guangdong/1/96 (H5N1) circulating in 2003 in Southern China replicate in the trachea of quail, transmitted by aerosol and are highly pathogenic in the quail (Webster et al., 2002).

In a study done by Perez et al. (2003) quail were infected with six H9N2 strains isolated from domestic ducks. Quail were susceptible to infections with all six viruses. Replication was predominant in the respiratory tract with little to no virus isolated in the fecal material. This is noteworthy since waterfowl shed most of the virus through the fecal-oral route (Webster et al., 1978). The infection in the respiratory tract promotes viral shedding in the aerosol, the same method of shedding and transmission that occurs in mammals, particularly humans. Thus influenza virus infections in quail can promote a change in the tissue tropism of avian strains, permitting the emergence of variants transmitted by aerosol (Perez et al., 2003a).

In contrast to the quail's susceptibility, chickens inoculated with the duck H9N2 viruses maintained infection in only 2 of the 6 viruses. Therefore quail are more susceptible to infection with H9N2 duck viruses than chickens, however, transmission studies show additional adaptations are required for the virus to transmit to uninfected quail. Perez et al. (2003) looked at the possibility of a previously adapted H9N2 quail virus transmitting to other species. This quail-adapted virus, A/Quail/Hong Kong/A28945/88 (H9N2), was able to transmit to other quail as well as from quail to chickens, demonstrating that a quail-adapted virus is capable of crossing the species barrier.

To understand the potential role played by quail in the live bird markets in southern China as well as in other areas of the world, Makarova et al. (2003) infected quail with all 15 hemagglutinin subtypes to see if each could establish an infection

and if it could then transmit to other quail. Viruses taken from North American and Eurasian lineages confirm quail could establish infection in both lineages. Quail replicated 14 of the 15 subtypes, the exception being a mallard H15N8. Replication occurred in the respiratory tract, one virus, a shorebird H10N4, led to clinical symptoms, killing 2 of the 6 quail infected. Although quail can be experimentally infected with 14 of the 15 HA subtypes, no virus was able to transmit to sentinel cage mates. Additional adaptation is required for adequate transmission among quail. Live quail are no longer permitted in the live birds markets in Hong Kong. Public authorities suspect quail transmit influenza viruses to other birds (Makarova et al., 2003). Unfortunately markets in Mainland China and parts of Europe and the United States continue to house many species of poultry, aquatic and terrestrial including quail, in one location. A market in Nanchang, China was surveyed and labeled by Liu and colleagues as an ideal environment for the maintenance and reassortment of influenza viruses. This surveillance highlights quail as susceptible to all virus subtypes isolated in the market (Liu et al., 2003a; Liu et al., 2003b; Makarova et al., 2003).

Human influenza virus has been isolated in the trachea of one quail (Liu et al., 2003a). In experimental infections with human H1N1 and H3N2 viruses, quail have limited replication and replication is highly unlikely to occur in nature, without prior molecular modification (Makarova et al., 2003). In contrast to the human viruses, swine influenza viruses (H1N1, H3N2, H1N2) replicate in quail, although inefficiently, the location is in the respiratory tract.

The host range of influenza A viruses is polygenic, determined by surface and internal gene products (Ito and Kawaoka, 2000). Interspecies transmission of influenza A viruses among non-natural hosts, in particular land-based birds, and the factors that determine host range are not clear. This project sets out to define changes at the molecular level that determine adaptation of mallard viruses in quail and what changes are necessary for the transmission to other species, in particular chickens.

This research project determined the ability of an aquatic avian influenza virus, A/Mallard/Potsdam/178-4/83 (H2N2), to replicate and transmit in quail. Influenza viruses of the H2 cause the Asian pandemic of 1957 and then disappeared from the human population 11 years later. The pandemic resulted from a reassortant of the circulating human H1N1 and avian H2N2, most closely related to the Eurasian avian H2 HAs (Schafer et al., 1993). The new virus contained avian HA, NA and PB1 genes (Kawaoka, Krauss, and Webster, 1989; Scholtissek et al., 1978). Avian H2N2 isolates were first noted in 1972 and surveillance of avian influenza and strain isolation was not set in motion until 1970. This H2N2 subtype continues to circulate in avian species. It has been almost 40 years since it has last circulated in humans, resulting in a population whose majority is immune naive and / or lacks the memory B cell response to battle this potential pandemic reemergence (Govorkova et al., 1993). H2 influenza disappeared from the human population in 1968 with the emergence of the H3N2 strain and pandemic, but H2N2 influenza viruses have been isolated from domestic and wild birds in recent years. Avian H2 influenza viruses are

divided into two distinct lineages, based on geography, North American and Eurasian (Liu et al., 2004). There was an increased prevalence of H2 influenza in wild ducks in 1988 in North America leading to the appearance of H2N2 in domestic fowl (Schafer et al., 1993). Antigenic analysis indicates that the majority of avian H2N2 isolates from wild birds up through 1991 are closely related to the early human H2N2 viruses, demonstrating that H2N2 pandemic-like virus strains are continually circulating. Antigenically distinct H2N2 viruses have been isolated from shorebirds in Delaware in 1993 and 1997. The H2 HA genes of the Eurasian lineage were transmitted to North American birds, however no internal genes have been reported in the North American lineages (Makarova et al., 1999; Schafer et al., 1993). In contrast, Eurasian strains can gain the internal genes from the North American lineage, proving interregional transmission of the internal protein genes of influenza occur between North American and Eurasian migratory ducks (Liu et al., 2004). As the prevalence of avian H2N2 influenza viruses increase, greater numbers come into direct contact with susceptible humans, highlighting the importance of surveillance and characterization of H2 influenza viruses in wild and domestic avian species.

Though no H2 viruses have been isolated from swine, transmission of influenza from bird to human has occurred (Claas et al., 1998; Subbarao et al., 1998; van Kolfschooten, 2003). In 1997 and 1999, human infections with H5N1 and H9N2 strains respectively (Shortridge, 1999) have resulted from direct transmission by land-based birds, indicating their possible role as a vehicle capable of crossing the species barrier. Quail are a member of that group. Our goal is to determine how much

change is needed to adapt a strain, from its natural reservoir, to a species that can transmit within its species and across to other species, including mammals.

CHAPTER 3, MATERIALS AND METHODS

3.1 Influenza Virus

The H2N2 virus, A/Mallard/Potsdam/178-4/83, was provided by the repository at St. Jude Children's Research Hospital, Memphis, Tennessee. The virus was propagated in 10-day-old embryonated SPF chicken eggs and stored at -70^0C. The virus was titrated to determine the egg infectious dose 50 by the Reed and Muench method (1938). The stock virus yielded a concentration of 5×10^7 EID_{50} / ml when titrated in eggs. Hemagglutinin Assay revealed a titer of 1:1024.

3.2 Animals and experimental infections

Four to six-week old Japanese quail (*Coturnix coturnix*) (Department of Animal and Avian Sciences, University of Maryland, College Park), three to four-week White Leghorn chickens (Charles River Laboratories, Wilmington, MA) and two to three-week old mallard ducks (M^cMurray Hatchery, Webster City, IA) were used. Birds were fed Purina Mills game bird diet and food and water was provided *ad lib* for all studies.

Virus was administered in doses of 600 µl at a concentration of 5×10^6 EID_{50}/ ml. Infection routes were through the nares, mouth and eyes with roughly 100 µl given through the nares and eyes and the remaining 500 µl through the trachea. Feeding gavages were used to administer the virus. The inoculum was prepared in a 10-fold dilution using a solution of PBS with 1% antibiotic/ antimycotics (100X

Sigma). Tracheal swabs were collected days 1, 3, 5, 7, 9 post-infection and stored in glass vials containing 1 ml glycerol medium at -70^0C (50% sterile glycerol, 50% PBS, 1 ml/ 200 ml total volume gentamicin, 10 ml 100X antibiotic/antimycotics) (Guan et al., 2000). Swab samples were tested for virus by passing swab medium in 10-day-old embryonated chicken eggs (Palmer et al., 1975). Three eggs were used for each swab collected and 200 µl of medium was injected into each egg, incubated for 48 hours and chilled at 4^0C for no more than 24 hours, or 30-60 minutes at -20^0C. Allantoic fluid was collected and a hemagglutinin assay was performed to determine virus presence. Samples showing agglutination were scored as positive.

Clinical symptoms that are usually associated with influenza infections in poultry include ruffled feathers, excess mucous, cyanosis (usually on the tongue), appetite and weight loss and a drop in egg production. None of the females in any of the passages in this experiment were at sexually maturity. Additional symptoms are listed in Table 1. Birds were observed and scored daily for clinical signs of illness and general well being. Animals were evaluated on appetite, activity, fecal output and signs of distress or clinical illness including cyanosis of tongue or legs, ruffled feathers and respiratory distress. Birds were scored on a scale of 1 to 4, 4 requiring euthanasia. Table 2 lists the categories each symptom was grouped into and the appropriate score associated with the symptom.

Experiments were carried out under BSL2 conditions with investigators wearing appropriate protective equipment and compliant with all IACUC approved protocols (R-03-13) and under AWA regulations.

Table 1 Infection Study Endpoints

Infection Study Endpoints: Clinical Signs associated with influenza infection in poultry

Typical of low pathogenic avian influenza

- Ruffled feathers
- Depression and lethargy
- Loss of appetite / weight loss
- Respiratory distress
- Diarrhea

Typical of high pathogenic avian influenza

- Cyanosis (purple-blue coloring) of wattles and comb and tongue Edema and swelling of head, eyelids, comb, wattles, and hocks
- Blood-tinged discharge from nostrils
- Incoordination, including loss of ability to walk and stand
- Pin-point hemorrhages (most easily seen on the feet and shanks)

Table 2, Score Table for Daily Observations in Poultry

Scores and Endpoints

	Category of	Clinical	Symptoms
Score/Endpoints	1) Ruffled feathers, depression, loss of appetite/weight loss, respiratory distress.	2) Diarrhea, cyanosis of tongue, pinpoint hemorrhages.	3) Edema and swelling of head, eyelids, legs, blood-tinged discharge from nostrils, incoordination.

Score

1-1 or 2 clinical symptoms; from category 1

2-3 or 4 clinical symptoms; from category 1

3-5 or 6 clinical symptoms; from combination of categories 1 and 2

4-7 clinical symptoms; from combination of categories 1, 2, and 3 (if the bird does not appear to recover at the beginning of the 3rd day, it will be euthanized).

Table 2. Birds were observed and scored daily for clinical signs of illness and general well being. Animals were evaluated on appetite, activity, fecal output and signs of distress or clinical illness, including cyanosis of tongue or legs, ruffled feathers and respiratory distress. Birds were scored on a scale of 1 to 4, 4 requiring euthanasia.

3.3 Transmission of influenza virus in quail

For transmission experiments, three quail, four to six weeks old, were infected with virus at a concentration of 5 x 10^6 EID_{50} / ml, and volume of 600 µl, as previously described. Three contact quail were introduced at day one post-infection, into the cage with the infected quail. Water and food bowls as well as cage liners were changed in order to prevent transmission of virus via contaminated water or food. Therefore, if transmission occurred it was through direct contact with the quail and not because of virus contamination. Tracheal samples were collected day 1, 3, 5, 7, and 9 post infection and HA assays were run to determine viral shedding.

3.4 Adaptation if mallard H2N2 virus in quail

Adaptation experiments comprised of eight passages of A/Mallard/Potsdam/178-4/83 (H2N2) virus in quail. The first five passages consisted of three quail that were infected with a 600 µl dose of virus. The remaining three passages were groups of six quail and transmission studies were carried out. The first passage's dose was H2N2 viral stock (wild type) grown once in embryonated chicken eggs, as described above. Later passages were inoculated with either a 10% lung homogenate from the previous passage, a two-fold dilution of swab medium using

PBS/ antibiotic/antimycotic (abbreviated PBS/Antb) or a ten-fold dilution of allantoic fluid, collected from swab inoculation, into PBS/Antb from the previous passage. Tracheal swabs were collected on days 1, 3, 5, 7 post-infection and virus was detected through HA assays as outlined in Table 3.

Table 3, Dosing for Adaptation Study

Passage	Dose	Swab Collected	Lung Homogenate Collected
		Days	Days
1	1:10 dilution stock virus	3	3
2	1:10 dilution af[a] from lung homogenate, p1[b]	3	3
3	10% lung homogenate pooled from p2	3	3
4	10% lung homogenate pooled from p3	1,3,5	5
5	10% lung homogenate pooled from p4	1,3,5	5
6[c]	pooled swab medium, day 5, p5	1,3,5,7	7
7	1:10 dilution af from swabs, day 5, p6 contacts	1,3,5,7	7
8	1:10 dilution af from swabs, day 5, p7 contacts	1,3,5,7	7

a: af = allantoic fluid
b: p1-7 = passage 1, 2, 3, 4, 5, 6, 7
c: Transmission tested and confirmed

Table 3. Doses were given to quail in 600 µl volumes. Lung homogenate was pooled, 1 ml from each quail, and used as the dose for the following group. Tracheal swabs were passed in embryonated eggs and allantoic fluid was collected and used for doses in passages 7 and 8 because swab medium was directly used for PCR and analysis. Transmission was tested and confirmed at passage 6, this passage was repeated three times before proceeding to passage 7 to ensure and confirm transmission.

3.5 Dose Preparations

Lungs were collected on day 3, 5 or 7 post-infection, as outlined in Table 3.3. B-Euthanasia solution was administered intra-cardiac, 500 µl per quail**.**
Lungs were collected through the back of the quail. A midline incision was made from the neck to pelvic bones using a scalpel (Feather No10) and scissors were used to cut through the rib cage to expose the lungs. Each lobe was removed with forceps and washed two times in PBS. Lungs were then placed in tissue bags and weighed, PBS was then added 1X to lung weight. Lung tissue was homogenized using a stomacher (80 Biomaster, Wolf Laboratories Limited, UK). Lung tissue was completely homogenized for the next passage's dose.

A 10% lung homogenate was prepared by adding 10 ml of PBS to each tissue sample and then filtered through a 0.22 µm filter unit (Corning #430626) and double filter pad. The next passage was infected with the 10% lung homogenate; 1 ml from each quail was combined and used for dosing the next passage. Doses of 600 µl were administered, using sterile feeding gavages (Fisher Ref 7905,18x2" (50.8MM W/2-1/4). Roughly 100 µl was given in the eyes and nares while the remaining 500 µl was inoculated in the trachea. Homogenate from each quail was titrated separately in embryonated chicken eggs to confirm virus presence in each of the homogenate doses.

Preparations of swab medium were diluted two-fold in PBS/Antibiotics to yield the dose for passage 6. The medium was diluted to decrease the viscosity of the glycerol-based medium and to ease administration in quail. Allantoic fluid, used for infections in passages 7 and 8, was diluted in a 1:10 dilution of allantoic fluid with PBS/Antibiotic. The allantoic fluid was from tracheal swabs from passage 6 contact quail collected on day 5 post-infection.

3.6 Location of virus replication and shedding of WT and Quail-adapted H2N2

To confirm previous findings that quail replicate virus mainly in the trachea and shed via aerosol in contrast to the fecal-oral route established in wild aquatic birds (Makarova et al., 2003), both WT H2N2 and Quail-adapted H2N2 were administered to quail in order to identify the location of maximum shedding and replication. Quail were divided into groups of six, three infected and three contact, per virus. Each viral group was infected and monitored as described for the transmission and adaptation studies. Tracheal and cloacal swabs were collected day 1, 3, 5 and 7 post-infection. Swabs were passed in embryonated chicken eggs and HA assays were run to determine if WT and / or Quail-adapted virus shed in the cloaca as well as the trachea.

3.7 RT-PCR and sequencing

Viral RNA was extracted from allantoic fluid or swab medium using the RNeasy Mini Kit (Qiagen Sciences, MD) as directed by the manufacturer's protocol. RNA extraction, cDNA synthesis and RT-PCR amplification were performed as described by Hoffman (et al). Modifications were made when extracting RNA from swab medium. Briefly, 500 µl of swab medium was combined with 500 µl Buffer RLT/BME and 500 µl 70% ethanol and the remaining protocol was followed. For cDNA synthesis, 8 µl of swab-extracted RNA was used rather than the usual 4 µl when using allantoic fluid. Sequencing was performed using the BigDye-Terminator protocol V3.1 (Applied Biosystems, Foster City, CA). Minor modifications were made to the manufacturer's protocol. BigDye terminator solution volumes were decreased so that Sequence RR-100 was at 2 µl and the 5X Sequence Buffer was a 3 µl per reaction. To counter the change in volume Millipore water added was 10.5 µl, DNA was 3 µl and primer (40 ng/ µl) was 1.5 µl to bring the total volume to 20 µl. The PCR program was 96^{o}C for 4 min, (95^{o}C for 30 sec, 56^{o}C for 10 sec, 60^{o}C for 4 min) x 45 cycles, 4^{0}C forever. In addition to the universal primers described by Hoffmann, specific primers were designed and are listed in Table 4 (Hoffmann et al., 2001). Primers were designed through the alignment and analysis of sequences found on the Influenza Database website, http://www.flu.lanl.gov.

To sequence the quail-adapted virus, tracheal swabs from passage 5 were grown in embryonated chicken eggs, titrated, aliquoted and stored at -70^{0}C for stock. Passage 5 was chosen because it was the first to be used as the dose in the transmission study.

Table 4, Primers designed for sequencing WT H2N2 and Quail-adapted H2N2 viruses

GENE	PRIMER 5' to 3' DIRECTION
HA	
H2 362-386F	CCAGGCAGTTTCAATGATTATGAAG
H2 386-362R	CTTCATAATCATTGAAACTGCCTGG
H2 753-771F	GAGGTAGAATGGAATTCTC
H2 771-753R	GAGAATTCCATTCTACCTC
H2 1006-990R	TTTTACATATTTGGGGC
H2 1260-1283F	TTGGAAAAGAATTCAGTAACTTGG
H2 1473-1496F	GTTTTGAATTTTATCACAAATGTG
H2 1773-1753R	AGTAGAAACAAGGGTGTTTTT
NA	
N2 342-324R	AAAGCCGAATTGAATTGTCC
N2 845-867F	GAGGAATGTTCCTGTTATCCTCG
N2 977-999F	GGGCTCGTTGGTGATACACCCAG
N2 1283-1303F	TTTATGTGGAGTTGATAAGGG
N2 1467-1445R	AGTAGAAACAAGGAGTTTTTTTC
M	
M 431-410R	CCCATCCTGTTGTATATGAGAC
M 468-490F	TGTGCCACTTGTGAGCAGATTGC
M 758-727R	TGGTAGGCCTGCAAATTTTCAA
M 827-858F	TGATATTGTGGATTCTTGATCG
NP	
NP 431-412R	GCGTCTTCTCCATTGTTCGC
NP 529-551F	AGAATGTGCTCTCTGATGCAAGG
NP 716-697R	TTGCACATTCTCTCATATGC
NP 856-878F	GCCCATAAGTCCTGCTTGCCTGC
NP 1052-1031R	GCAGAGTGGCAGCCATCCATA
NP 1360-1380F	GACATGAGGACTGAAATCATA
PA	
PA 336-317R	CTTGGGCTTCTCAACCCCCG
PA 605-623F	CCGAAAGAGGCGAAGAGAC
PA 728-709R	CCATCCACATAGGCTCTAAA
PA 734-756F	AACCGAACGGCTGCATTGAGGGC
PA 1321-1340F	GCCCCAATTGAGCATATTGC
PA 1440-1420R	TTATGTACACTCCCTTCATTA
PA 1861-1840R	CAAAGAATTCTTTGGTCATGTC
PA 2039-2969F	TTAGGGACAACCTGGAACCTGG

GENE	PRIMER 5' to 3' DIRECTION
PB1	
PB1 419-400R	GTCCAGTCATAGGTCTGGCG
PB1 742-764F	GCAATTGCAACACCCGGAATGCA
PB1 960-940R	ATTCTCATTCCATTTGGTGTT
PB1 1240-1259F	GGAATGATGATGGGCATGTT
PB1 1360-1337R	CATCAGAGGATTGGAGTCCGTCCC
PB1 1642-1664F	GGACCAGCAACAGCTCAGATGGC
PB1 1811-1790R	GGTCCTCCATCTGAAACCAACA
PB1 1930-1952F	GAAATTGAGTCCGTAAACAATGC
PB2	
PB2 412-392R	CCATGTTTCAACCTTTCAACC
PB2 680-658R	CGAACCAGTTCTCTTTCCAACAT
PB2 879-900F	GCCATAGCACACAAATTGGCGG
PB2 900-879R	CCGCCAATTTGTGTGCTATGGC
PB2 1012-1034F	AGCTTTGGAGGCTTCACTTTCAA
PB2 1262-1242R	TCTTGTGAGAACACCATTGC
PB2 1430-1452F	TATTACCTGACATGACTCCCAGC
PB2 1774-1756R	CAAACTCCATCTTATTGTA
PB2 1972-1994F	GGAATGAGAATACTCGTAAGGGG

3.8 Hemagglutinin Assays

Hemagglutinin assays (HA) were performed for virus detection in swabs and lung homogenate. 10-day-old embryonated chicken eggs (B&E Eggs, Your Springs, PA) were inoculated with swab medium or lung homogenates (200 µl/egg) as previously described (Palmer et al., 1975). Positive and negative controls used were stock virus and PBS or uninfected allantoic fluid, respectively (Reed and Muench, 1938).

For titration of virus using lung homogenate or swab medium, serial dilutions with PBS/Antibiotics were prepared, 10^{-1} to 10^{-5}, and inoculated in eggs. Egg Infectious Dose (EID_{50}) was also determined through HA assays of swab samples, passed once in eggs, and used to compare concentrations of virus in wild type and adapted studies using both Japanese quail and White Leghorn chickens (Palmer et al., 1975).

3.9 Transmission of quail-adapted virus in chickens

After adapting the wild type H2N2 (WT H2N2) virus in quail, it was necessary to determine if that adaptation would allow the replication of the virus to other terrestrial birds, particularly chickens. White leghorn chickens, three to four-weeks of age, were used to test whether WT H2N2 or Quail-adapted H2N2 replicated and transmitted in chickens.

The chickens were inoculated with either WT or Quail-adapted virus. Three chickens were infected for each group and housed in separate BSL2 rooms. Doses were administered in the same manner as the quail although the doses were increased for the chickens to a volume of 1 ml. For the WT infection, viral dose was prepared from wild type stock at a 1:10 dilution in PBS/Antb. Therefore the chickens received the same amount of virus, $5x10^6$ /ml, as the quail. In the Quail-adapted group, virus was grown in embryonated chicken eggs from the tracheal swabs from quail in passage 5 and then diluted in 1:10 ratio with PBS/Antb. Viral titer from passage 5 stock was $2x10^9$ EID_{50} / ml so the dose given was diluted and prepared at 5 $x10^6$ EID_{50} / ml. To inocluate the chickens with virus, feeding gavages were used in the same manner as the quail. Drops were placed in the eyes and nares and the remaining volume, roughly 600 µl was administered through the trachea.

One-day post infection three contact chickens were introduced into each group. Feeders and water containers were cleaned and disinfected to prevent transmission to contacts through contaminated food and water. Cloacal and tracheal swabs were collected for the chickens. Adaptation studies with the quail created a virus that replicated and transmitted in the trachea of the quail, so to cover both areas of possible shedding, tracheal and cloacal swabs were taken. Swabs were collected days 1, 3, 5, 7, 9, 11 and 15 post-infection. Swabs were stored in the same conditions as quail tracheal swabs, 1 ml glycerol medium at -70^0C (Guan et al., 2000).

Daily observations were made for both groups. The chickens in the wild type and those in the Quail-adapted group were treated and observed in the same way. Transmission studies for the WT and Quail-adapted viruses were repeated twice in chickens as they were for the quail studies. Therefore, the experiment was carried out identically, in full three times to confirm all results and observations.

3.10 Weight Study

Clinical symptoms can be one indication of an influenza infection but when signs are not apparent other measures can be taken to monitor the stability of the bird. In addition to confirming transmission studies in both quail and chickens, the birds were weighed and monitored during the study to see if through the life of the infection quail and / or chickens lost weight in either of the viral groups. Quail and chickens were swabbed and weighed for 9 days.

3.11 Infectious Dose 50 Studies

To determine the dose that will infect half the quail, serial dilutions of the WT and Quail-adapted H2N2 were prepared. The dose for each virus in transmission studies of WT H2N2 was $5x10^6$ EID_{50}/ ml; therefore dilutions of $5x10^5$ to 50 EID_{50} / ml were prepared. Stock was diluted with PBS/Antb. Quail were assigned three to a viral dilution and placed in cages separate from other dilution groups. Tracheal swabs were collected on days 1 and 3 post-infection and HA assays were run to detect viral shedding. The dilution resulting in infection and shedding from half the quail in the group was selected as the Quail-ID_{50}. For the Quail-adapted virus

the dose at infection was 5 x10^6 EID_{50} / ml. Dilutions prepared for the study ranged from 5 x 10^4 to 5 x10^{-1} EID_{50} / ml. Quail were again grouped in three per dilution with ample space between cages to prevent transmission between dilution groups. Tracheal swabs were collected on days 1 and 3 and tested for virus through HA assays.

Chicken infectious dose 50 was performed for the WT H2N2 and the Quail-adapted H2N2. WT H2N2 was prepared in serial dilutions of 5x10^7 to 5x10^6 EID_{50} / ml. Quail-adapted virus was prepared in dilutions ranging from 5 x10^6 to 5 EID_{50} / ml. Chickens were observed for 5 days and cloacal swabs were collected day 1 and 3 post-infection.

3.12 Replication in Mallard Ducks

Once A/Mallard/Potsdam/178-4/83 (H2N2) had been adapted in quail and tested in chickens it was examined to see if the adaptation in quail led to a virus that replicated and transmitted successfully in mallard ducks. Two week-old mallard ducks were used to test WT H2N2 against Quail-adapted H2N2 to see if any changes in replication and shedding occurred. Ducks were infected in the same manner and with the same volume doses as the chickens and swab samples were collected from the trachea and cloaca of the ducks. Ducks were swabbed days 1, 3 and 5 post-infection. The objective of this experiment was to see whether adaptation of a

mallard strain in quail could lead to a virus that would still be able to replicate and infect the natural host.

3.13 Plaque Assays

Plaque assays were performed on select passages to compare plaque size and number. Three passages were chosen, Passages 1, 5 and 8 and were compared to viral stock. The assay dilutions from swab medium were 10^{-1} to 10^{-6} while allantoic fluid from stock was diluted 10^{-4} to 10^{-9}, based on the amount of virus in a swab sample versus allantoic fluid. Confluent MDCK cell monolayers in 6-well plates were infected with 10-fold dilutions of virus in a total volume of 1 ml PBS at 4^0C for 15 minutes and then 45 minutes at 37^0C. Cells were washed twice with PBS and covered with an overlay of MEM containing 1.8% agar, 0.02% BSA, 1% glutamine and 1 μg/ml TPCK trypsin. After three days of incubation at 37^0C under 5% CO_2, the overlay was removed and cells were stained with 0.1% crystal violet in a 10% formaldehyde solution.

CHAPTER 4, RESULTS

4.1 Transmission of A/Mallard/Potsdam/178-4/83 (H2N2), in Quail

To determine if quail can replicate and transmit A/Mallard/Potsdam/178-4/83 a transmission experiment was performed. As shown in Table 5, there was no transmission of the H2N2 virus to contact quail. Viral replication occurred in the trachea of the infected birds to titers of $5x10^4$ EID_{50} / ml. Viral shedding peaked at day 3 post-infection and virus undetectable by day 5. There were no clinical symptoms observed in any of the quail. Quail gained weight by the end of the study.

4.2 Adaptation of A/Mallard/Potsdam/178-4/83 (H2N2) in Quail

The inability of the virus to transmit to sentinel quail was the basis for further experiments. Virus shedding peaked on day 3 post-infection with a titer of $5 x10^4$ EID_{50} / ml for the quail infected with WT H2N2. In order to adapt this virus, lungs were collected from quail infected with the WT H2N2 and serially passed to groups of three quail. Swabs were collected from the trachea and at the day of peak viral shedding, lungs were collected and homogenated. Eight passages of quail were involved in this adaptation study. Table 3 in chapter 3, lists the doses administered to each passage of quail involved in the adaptation study. Table 6 highlights the shedding of virus, days of lung collection and viral detection in the lung homogenate

for passages 1 through 8. At passage 6, transmission was tested and confirmed. Table 5 shows transmission in contacts on day 3 post-infection. Quail shed this virus longer than previous passages. Transmission occurred between day 2 and 3 post-infection in 70% of the contacts and by day 5 in the remaining quail. As with the WT H2N2 infections, no clinical signs of illness or distress were noted in any of the quail.

Passage 6 was repeated two additional times and transmission was confirmed yielding the same results (Table 5). Lungs were collected and tested from passage 6 for virus, however once transmission occurred, swab medium was used to dose the next two passages. Swabs from passage 6, day 5, contacts were used for PCR as well as HA assays. Therefore, allantoic fluid collected from the HA assays on contact quail, on day 5 were used to infect passages 7 and 8. Transmission was tested and observed in passages 7 and 8. Thus, a constant addition of sentinel quail every few days would ensure viral success.

Table 5, Transmission of WT H2N2 and Quail-adapted Virus in Quail

Transmission Study	Group	Day (number of positive swab samples)					
		1	3	5	7	9	11
WT H2N2							
	Infected Quail	9/9	6/9	0/9	0/9	0/9	
	Contact Quail	0/9	0/9	0/9	0/9	0/9	
Passage 6 H2N2 Quail-adapted	Infected Quail	8/9	9/9	9/9	0/9	0/9	
	Contact Quail	0/9	7/9	9/9	3/9	0/9	
Passage 8 H2N2 Quail-adapted	Infected Quail	3/3	3/3	3/3	0/3	0/3	0/3
	Contact Quail	0/3	0/3	3/3	3/3	2/3	0/3

Table 5. Transmission studies of quail inoculated with the WT H2N2 virus and the same H2N2 virus serially passed through six groups of quail. Tracheal swabs collected days 1, 3, 5, 7 and 9 post infection show that A/Mallard/Potsdam/178-4/83 (H2N2) does not transmit to contact quail. Quail infected with virus shed for 3 to 4 days with peak titers on day 3. WT H2N2 was then serially passed in quail. Lungs were collected and a 10% homogenate was prepared to use as a dose for the following group of quail. After five passages, the sixth group was tested for transmission and transmission of the virus to contact quail was confirmed. After transmission at passage 6, the virus continued to adapt to the quail, improving the length of shedding of the virus in contact quail. In neither the WT nor Quail-adapted studies did quail show any clinical signs of infection. Transmission experiments were run at three separate times to confirm results.

Table 6, Viral Shedding in Quail, Passages 1-8

Passage	Swab Collection Day (positive out of 3)						Lung Collection Day (positive out of 3)			
	1	3	5	7	9	11	3	5	7	9
1	3/3	3/3					3/3			
2	3/3	3/3					3/3			
3	3/3	3/3					3/3			
4	3/3	3/3	2/2					1/2*		
5	3/3	3/3	3/3					2/3		
6	8/18	**16/18**	**18/18**	**6/18**	**0/18**				**1/3**	
7	3/6	**6/6**	**6/6**	**3/6**	**1/6**				**3/3**	
8	3/6	3/6	**6/6**	**3/6**	**2/6**	**0/6**				**1/3**

- One quail from passage 4 was euthanized due to pecking from cage mates
- Bold numbers include contact quail

Table 6. Tracheal swabs were collected and tested for virus shedding for each passage. At passage 4 shedding was observed at day 5 and tracheal swabs collected. Lungs were then collected on day 5 for passages 4 and 5. At transmission for passage 6, contact quail shed virus until day 7. Contact shedding extended to day 9 post-infection for passages 7 and 8.

4.3 Location of viral replication and shedding

To confirm the location of viral shedding both WT and Quail-adapted viruses were tested. Cloacal and tracheal swabs were collected and HA assays were run to determine viral presence. As anticipated, quail did not successfully shed WT or Quail-adapted virus via the fecal-oral route. Out of 24 quail tested (groups of 6 quail/virus repeated twice each) only one quail shed virus via the cloaca. This quail was a contact quail in the quail-adapted virus group and the only positive cloacal swab taken from this quail occurred on day 5. Viral shedding correlated to previous transmission studies, with the WT group shedding virus until day 3 post-infection without transmission and the Quail-adapted virus shedding until day 7 in contacts and day 5 in infected birds.

4.4 Adaptation of mallard influenza virus creates a strain capable of transmitting to other terrestrial avian species

Quail's role as an intermediate host in which an influenza virus can change its phenotypic characteristics and increase its host range was investigated.

White Leghorn chickens were chosen to test whether the newly quail-adapted virus could lead to infection and transmission in chickens. The WT, A/Mallard/Potsdam/178-4/83 (H2N2) was also tested to compare infectivity and transmissibility with the quail-adapted virus.

Chickens infected with the WT H2N2 virus did not establish an infection in the trachea or cloaca. No virus was detected in either swab sample from infected chickens and thus no transmission was noted in contact chickens from the WT group. In contrast, chickens infected with the quail-adapted H2N2 shed virus, transmitted to contact chickens and established an infection. Infected and contact chickens displayed clinical symptoms including lethargy, excess mucous and diarrhea. Infected chickens shed virus up to day 9 while contact chickens began shedding virus on day 3 post-infection and shed up until day 15. All positive samples were detected through cloacal swabs, no tracheal swabs were positive for virus in either group, Table 7.

Quail-adapted A/Mallard/Potsdam/178-4/83 (H2N2) did not loose its ability to replicate in mallard ducks. Table 8 lists the days of shedding in both WT and quail-adapted groups. Adaptation of the mallard virus in quail led to a virus that shed in the cloaca and trachea of mallard ducks, albeit less than the WT H2N2. Ducks from both groups showed no signs of infection.

Viral titers were measured in swabs collected from both infected and contact quail, chickens and mallard ducks to determine if the adaptation in quail led to a virus that improved its ability to shed to contacts. Table 9 outlines the titers produced from swabs, tracheal for quail and cloacal for chickens and mallard ducks, that were collected on peak days of shedding. The adaptation created a virus that sheds better than the WT H2N2.

Table 7, Replication and Transmission of WT and Quail-adapted H2N2 in White Leghorn Chickens

Transmission Study	Group	Day (number of positive swab samples)						
		1	3	5	7	9	11	15
WT H2N2	Infected Chicken	0/9	0/9	0/9	0/9	0/9	0/9	ND
	Contact Chicken	0/9	0/9	0/9	0/9	0/9	0/9	ND
Passage 6 H2N2 Quail-adapted	Infected Chicken	3/9	8/9	9/9	9/9	8/9	0/9	0/9
	Contact Chicken	0/9	3/9	4/9	9/9	9/9	3/9	1/9

Table 7. WT A/Mallard/Potsdam/178-4/83 (H2N2) does not replicate or transmit in White Leghorn chickens. Infection with quail-adapted virus from passage 6 leads to replication and transmission in chickens. Infection leads to disease symptoms including lethargy, excess mucous and diarrhea. Experiments were run at three separate times to confirm results.

Table 8, Replication of WT H2N2 versus Quail-adapted H2N2 in mallard ducks

Virus	Day	Trachea Swabs Positive (out of 2)	Cloacal Swabs Positive (out of 2)
WT H2N2	1	1/2	2/2
	3	1/2	2/2
	5	1/2	2/2
	7	1/2	1/2
Passage 6 Quail-adapted	1	0/2	0/2
	3	0/2	1/2
	5	0/2	1/2
	7	1/2	1/2

Table 8. Mallard ducks were infected with WT H2N2 and Quail-adapted H2N2 to determine the ability of quail-adapted H2N2 to replicate in the virus' natural host. Although the Quail-adapted virus replicated in the trachea and cloaca of mallards it did so at decreased abilities compared to the WT H2N2.

Table 9, Viral Titers in Tracheal and Cloacal Swabs from Quai, Chickens and Ducks

Species	Virus	Day	Infected (EID_{50}/ml)	Contact (EID_{50}/ml)
Quail	WT H2N2	3	5×10^4	N/A
	Quail-adapted H2N2	3	1.6×10^4	2×10^3
		5	9×10^3	9×10^4
		7		9×10^3
Chicken	Quail-adapted H2N2	3	5×10^2	
		5	3×10^4	
		7	2×10^5	2×10^5
		9	8×10^4	2×10^5
		11		9×10^4
Mallard Ducks	WT H2N2	3	2.8×10^4 /ml	N/A
	Quail-adapted H2N2	3	9×10^3/ml	N/A

Table 9. Tracheal (quail) and cloacal (chicken and ducks) swabs were diluted (10^{-1} to 10^{-5}) and passed in embryonated chicken eggs to determine the EID_{50} for each swab. Three swab titers were averaged for each day when possible.

4.5 Infectious Dose 50 Studies

The Egg Infectious Dose 50 (EID_{50}) was measured for WT and quail-adapted H2N2 viruses. The WT virus gave 5 $x10^7$ EID_{50} /ml and the quail-adapted H2N2 gave1.6 $x10^9$ EID_{50} /ml. Quail and chickens were infected to determine the minimal amount of WT and quail-adapted virus needed to establish an infection in 50% of the birds. The quail-adapted virus required a smaller dose than the WT to establish an infection in 50% of the birds, chickens or quail. For quail, the dose of WT H2N2 required was 5 $x10^2$ EID_{50} /ml while the quail-adapted virus required a dose of 50 EID_{50}/ml. Concentrating the WT H2N2 virus did not aid in infecting the chickens. The dose of quail-adapted virus needed to infect half the chickens was 5 x 10^4 EID_{50} /ml.

4.6 RT-PCR and Sequencing of the WT A/Mallard/Potsdam/178-4/83 (H2N2) and Quail-adapted Virus

A single amino acid change was observed on the surface proteins of the quail-adapted virus and five other amino acid changes were detected in the internal proteins of the virus. The change on the surface occurred at position 170 of the HA protein in which asparagine in the mallard virus was changed to aspartic acid in the quail-adapted virus (N170D). Other amino acid changes occurred in the internal proteins of the virus, specifically PB2, PB1 and NP. There was one change in PB2 alanine to valine at position 588 (A588V). Three amino acid changes occurred in PB1,

glutamine to arginine, aspartic acid to glutamic acid and serine to isoleucine (Q268R, D398E, S654I) and there was one in NP, alanine to threonine (A234T). All changes are listed in Table 10. Neither amino acid changes nor nucleotide changes were detected in the PA and M genes. Silent changes were observed in the NA and NS genes. Synonymous changes observed are listed in Table 11.

4.7 Amino Acid Changes in Adaptation: Comparison to Contact Quail in Passage 8 and Contact Chickens Infected with Quail-adapted H2N2

After two additional passages in quail, the adapted H2N2 virus did not show any indications of further mutations at the nucleotide or amino acid level. When comparing the virus extracted from contact chicken swab medium passed in embryonated chicken eggs, there was one mutation leading to an amino acid change in the HA region, serine to tyrosine (S152Y) which is close to the change observed during the quail adaptation (N170D).

Table 10, Comparison of Amino Acid changes in Quail-adapted H2N2 to WT H2N2 and Influenza Viruses from Different Animal Species

Gene	Position	WT	Quail-adapted	Avian	Swine	Human	Equine
PB2	588	A	V	A,V,T	T,I,V	I,V	T
PB1	268	Q	R	Q	Q	Q	Q
	398	D	E	D,E*	D	D,E*	E
	654	S	I	S	S	S,I	S
HA	170	N	D	N,D	N/A	N,D	N/A
NP	234	A	T	A	A	A	A

* Most quail viruses have E at this position. The 1997 H5N1 viruses that crossed to humans have E at this position. N/A: not applicable

Table 10. Changes associated with the adaptation in quail are changes that correspond to the sequences of virus from passage 5. No additional mutations occurred after passaging the virus two more times. Sequences were preformed with virus from passage 8 in quail. Synonymous changes observed are listed in Table 11.

Table 11, Synonymous Changes from Adaptation in Quail

Gene	No. of Nucleotide Changes	Position	WT	Quail-adapted
PB2	2	402	G	A
		1251	T	C
PB1	0			
PA	0			
HA	1	739	A	G
NP	2	513	T	C
		1117	C	T
NA	1	541	A	G
M	0			
NS	1	371	T	C

Table 11. Nucleotide changes were observed in all genes except M and PA. PB was the only gene to have nonsynonymous changes without any synonymous changes.

4.8 Clinical Symptoms Associated with Infection of Quail-adapted H2N2 in quail and chickens

Quail infected with WT H2N2 and their cage mates showed no significant signs of weight loss, Figure 2A. Quail infected with the quail-adapted virus showed a steep decrease in weight loss ranging from 2-4% at day 1 and up to 7% on day 3. There was a noted increase of weight between day 4 and 5, Figure 2B.

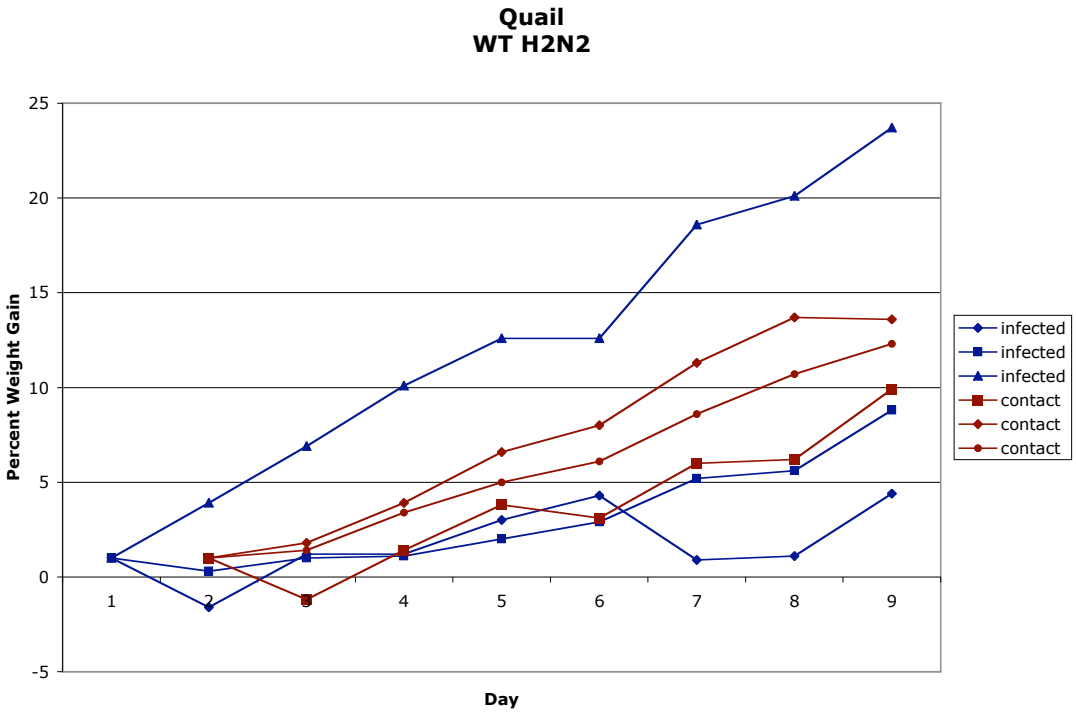
Chickens infected with WT H2N2 shed no virus and gained weight throughout the nine days. There is a linear relationship shown in Figure 3A for the chickens. Chickens infected with the quail-adapted virus had a similar weight gain to the WT chickens up until day 7. A few chickens lost weight but there was only one to lose more than 2%, Figure 3B. There is less of a linear relationship in chickens infected with the quail-adapted virus however there is a significant increase, on average 30% between days 7 and 8.

4.9 Plaque Assays

There were no signs of changes in plaque morphology from the adaptation in quail and subsequent infection in chickens. Plaque size and quantity did not vary to a significant extent when comparing the WT H2N2 to the plaques from passage 6 and contact chickens.

Figure 2, Weight Study in Quail Infected with Quail-adapted H2N2

A.



B.

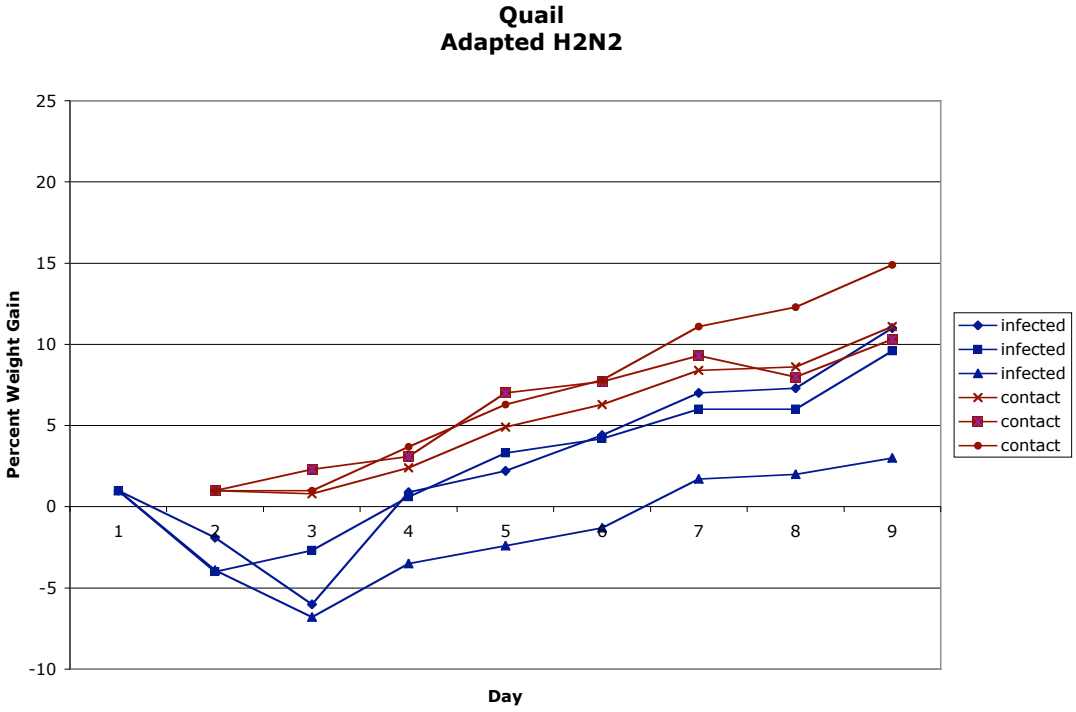
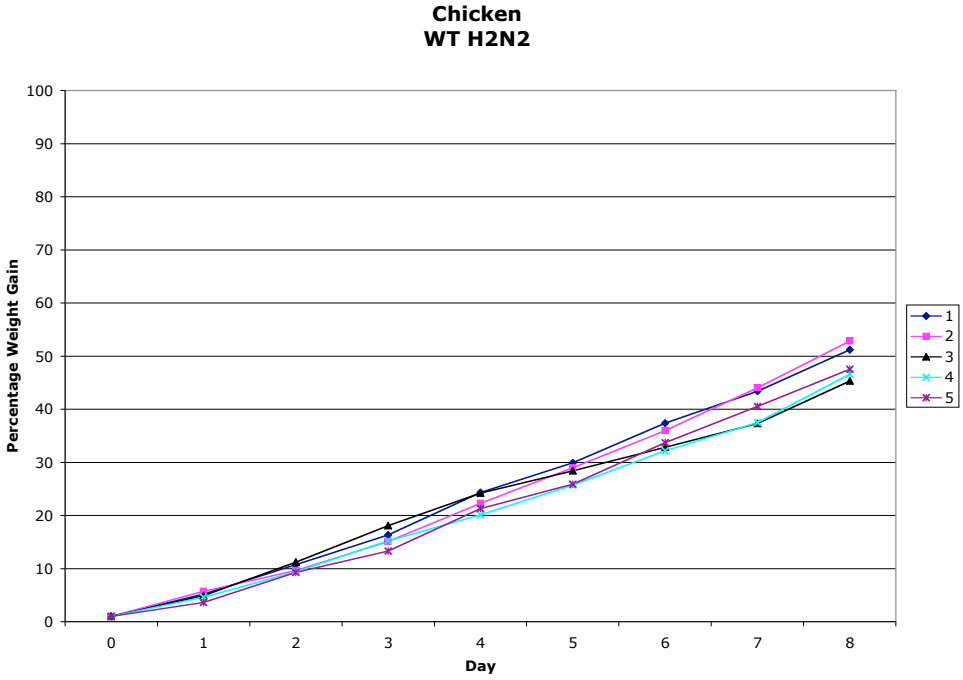
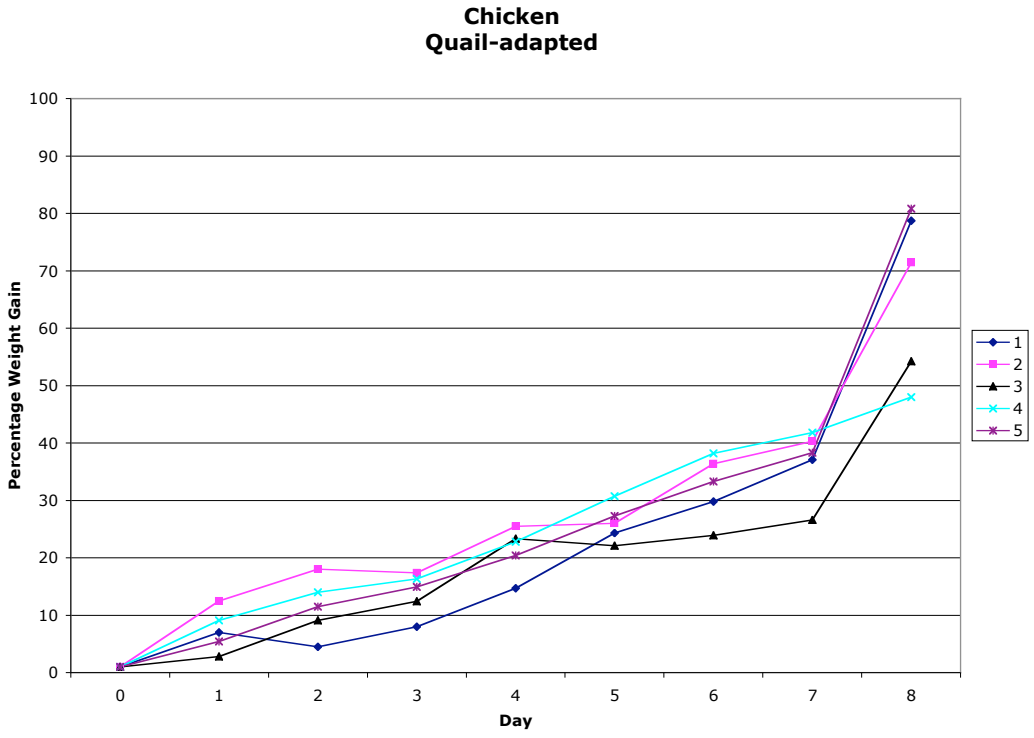


Figure 3, Weight Study in Chickens Infected with Quail-adapted H2N2

A.



B.



CHAPTER 5, DISCUSSION

Influenza viruses of the H2 subtype caused a major pandemic in 1957 and then disappeared in humans in 1968. Similar H2N2 influenza viruses appear to have caused an epidemic in the last quarter of the 19th century, a possible cause of the pandemic of 1889-1890 (Mulder and Masurel, 1958). Influenza A viruses circulating in aquatic birds are the progenitors for most influenza A viruses in other species. They are considered to be the most likely source of the Hemagglutinin (HA) genes introduced into the human population (Webster et al., 1992). Thus, pandemic strains of the H2 subtype would appear to have risen from avian sources. Hemagglutinin genes of avian H2 influenza viruses are moderately variable, the largest difference in the amino acid sequence being 15.6% (Schafer et al., 1993). Avian H2 influenza viruses are categorized into two distinct lineages, North America and Eurasian. Human H2 viruses form a separate lineage, strong evidence that there was a single introduction of a H2 gene into humans pre-1957. Human H2 viruses are most similar to Eurasian strains suggesting that the human pandemic H2N2 virus was derived from an Eurasian avian virus reassorting with the human H1N1 influenza circulating in the population at that time (Kawaoka, Krauss, and Webster, 1989; Scholtissek et al., 1978).

Antigenic drift of human H2 influenza viruses began in 1962. Amino acid changes became clustered in two regions of HA1 that corresponded to antigenic sites

while the antigenic profiles of the majority of avian H2s were conserved through 1991, closely resembling the prototype Japan 57 (H2N2) strain (Schafer et al., 1993). Amino acid changes in avian H2 influenza strains occur throughout the HA1, providing evidence that antibodies do not play a large role in the selection of avian H2 viruses, much like influenza B viruses (Air et al., 1990). Thus avian H2 virus like that of the human pandemic strain can circulate and evolve with the avian species, surviving to a point now where most of the human population is immune naive to H2 influenza. Avian species have a humoral and cell mediated response to influenza infections but antibody responses are not long lasted (Kida, Yanagawa, and Matsuoka, 1980). Therefore, antibody titers may be too low to allow the selection of variants. The H3 of the 1968 and the H2 of the 1957 pandemic are unlikely to have been very pathogenic for poultry (Li et al., 2003). Therefore, two-way transmission between different types of poultry, particularly in southern China increases the opportunity to generate an avian influenza virus with human pandemic potential.

Quail serve as an intermediate host in the formation and perpetuation of influenza viruses crossing the species barrier. They have been proposed to be the "route modulator" that alters the mode of transmission from that of fecal-oral to aerosol (Liu et al., 2003a). Quail may have a role in the history of influenza because of the ability of influenza viruses to replicate in trachea of quail and the quail's susceptibility to multiple subtypes. It is close to impossible to confirm because surveillance to isolate influenza from the natural pool of avian species began in the early 1970s.

The 1997 and 1999, human infections with H5N1 and H9N2 strains respectively have resulted from direct transmission by land-based birds, indicating their possible role as hosts for potential human influenza. Quail are a member of that group and can become infected with avian viruses and adapting them thus, permitting replication and transmission within quail and throughout species, particularly land based birds. Quail infected with the highly pathogenic A/Turkey/Ontario/7732/66 (H5N9) show no clinical symptoms but can transmit the virus to chickens causing morbidity and mortality (Slemons and Easterday, 1972; Tashiro, Reinacher, and Rott, 1987). Quail are also more susceptible to infection with goose H5N1 than chickens. The virus has a longer incubation in the quail, displaying clinical symptoms and death later, therefore increasing the likelihood of transmission (Webster et al., 2002). Viruses like A/Goose/Guangdong/1/96 (H5N1) circulating in 2003 in Southern China replicate in the trachea of quail, transmitted by aerosol and are highly pathogenic in the quail (Webster et al., 2002). This particular goose H5N1 can be reintroduced in the duck generating multiple reassortants with new aquatic gene segments (Guan et al., 2002). These new reassortants may be able to transmit to other hosts, particularly terrestrial poultry, Figure 4, (Li et al., 2003).

Quail are also susceptible to mallard H9N2 influenza viruses, establishing replication in the respiratory tract. In contrast, chickens infected with the same 6 viruses became infected with only 2 (Perez et al., 2003a). The infection in the respiratory tract promotes viral shedding in the aerosol, the same method of shedding

and transmission that occurs in mammals, particularly humans. Thus influenza virus infections in quail can promote a change in the tissue tropism of avian strains, permitting the emergence of variants transmitted by aerosol (Perez et al., 2003a). Quail have maintained a stable lineage of H9N2 influenza viruses with human virus-like receptor specificity with the ability to infect different species of poultry (Matrosovich, Krauss, and Webster, 2001). Therefore, strict receptor requirements may not be a necessity for influenza replication in other avian species.

Figure 4, Two-way Transmission of Avian Influenza Viruses

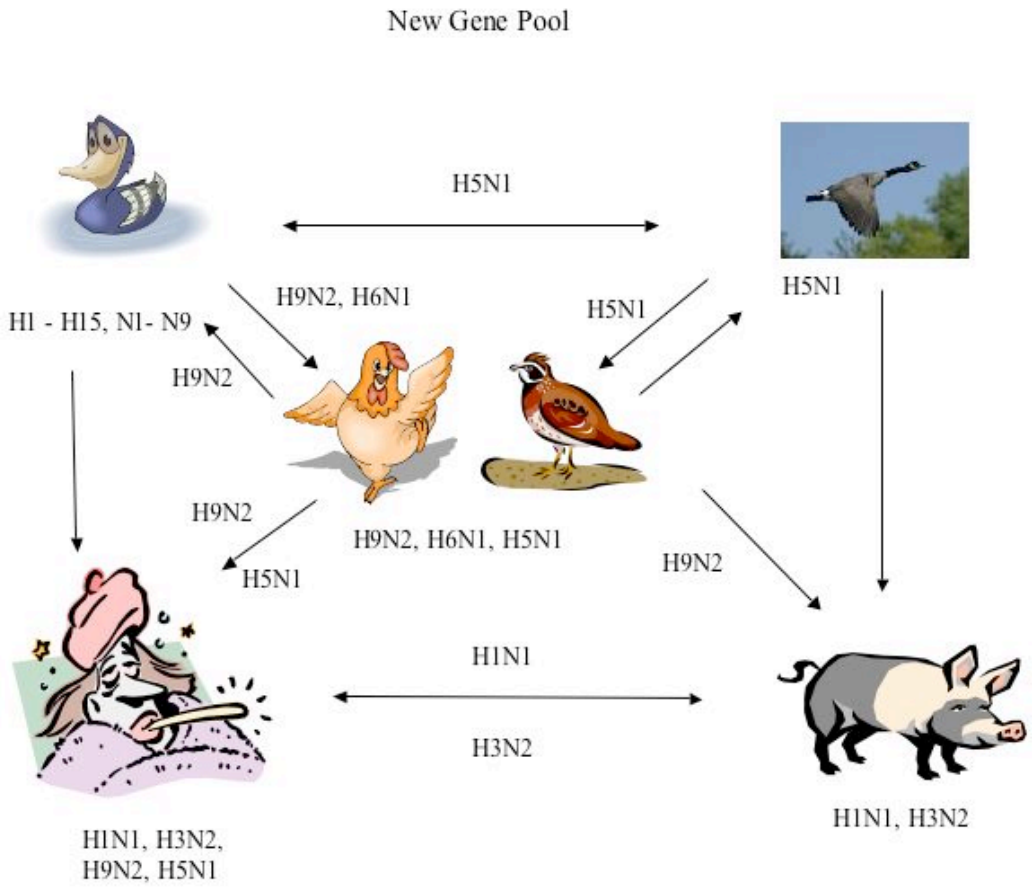


Figure 4. The concept of the classical gene pool and interaction between aquatic birds and influenza viruses may be explained by interspecies transmission between terrestrial and aquatic poultry.

Quail can be infected with viruses from North American and Eurasian lineages. Quail replicated 14 of the 15 HA subtypes, the exception was a mallard H15N8. Replication occurred in the respiratory tract, however no virus was able to transmit to sentinel cage mates. Thus, additional adaptation is required for adequate transmission among quail (Makarova et al., 2003). Human influenza virus has been isolated in the trachea of quail (Liu et al., 2003b). However, experimental infections with human H1N1 and H3N2 viruses have limited replication in quail (Makarova et al., 2003). This infection, without prior molecular modification, would be highly unlikely in nature. In contrast to the human viruses, swine influenza viruses (H1N1, H3N2, H1N2) replicate in quail, although inefficiently, the location is in the respiratory tract. Therefore quail could possibly be a vehicle for avian and swine reassortant viruses.

Though H2 influenza viruses have not been isolated from swine, transmission of influenza from bird to human has occurred (Claas et al., 1998; Subbarao et al., 1998; van Kolfschooten, 2003). Interspecies transmission of influenza A viruses among non-natural hosts, in particular land-based birds, and the factors that determine host range are not clear. This project sets out to define changes at the molecular level that determine adaptation of mallard viruses in quail and what changes are necessary for the transmission to other species, in particular chickens.

In this study we demonstrate that through adaptation in quail, a mallard influenza virus can gain the capacity to cross species barriers into terrestrial poultry,

an action the WT virus was unable to do. This adaptation creates a virus that can transmit within the quail as well as to chickens, enabling transmission to cage mates. The genotypic changes from adaptation occur in both surface and internal genes, confirming previous studies implicating roles for both HA and polymerase genes in host range. What is interesting to note is for the 1957 and 1968 human pandemics, both surface and internal genes were exchanged, the HA, NA and PB1 for the H2N2 and the HA and PB1 for the H3N2 pandemic (Kawaoka, Krauss, and Webster, 1989; Scholtissek et al., 1978). The possibility of a quail-adapted virus transmitting to other species is not new. A/Quail/Hong Kong/A28945/88 (H9N2) was able to transmit to other quail as well as from quail to chickens, demonstrating that a quail-adapted virus is capable of crossing the species barrier (Perez et al., 2003a).

Quail acted as a silent host, establishing an infection of A/Mallard/Potsdam/178-4/83 (H2N2) and maintained the role during adaptation of that virus without clinical symptoms. To the observer, the quail were healthy and gaining weight. This silent host mechanism provided insight in the virus' ability to go undetected in an intermediate host, much like it does in its natural host and therefore allows the mutation and perpetuation of the virus. In a silent and healthy host, the virus is established and able to interact with the environment and therefore other potential carriers.

The adaptation of the mallard virus by dosing with lung homogenate was used to accelerate the process in nature by giving the quail concentrated virus that was able

to replicate and establish an infection the previous group. In this manner each passage would be receiving a dose of virus, targeted to replicate and shed in the respiratory tract of quail. The adaptation of A/Mallard/Potsdam/178-4/83 (H2N2) did not loose its ability to replicate in mallard ducks, a finding consistent with observations that avian influenza viruses, like H9 and H5, adapted to terrestrial birds may display a broader host range (Perez et al., 2003a). The two-way transmission theory proposed for avian H9N2 viruses from Li et al, 2003 can be applied here (Figure 4). With the ability of the quail-adapted H2N2 virus to re-infect ducks, the virus has the potential to reassort, creating a new virus, that contains some genes of aquatic avian origin with the potential to cross the species barrier.

The adaptation created a virus that sheds better than the WT H2N2 in quail and chickens. Therefore, it would be possible to maintain an infected population as long as uninfected birds were introduced to the population whether they are quail, chickens or ducks. This is a huge implication that the role of live bird markets play in the promiscuity and prevalence of influenza viruses. The quail-adapted virus sheds more than the WT and requires less virus for infection in both chicken and quail, implying the amount of virus transmitting in the aerosol and fecal route for the quail and chickens is more than enough for infection. Efforts to concentrate the WT H2N2 virus to establish an infection in chickens proved unsuccessful indicating the changes that occurred in the adaptation of the virus in quail are completely necessary for the transmission to the chickens.

Clinical symptoms of infection can aid in diagnosis and surveillance of avian influenza however when infections involve a silent host, detection is possible only through sampling. Weight loss is a clinical symptom and often involved with influenza infections. Quail infected with the WT virus showed an overall increase in weight and weight was gained throughout the infection. Quail infected with the quail-adapted virus did not respond as well. The most drastic weight loss was on the day of transmission and peak shedding for infected quail. Infected quail regained weight by day 4 and returned to their pre-infection weights by day 5, around the same day that antibodies can be detected. Quail that became infected with either WT or quail-adapted virus through infection or transmission lost weight at initial days of infection, however the quail-adapted group lost 2-5% more weight.

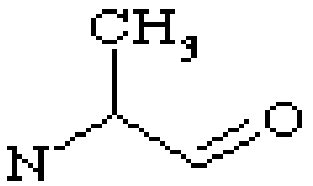
Weight studies in chickens displayed normal linear growth for those in the WT group. This is typical growth for the chickens since no infection was established. The weight gain in the quail-adapted group was not as clear as the WT group with some weight loss, although minimal. What is interesting to note is that on day 7 the chickens gained a drastic amount of weight, the majority increasing by 30% compared to the previous day. This weight gain may be due to two observations; a humoral response was active and symptoms of diarrhea had ceased by 7 days post-infection. The chickens in the adapted group had increased their weight anywhere from 50-80% their original weight at the end of the study whereas the WT group had uniformly gained 45-50% of their original weight.

Sequence analysis was performed to determine the molecular changes associated with the transmission phenotype of the quail-adapted H2N2 virus. Different subtypes of avian influenza have been studied to determine the minimal changes necessary in the adaptation of influenza from waterfowl to land-based birds. An increased number of glycosylation sites on the HA surface protein have been associated with adaptation of H5 and H7 viruses to land-based poultry (Matrosovich et al., 1999). In H9 viruses there are seven amino acid positions on HA that correspond to adaptation (Perez et al., 2003a).

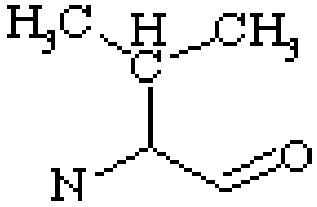
For the adaptation of A/Mallard/Potsdam/178-4/83 (H2N2), a single amino acid change was observed on the surface proteins and five other amino acid changes were detected in the internal proteins of the virus (Table 10). The change on the viral surface occurred at position 170 of the HA protein in which asparagine in the mallard virus was changed to aspartic acid in the quail-adapted virus (N170D). This change occurs in a region located in close proximity to the receptor-binding pocket of the virus, nine amino acids away from glycosylation site on the HA1 subunit at position 179 (Schafer et al., 1993). This change is not unique to the quail-adapted virus. Human H2N2 viruses from 1962 (A/Taiwan/1/62 and A/Yokosuka/3/62) and 1967 (A/Johanesburg/567/67 and A/Montevideo/2208/67) as well as A/Japan/305/57 (H2N2) have aspartic acid in position 170. Other amino acid changes occurred in the internal proteins of the virus; specifically, changes were identified in the PB2, PB1, and NP proteins. These changes have previously been suggested to play a role in host range in influenza viruses in mammalian and avian species (Hatta et al., 2002; Ito and

Kawaoka, 2000; Perez and Donis, 2001; Reid et al., 2004). Interestingly, some of the amino acid changes detected share commonalities with amino acids found in other terrestrial birds and with some human viruses. Amino acid residue 588 in PB2 appears to be under selective pressure. The majority of avian influenza viruses have alanine in this position, whereas all human influenza viruses sequenced to date have isoleucine. Our quail-adapted virus contains valine in position 588 (A588V), also found in the 2002 avian H5N1 influenza viruses that display an unusual lethality for ducks and in the mouse adapted neurotropic A/WSN/33 (H1N1) strain. Whether the change in the WSN virus is a consequence of its adaptation for growth in chicken eggs is not known. It is important to note that valine contains an intermediate structure between alanine and isoleucine (Figure 5). Thus, viruses having valine at position 588 may be at a transition state that could later favor a change in either direction. Further evidence that position 588 is under host selective pressure comes from the observation that all equine influenza PB2 sequences available to date contain threonine at this position, a feature that is shared with few duck influenza viruses and few swine influenza viruses.

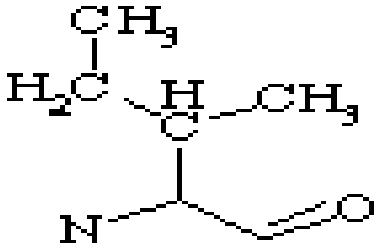
Figure 5, Structures of Alanine (A), Valine (V) and Isoleucine (I)



[Ala]



[Val]



[Ile]

Changes at amino acid residues 268 in PB1, glutamine to arginine, and 234 in NP, alanine to threonine, appear to be unique to the H2N2 quail-adapted virus and further studies will be needed before any conclusion can be made about these changes. In contrast, amino acid position 398 in PB1 has been changed from an aspartic acid in the mallard virus to a glutamic acid in the quail-adapted virus. Interestingly, the majority of quail influenza isolates sequenced to date have glutamic acid at this position, suggesting that this change may confer some selective advantage to the virus for replication in quail. Glutamic acid at position 398 is also found in the PB1 gene of the 1997 H5N1 viruses that crossed to humans and in influenza A/Puerto Rico/8/34 (H1N1) virus. An additional change in PB1 was observed in position 654 in which serine in the mallard virus has been replaced by isoleucine in the quail-adapted virus. This type of change is not unique to the quail-adapted virus with at least two human H1N1 isolates having the same amino acid: they are from 1983, influenza A/Chile/1/83 (H1N1) and A/Fiji/15899/83 (H1N1). This A/Chile/1/83 (H1N1) was the major influenza strain reported in the 1983-84 season in the United States. Of the 2,130 isolates obtained by collaborating laboratories, 51% were of the H1N1 subtype. The number of isolates reported was the largest since the 1976-77 season (MMWR, CDC). The mortality rate for this H1N1 outbreak was minimal and hardly cross the epidemic threshold but could be due to the lack of reported illnesses among the elderly, the group typically most vulnerable to severe influenza infection.

Neither amino acid changes nor nucleotide changes were detected in the PA and M genes. Silent changes were observed in the NA and NS genes. Synonymous changes observed are listed in Table 11.

In summary, minor changes are responsible for the transmission phenotype of the mallard H2N2 virus in quail and chickens. While two of these changes appear to be unique to the quail-adapted virus, other changes show a trend during adaptation of avian influenza in terrestrial poultry. The results are consistent with the notion that changes on the surface glycoproteins alone are not sufficient for transmission of avian influenza in chickens (Makarova et al., 2003; Perez et al., 2003a; Perez et al., 2003b). Four of the six amino acid changes are found in nature, therefore it is highly unlikely that this adaptation was artificially created in the lab, instead it mimics what could happen in nature. Although two changes are not found in current sequences it does not mean these changes are irrelevant, instead they are undefined. Increasing the database of avian and mammalian influenza sequences will help to highlight amino acids that are host specific.

Sequence analysis from passage 8 in quail showed no further amino acid changes. There is no pressure for further mutations, indicating a virus well adapted to its host. In contrast, review of virus extracted from cloacal swabs from contact chickens contained one additional change on the HA gene at position 152, serine to tyrosine, (S152Y) which is close to the change observed during the quail adaptation (N170D). This change is close to the receptor binding area, but tyrosine does not

appear to be a common amino acid in position 152 for the HAs of avian or human influenza viruses. Therefore, although current sequenced isolates for chickens do not show tyrosine prevalence at this position it does not mean this mutation is irrelevant.

Live quail are no longer permitted in the live bird markets in Hong Kong. Public authorities suspect quail transmit influenza viruses to other birds (Makarova et al., 2003). Unfortunately markets in Mainland China and parts of Europe and the United States continue to house many species of poultry, aquatic and terrestrial including quail, in one location. A market in Nanchang, China was surveyed and labeled by Liu and colleagues as an ideal environment for the maintenance and reassortment of influenza viruses. This surveillance highlights quail as susceptible to all virus subtypes isolated in the market (Liu et al., 2003b; Makarova et al., 2003). Live bird markets pose the debate of human and animal health versus lifestyle and culture that has been around for centuries. In order to improve on the current situation in Asia as well as around the world, live bird markets need increased surveillance, proper sanitation, record keeping and separation of avian species to prevent quail and other land-based birds from taking an active role in the perpetuation of avian influenza strains that are capable of crossing the species barrier.

The mechanism for adaptation of mallard viruses in quail and their transmission to other species is ill defined. More adaptation studies like this are needed to fully evaluate whether the changes identified here represent a trend in more than one influenza virus strain or whether similar changes will occur in other animal

species. It is important to identify potential intermediate hosts and study the mechanism to which the alteration of viruses leads to a new virus capable of crossing the species barrier to other avian species or mammals where further mutations and reassortment can take place.

CHAPTER 6, CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

Respiratory replication and shedding of influenza A viruses in quail promotes changes in tissue tropism of avian influenza A viruses, thus allowing the existence of variants that transmit by aerosol.

The key factors of influenza are six-fold; 1) wild aquatic birds are the natural reservoir, 2) influenza viruses in these birds replicate predominantly in the respiratory tract and shed by fecal-oral transmission, often through water, 3) there are two established lineages for wild aquatic birds; North American and Eurasian, 4) influenza viruses in aquatic birds have reached an evolutionary stasis while rapid evolution occurs after transmission of influenza to a new host, 5) most interspecies transmissions are unstable and do not lead to stable lineages, and 6) intermediate hosts include pigs, chickens and quail.

The major findings of this research are that A/Mallard/Potsdam/178-4/83 (H2N2) established an infection in quail directly inoculated with the virus and shed until day 3 post-infection. No transmission to sentinel cage mates was observed. After six passages of lung homogenate a virus arose that was adapted to quail. Transmission to contact quail occurred on day 3 post-infection and all contact quail became infected shedding just as much virus as the infected quail.

Quail-adapted H2N2 virus established an infection and transmitted to White Leghorn chickens while in contrast the original, wild type, A/Mallard/Postdam/178-4/83 (H2N2) was unable to establish an infection even in chickens directly inoculated with virus. Quail-adapted H2N2 was able to establish an infection in mallard ducks. Changes occurred at both surface and internal genes, including the HA, PB2, PB1 and NP genes.

Live bird markets like those in Southeast Asia, as well as other parts of the world, including Europe and North America need to be updated to fit the needs of the animals as well as the humans partaking in them. It is a cultural barrier necessary to cross in order to protect both poultry and humans from the optimal breeding ground for influenza virus reassortment and interspecies transmission. We have highlighted in this study how quail can take a mallard virus and adapt it to become a virus that establishes infection in quail and can transmit from the quail to chickens and ducks, enabling the virus the potential to reassort and spread. Governments across the world have the responsibility to set plans in action to increase surveillance and education of avian influenza to their public. Further research is necessary to outline methods of transmission of influenza viruses from avian species to humans, across all potential influenza subtypes, not just the current issues of H5N1 influenza viruses.

6.2 Future Work

Future work for determining key genes in the transmission of A/Mallard/Potsdam/178-4/83 (H2N2), to quail as well as to chickens includes cloning the WT and Quail-adapted viruses using reverse genetics outlined by Hoffmann, (Hoffmann et al., 2002)

Immediate work would involve cloning all eight genes of the WT H2N2 influenza virus and substituting each gene that contained amino acid changes in the adaptation in quail. Viruses prepared through reverse genetics are listed below:

1) WT + PB2, PB1, HA quail-adapted
2) WT + PB1, HA quail-adapted
3) WT + HA quail-adapted

If one of the combinations above proved to be a reassortant capable of transmission then with each quail-adapted gene included one at a time would be replaced by a WT gene to see what composition was responsible for transmission. If the gene highlighted was one where multiple amino acid changes occurred, each change would be reverted back to the WT amino acid at a time to determine if one or more amino acid was implicated in the adaptation. When the composition for transmission in quail is identified that same virus will be tested in chickens to determine if the minimal changes necessary for quail are the same for chickens.

Later work important to understanding H2N2 influenza viruses would include testing other mallard viruses from both lineages, Eurasian and North American, to determine if similar amino acid changes are necessary for adaptation in quail. It is also important to determine if similar adaptations can occur from mallard ducks to chickens and if those chicken-adapted viruses can transmit to quail. Other influenza subtypes that pose a threat to humans such as the H5, H7 and H9 viruses should also be studied to determine the ability of these viruses to transmit across avian species and what changes occur at the protein level when adapting these viruses in quail.

Additional studies should include developing a mammalian model, such as ferrets, to determine what changes occur at the molecular level to allow these quail-adapted and avian H2N2 influenza viruses to infect and transmit in mammals. Identifying the necessary changes will aid in combating and preventing future human infections with H2N2 influenza viruses.

REFERENCES

Air, G. M., Gibbs, A. J., Laver, W. G., and Webster, R. G. (1990). Evolutionary changes in influenza B are not primarily governed by antibody selection. *Proc Natl Acad Sci U S A* **87**(10)**,** 3884-8.

Area, E., Martin-Benito, J., Gastaminza, P., Torreira, E., Valpuesta, J. M., Carrascosa, J. L., and Ortin, J. (2004). 3D structure of the influenza virus polymerase complex: localization of subunit domains. *Proc Natl Acad Sci U S A* **101**(1)**,** 308-13.

Bancroft, C. T., and Parslow, T. G. (2002). Evidence for segment-nonspecific packaging of the influenza a virus genome. *J Virol* **76**(14)**,** 7133-9.

Beare, A. S., and Webster, R. G. (1991). Replication of avian influenza viruses in humans. *Arch Virol* **119**(1-2)**,** 37-42.

Biswas, S. K., and Nayak, D. P. (1994). Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. *J Virol* **68**(3)**,** 1819-26.

Braam, J., Ulmanen, I., and Krug, R. M. (1983). Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell* **34**(2)**,** 609-18.

Bron, R., Kendal, A. P., Klenk, H. D., and Wilschut, J. (1993). Role of the M2 protein in influenza virus membrane fusion: effects of amantadine and monensin on fusion kinetics. *Virology* **195**(2)**,** 808-11.

Brown, I. H. (2000). The epidemiology and evolution of influenza viruses in pigs. *Vet Microbiol* **74**(1-2)**,** 29-46.

Brown, P., Gajdusek, D. C., and Morris, J. A. (1966). Epidemic A2 influenza in isolated Pacific island populations without pre-epidemic antibody to influenza virus types A and B, and the discovery of other still unexposed populations. *Am J Epidemiol* **83**(1)**,** 176-88.

Buonagurio, D. A., Nakada, S., Desselberger, U., Krystal, M., and Palese, P. (1985). Noncumulative sequence changes in the hemagglutinin genes of influenza C virus isolates. *Virology* **146**(2)**,** 221-32.

Chin, P. S., Hoffmann, E., Webby, R., Webster, R. G., Guan, Y., Peiris, M., and Shortridge, K. F. (2002). Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. *J Virol* **76**(2)**,** 507-16.

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)**,** 472-7.

Colman, P. M., and Lawrence, M. C. (2003). The structural biology of type I viral membrane fusion. *Nat Rev Mol Cell Biol* **4**(4)**,** 309-19.

Colman, P. M., Tulip, W. R., Varghese, J. N., Tulloch, P. A., Baker, A. T., Laver, W. G., Air, G. M., and Webster, R. G. (1989). Three-dimensional structures of influenza virus neuraminidase-antibody complexes. *Philos Trans R Soc Lond B Biol Sci* **323**(1217)**,** 511-8.

Cox, N. J., Bai, Z. S., and Kendal, A. P. (1983). Laboratory-based surveillance of influenza A(H1N1) and A(H3N2) viruses in 1980-81: antigenic and genomic analyses. *Bull World Health Organ* **61**(1)**,** 143-52.

Cox, N. J., and Subbarao, K. (2000). Global epidemiology of influenza: past and present. *Annu Rev Med* **51,** 407-21.

Easterday, B. C. (1975). Antigens (and recombinants) and immune response to avian influenza. *Am J Vet Res* **36**(4 Pt 2)**,** 503-4.

Enami, M., Sharma, G., Benham, C., and Palese, P. (1991). An influenza virus containing nine different RNA segments. *Virology* **185**(1)**,** 291-8.

Gambaryan, A., Webster, R., and Matrosovich, M. (2002). Differences between influenza virus receptors on target cells of duck and chicken. *Arch Virol* **147**(6)**,** 1197-208.

Govorkova, E. A., Kizina, A. A., Krylov, V. F., and Smirnov Iu, A. (1993). [The level of population immunity to influenza A viruses with hemagglutinin subtype H2]. *Zh Mikrobiol Epidemiol Immunobiol*(6)**,** 58-9.

Guan, Y., Peiris, M., Kong, K. F., Dyrting, K. C., Ellis, T. M., Sit, T., Zhang, L. J., and Shortridge, K. F. (2002). H5N1 influenza viruses isolated from geese in Southeastern China: evidence for genetic reassortment and interspecies transmission to ducks. *Virology* **292**(1)**,** 16-23.

Guan, Y., Shortridge, K. F., Krauss, S., Chin, P. S., Dyrting, K. C., Ellis, T. M., Webster, R. G., and Peiris, M. (2000). H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. *J Virol* **74**(20)**,** 9372-80.

Guan, Y., Shortridge, K. F., Krauss, S., and Webster, R. G. (1999). Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc Natl Acad Sci U S A* **96**(16)**,** 9363-7.

Guo, Y., Wang, M., Kawaoka, Y., Gorman, O., Ito, T., Saito, T., and Webster, R. G. (1992). Characterization of a new avian-like influenza A virus from horses in China. *Virology* **188**(1)**,** 245-55.

Guo, Y. J., Krauss, S., Senne, D. A., Mo, I. P., Lo, K. S., Xiong, X. P., Norwood, M., Shortridge, K. F., Webster, R. G., and Guan, Y. (2000). Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology* **267**(2)**,** 279-88.

Hatta, M., Halfmann, P., Wells, K., and Kawaoka, Y. (2002). Human influenza a viral genes responsible for the restriction of its replication in duck intestine. *Virology* **295**(2)**,** 250-5.

Hay, A. J. (1974). Studies on the formation of the influenza virus envelope. *Virology* **60**(2)**,** 398-418.

Hinshaw, V. S., Bean, W. J., Geraci, J., Fiorelli, P., Early, G., and Webster, R. G. (1986). Characterization of two influenza A viruses from a pilot whale. *J Virol* **58**(2)**,** 655-6.

Hinshaw, V. S., Bean, W. J., Webster, R. G., and Sriram, G. (1980a). Genetic reassortment of influenza A viruses in the intestinal tract of ducks. *Virology* **102**(2)**,** 412-9.

Hinshaw, V. S., Webster, R. G., Bean, W. J., and Sriram, G. (1980b). The ecology of influenza viruses in ducks and analysis of influenza viruses with monoclonal antibodies. *Comp Immunol Microbiol Infect Dis* **3**(1-2)**,** 155-64.

Hinshaw, V. S., Webster, R. G., Naeve, C. W., and Murphy, B. R. (1983). Altered tissue tropism of human-avian reassortant influenza viruses. *Virology* **128**(1)**,** 260-3.

Hobman, T. C. (1993). Targeting of viral glycoproteins to the Golgi complex. *Trends Microbiol* **1**(4)**,** 124-30.

Hoffmann, E., Krauss, S., Perez, D., Webby, R., and Webster, R. G. (2002). Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* **20**(25-26)**,** 3165-70.

Hoffmann, E., Stech, J., Guan, Y., Webster, R. G., and Perez, D. R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* **146**(12)**,** 2275-89.

Hoffmann, E., Stech, J., Leneva, I., Krauss, S., Scholtissek, C., Chin, P. S., Peiris, M., Shortridge, K. F., and Webster, R. G. (2000). Characterization of the influenza A virus gene pool in avian species in southern China: was H6N1 a derivative or a precursor of H5N1? *J Virol* **74**(14)**,** 6309-15.

Honda, A., Mukaigawa, J., Yokoiyama, A., Kato, A., Ueda, S., Nagata, K., Krystal, M., Nayak, D. P., and Ishihama, A. (1990). Purification and molecular structure of RNA polymerase from influenza virus A/PR8. *J Biochem (Tokyo)* **107**(4)**,** 624-8.

Honda, A., Ueda, K., Nagata, K., and Ishihama, A. (1988). RNA polymerase of influenza virus: role of NP in RNA chain elongation. *J Biochem (Tokyo)* **104**(6)**,** 1021-6.

Horimoto, T., and Kawaoka, Y. (1995). The hemagglutinin cleavability of a virulent avian influenza virus by subtilisin-like endoproteases is influenced by the amino acid immediately downstream of the cleavage site. *Virology* **210**(2)**,** 466-70.

Huang, T. S., Palese, P., and Krystal, M. (1990). Determination of influenza virus proteins required for genome replication. *J Virol* **64**(11)**,** 5669-73.

Ito, T., Couceiro, J. N., Kelm, S., Baum, L. G., Krauss, S., Castrucci, M. R., Donatelli, I., Kida, H., Paulson, J. C., Webster, R. G., and Kawaoka, Y. (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol* **72**(9)**,** 7367-73.

Ito, T., and Kawaoka, Y. (2000). Host-range barrier of influenza A viruses. *Vet Microbiol* **74**(1-2)**,** 71-5.

Kawaoka, Y., Krauss, S., and Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol* **63**(11)**,** 4603-8.

Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K. F., Kawaoka, Y., and Webster, R. G. (1994). Potential for transmission of avian influenza viruses to pigs. *J Gen Virol* **75 (Pt 9),** 2183-8.

Kida, H., Yanagawa, R., and Matsuoka, Y. (1980). Duck influenza lacking evidence of disease signs and immune response. *Infect Immun* **30**(2)**,** 547-53.

Kido, H., Murakami, M., Oba, K., Chen, Y., and Towatari, T. (1999). Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. *Mol Cells* **9**(3)**,** 235-44.

Kilbourne, E. D. (1963). Influenza. *Med Sci* **13,** 527-40.

Klenk, H. D. (1974). Viral envelopes and their relationship to cellular membranes. *Curr Top Microbiol Immunol*(68)**,** 29-58.

Kobayashi, M., Toyoda, T., and Ishihama, A. (1996). Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. *Arch Virol* **141**(3-4)**,** 525-39.

Li, K. S., Xu, K. M., Peiris, J. S., Poon, L. L., Yu, K. Z., Yuen, K. Y., Shortridge, K. F., Webster, R. G., and Guan, Y. (2003). Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? *J Virol* **77**(12)**,** 6988-94.

Li, M. L., Rao, P., and Krug, R. M. (2001). The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. *Embo J* **20**(8)**,** 2078-86.

Lin, Y. P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N., and Hay, A. (2000). Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. *Proc Natl Acad Sci U S A* **97**(17)**,** 9654-8.

Liu, C., Eichelberger, M. C., Compans, R. W., and Air, G. M. (1995). Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. *J Virol* **69**(2)**,** 1099-106.

Liu, J. H., Okazaki, K., Bai, G. R., Shi, W. M., Mweene, A., and Kida, H. (2004). Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. *Virus Genes* **29**(1)**,** 81-6.

Liu, M., Guan, Y., Peiris, M., He, S., Webby, R. J., Perez, D., and Webster, R. G. (2003a). The quest of influenza A viruses for new hosts. *Avian Dis* **47**(3 Suppl)**,** 849-56.

Liu, M., He, S., Walker, D., Zhou, N., Perez, D. R., Mo, B., Li, F., Huang, X., Webster, R. G., and Webby, R. J. (2003b). The influenza virus gene pool in a poultry market in South central china. *Virology* **305**(2)**,** 267-75.

Mahy, B. W., Siddell, S., Wege, H., and ter Meulen, V. (1983). RNA-dependent RNA polymerase activity in murine coronavirus-infected cells. *J Gen Virol* **64 (Pt 1),** 103-11.

Makarova, N. V., Kaverin, N. V., Krauss, S., Senne, D., and Webster, R. G. (1999). Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. *J Gen Virol* **80 (Pt 12),** 3167-71.

Makarova, N. V., Ozaki, H., Kida, H., Webster, R. G., and Perez, D. R. (2003). Replication and transmission of influenza viruses in Japanese quail. *Virology* **310**(1)**,** 8-15.

Matrosovich, M., and Klenk, H. D. (2003). Natural and synthetic sialic acid-containing inhibitors of influenza virus receptor binding. *Rev Med Virol* **13**(2)**,** 85-97.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M. R., Donatelli, I., and Kawaoka, Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol* **74**(18)**,** 8502-12.

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)**,** 1146-55.

Matrosovich, M. N., Krauss, S., and Webster, R. G. (2001). H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* **281**(2)**,** 156-62.

Mori, I., Komatsu, T., Takeuchi, K., Nakakuki, K., Sudo, M., and Kimura, Y. (1995). Viremia induced by influenza virus. *Microb Pathog* **19**(4)**,** 237-44.

Mulder, J., and Masurel, N. (1958). Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands. *Lancet* **1**(7025)**,** 810-4.

Muller, R., Poch, O., Delarue, M., Bishop, D. H., and Bouloy, M. (1994). Rift Valley fever virus L segment: correction of the sequence and possible functional role of newly identified regions conserved in RNA-dependent polymerases. *J Gen Virol* **75 (Pt 6),** 1345-52.

Nardelli, L., Rinaldi, A., Pereira, H. G., and Mandelli, G. (1970). [Influenza virus infections in Japanese quails]. *Arch Exp Veterinarmed* **24,** 231-49.

Nayak, D. P., and Barman, S. (2002). Role of lipid rafts in virus assembly and budding. *Adv Virus Res* **58,** 1-28.

Nayak, D. P., and Hui, E. K. (2004). The role of lipid microdomains in virus biology. *Subcell Biochem* **37,** 443-91.

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)**,** 6751-8.

Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L., Lai, R. W., Orr, W. K., and Shortridge, K. F. (1999). Human infection with influenza H9N2. *Lancet* **354**(9182)**,** 916-7.

Perales, B., Sanz-Ezquerro, J. J., Gastaminza, P., Ortega, J., Santaren, J. F., Ortin, J., and Nieto, A. (2000). The replication activity of influenza virus polymerase is linked to the capacity of the PA subunit to induce proteolysis. *J Virol* **74**(3)**,** 1307-12.

Perez, D. R., and Donis, R. O. (2001). Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. *J Virol* **75**(17)**,** 8127-36.

Perez, D. R., Lim, W., Seiler, J. P., Yi, G., Peiris, M., Shortridge, K. F., and Webster, R. G. (2003a). Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. *J Virol* **77**(5)**,** 3148-56.

Perez, D. R., Webby, R. J., Hoffmann, E., and Webster, R. G. (2003b). Land-based birds as potential disseminators of avian mammalian reassortant influenza A viruses. *Avian Dis* **47**(3 Suppl)**,** 1114-7.

Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992). Influenza virus M2 protein has ion channel activity. *Cell* **69**(3)**,** 517-28.

Poon, L. L., Pritlove, D. C., Fodor, E., and Brownlee, G. G. (1999). Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. *J Virol* **73**(4)**,** 3473-6.

Portela, A., and Digard, P. (2002). The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J Gen Virol* **83**(Pt 4)**,** 723-34.

Reid, A. H., Fanning, T. G., Hultin, J. V., and Taubenberger, J. K. (1999). Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A* **96**(4)**,** 1651-6.

Reid, A. H., Fanning, T. G., Janczewski, T. A., Lourens, R. M., and Taubenberger, J. K. (2004). Novel origin of the 1918 pandemic influenza virus nucleoprotein gene. *J Virol* **78**(22)**,** 12462-70.

Reid, A. H., Fanning, T. G., Janczewski, T. A., McCall, S., and Taubenberger, J. K. (2002). Characterization of the 1918 "Spanish" influenza virus matrix gene segment. *J Virol* **76**(21)**,** 10717-23.

Reid, A. H., Fanning, T. G., Janczewski, T. A., and Taubenberger, J. K. (2000). Characterization of the 1918 "Spanish" influenza virus neuraminidase gene. *Proc Natl Acad Sci U S A* **97**(12)**,** 6785-90.

Rogers, G. N., and Paulson, J. C. (1983). Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**(2)**,** 361-73.

Saito, T., Kawaoka, Y., and Webster, R. G. (1993). Phylogenetic analysis of the N8 neuraminidase gene of influenza A viruses. *Virology* **193**(2)**,** 868-76.

Schafer, J. R., Kawaoka, Y., Bean, W. J., Suss, J., Senne, D., and Webster, R. G. (1993). Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *Virology* **194**(2)**,** 781-8.

Scholtissek, C., Burger, H., Bachmann, P. A., and Hannoun, C. (1983). Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. *Virology* **129**(2)**,** 521-3.

Scholtissek, C., Hinshaw, V. S., and Olsen, C. W. (1998). Influenza in Pigs and their role as the intermediate host. *In* "Textbook of Influenza" (A. J. Hay, Ed.), pp. 137-145. Blackwell Science Ltd., Oxford.

Scholtissek, C., Rohde, W., Von Hoyningen, V., and Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* **87**(1)**,** 13-20.

Schulman, J. L., and Palese, P. (1977). Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. *J Virol* **24**(1)**,** 170-6.

Shapiro, G. I., Gurney, T., Jr., and Krug, R. M. (1987). Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. *J Virol* **61**(3)**,** 764-73.

Shortridge, K. F. (1999). Poultry and the influenza H5N1 outbreak in Hong Kong, 1997: abridged chronology and virus isolation. *Vaccine* **17 Suppl 1,** S26-9.

Shortridge, K. F., Webster, R. G., Butterfield, W. K., and Campbell, C. H. (1977). Persistence of Hong Kong influenza virus variants in pigs. *Science* **196**(4297)**,** 1454-5.

Shortridge, K. F., Zhou, N. N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K. G., Norwood, M., Senne, D., Sims, L., Takada, A., and Webster, R. G. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* **252**(2)**,** 331-42.

Skehel, J. J. (1972). Polypeptide synthesis in influenza virus-infected cells. *Virology* **49**(1)**,** 23-36.

Skehel, J. J., and Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69,** 531-69.

Slemons, R. D., and Easterday, B. C. (1972). Host response differences among 5 avian species to an influenzavirus--A-turkey-Ontario-7732-66 (Hav5N?). *Bull World Health Organ* **47**(4)**,** 521-5.

Smith, G. L., and Hay, A. J. (1982). Replication of the influenza virus genome. *Virology* **118**(1)**,** 96-108.

Stegmann, T. (2000). Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implications for intracellular fusion. *Traffic* **1**(8)**,** 598-604.

Suarez, D. L., Garcia, M., Latimer, J., Senne, D., and Perdue, M. (1999). Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. *J Virol* **73**(5)**,** 3567-73.

Subbarao, K., and Katz, J. (2000). Avian influenza viruses infecting humans. *Cell Mol Life Sci* **57**(12)**,** 1770-84.

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)**,** 393-6.

Sugiura, A., and Ueda, M. (1980). Neurovirulence of influenza virus in mice. I. Neurovirulence of recombinants between virulent and avirulent virus strains. *Virology* **101**(2)**,** 440-9.

Sugrue, R. J., and Hay, A. J. (1991). Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology* **180**(2)**,** 617-24.

Tashiro, M., Reinacher, M., and Rott, R. (1987). Aggravation of pathogenicity of an avian influenza virus by adaptation to quails. *Arch Virol* **93**(1-2)**,** 81-95.

Ulmanen, I., Broni, B. A., and Krug, R. M. (1981). Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. *Proc Natl Acad Sci U S A* **78**(12)**,** 7355-9.

van Kolfschooten, F. (2003). Dutch veterinarian becomes first victim of avian influenza. *Lancet* **361**(9367)**,** 1444.

Webby, R., Hoffmann, E., and Webster, R. (2004). Molecular constraints to interspecies transmission of viral pathogens. *Nat Med* **10**(12 Suppl)**,** S77-81.

Webby, R. J., and Webster, R. G. (2001). Emergence of influenza A viruses. *Philos Trans R Soc Lond B Biol Sci* **356**(1416)**,** 1817-28.

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)**,** 152-79.

Webster, R. G., Guan, Y., Peiris, M., Walker, D., Krauss, S., Zhou, N. N., Govorkova, E. A., Ellis, T. M., Dyrting, K. C., Sit, T., Perez, D. R., and Shortridge, K. F. (2002). Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. *J Virol* **76**(1)**,** 118-26.

Webster, R. G., Hinshaw, V. S., Bean, W. J., Van Wyke, K. L., Geraci, J. R., St Aubin, D. J., and Petursson, G. (1981). Characterization of an influenza A virus from seals. *Virology* **113**(2)**,** 712-24.

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)**,** 268-78.

Wilson, R., Miller, J., Greene, H., Rankin, R., Lumeng, L., Gordon, D., Nelson, D., and Noble, G. (1980). Reye's syndrome in three siblings. Association with type A influenza infection. *Am J Dis Child* **134**(11)**,** 1032-4.

Yamashita, M., Krystal, M., Fitch, W. M., and Palese, P. (1988). Influenza B virus evolution: co-circulating lineages and comparison of evolutionary pattern with those of influenza A and C viruses. *Virology* **163**(1)**,** 112-22.

Yuen, K. Y., Chan, P. K., Peiris, M., Tsang, D. N., Que, T. L., Shortridge, K. F., Cheung, P. T., To, W. K., Ho, E. T., Sung, R., and Cheng, A. F. (1998). Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet* **351**(9101)**,** 467-71.

Zebedee, S. L., and Lamb, R. A. (1988). Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J Virol* **62**(8)**,** 2762-72.

Zheng, H., Lee, H. A., Palese, P., and Garcia-Sastre, A. (1999). Influenza A virus RNA polymerase has the ability to stutter at the polyadenylation site of a viral RNA template during RNA replication. *J Virol* **73**(6)**,** 5240-3.