

## ABSTRACT

Title of Document: MOLECULAR MARKERS OF  
INTERSPECIES TRANSMISSION OF H2N2  
AND H9N2 INFLUENZA A VIRUSES

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Most avian influenza viruses do not replicate or transmit efficiently in mammals. The events that lead to interspecies transmission and host adaptation are unknown. Part one of this project set out to establish quail as an intermediate host of influenza. Our results indicate that adapting a mallard H2N2 virus in quail leads to expanded host range in chickens. The molecular changes, which occur during the adaptation in quail, are crucial for viral replication and transmission in chickens. Further adaptation of this quail-adapted virus in chickens leads to a 27 amino acid-deletion in the stalk region of the NA, changing the tissue tropism and temperature phenotype of the virus.

H9N2 influenza viruses have created in poultry an endemic situation in much of Asia, Europe and the Middle East. This subtype, albeit low pathogenic, carries

with its human receptor specificity and the ability to infect humans without prior adaptation. The generation of an influenza pandemic requires interspecies transmission of a novel strain, which can adapt to its new host through either reassortment or point mutations. Given that two previous pandemics were the result of reassortment between low pathogenic avian viruses and human subtypes of that period, and given the endemic situation of avian H9N2 viruses in Eurasia, for part two of this project, we wanted to determine if adaptation of an avian-human H9N2 reassortant in ferrets could support mammalian respiratory droplet transmission. Here we show for the first time that a reassortant virus carrying the HA and NA of an avian H9N2 virus can transmit in respiratory droplets. This is the first report of respiratory droplet transmission of H9N2 influenza, which carries profound implications for pandemic preparedness. The amino acid changes on the HA might identify critical, adaptive mutations necessary for respiratory transmission in subsequent pandemic avian influenza strains. Using reverse genetics we identified key combinations of this adapted reassortant that support respiratory droplet transmission.

MOLECULAR MARKERS OF INTERSPECIES TRANSMISSION OF H2N2 AND  
H9N2 AVIAN INFLUENZA A VIRUSES

By

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## **Dedication**

I dedicate this dissertation to my grandmothers, Sara Moran and Mary Alice Sorrell. Each was ahead of her time in thought and action. They showed me, in their own way, how to live life without boundaries and to never limit myself. I thank them both from the bottom of my heart.

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## List of Abbreviations

AI	avian influenza
<sup>0</sup> C	degrees Celsius
cDNA	complement DNA
EID <sub>50</sub>	egg infectious dose 50
HA Assay	hemagglutinin assay
HEF	hemagglutinin esterase-fusion
HI Assay	hemagglutinin inhibition assay
HPAI	highly pathogenic avian influenza
LPAI	low pathogenic avian influenza
MDCK	Madin Darby Canine Kidney
ml	milliliter
mRNA	messenger RNA
nt	nucleotide
PBS	phosphate-buffered saline
RBS	receptor binding site
RT-PCR	reverse transcriptase-polymerase chain reaction
TCID <sub>50</sub>	tissue culture infectious dose 50
μl	microliter
vRNP	viral ribonucleoprotein
vRNA	viral RNA
WT	wild type

## Table of One- and Three-Letter Abbreviations for Amino

### Acids

G	Glycine	Gly
A	Alanine	Ala
V	Valine	Val
L	Leucine	Leu
I	Isoleucine	Ile
M	Methionine	Met
F	Phenylalanine	Phe
W	Tryptophan	Trp
P	Proline	Pro
S	Serine	Ser
T	Threonine	Thr
C	Cysteine	Cys
Y	Tyrosine	Tyr
N	Asparagine	Asn
Q	Glutamine	Gln
K	Lysine	Lys
R	Arginine	Arg
H	Histidine	His
D	Aspartic Acid	Asp
E	Glutamic Acid	Glu

## Table of Influenza Genes: Genomic Size and Function

Gene	Segment	Size (nt)	Major Function
PB2	1	2341	Transcriptase: cap binding
PB1	2	2341	Transcriptase: elongation
PA	3	2231	Transcriptase: vRNA replication
HA	4	H2 1740	Hemagglutinin: Host cell attachment; receptor binding, membrane fusion glycoprotein
		H9 1766	
NP	5	1565	Nucleoprotein: RNA binding, part of transcriptase complex, nuclear/cytoplasmic transport of vRNA
NA	6	N2 1467	Neuraminidase: receptor destroying enzyme/viral release
M	7	1027	Matrix: M1 major component of virion. M2 integral membrane protein-ion channel
NS	8	890	Nonstructural: NS1 cellular RNA transport, splicing, translation, anti-interferon protein. NS2 (NEP) nuclear export protein

# Chapter 1: Introduction

## 1.1 General Introduction

Influenza A viruses have been isolated from wild and domestic avian species worldwide. Representatives from each of the known subtypes of influenza A have been isolated in aquatic birds, the virus' natural reservoir. The largest number of viruses have been gathered from feral water birds including duck, geese, terns and gulls as well as from a large group of domestic terrestrial birds like turkeys, chickens, quail and pheasants (Gambaryan et al., 2002b).

In aquatic species, the majority of viruses replicate in the cells lining the intestinal tract while there is some replication in the epithelial cells of the lung (Webster et al., 1978). The virus gains access by passage in the digestive tract, despite the low pH of the gizzard, and is shed in high concentrations in the feces. The mechanism to 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 asymptotically up to 30 days thus requiring few passages to maintain the virus in the 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, leading to 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 et al., 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) and that influenza evolution in avian reservoirs has reached stasis therefore, the source of the next pandemic virus exists phenotypically unchanged in the aquatic bird (Reid et al., 2004).

Influenza is a difficult disease to contain. The segmented nature of the genome provides the virus the ability to reassort with other influenza A strains, creating new viruses unrecognized by pre-existing immunity (pandemic) as well as mutating within its own subtype to circumvent antibodies made against particular epitopes (epidemic), also known as antigenic shift and drift, respectively. Influenza viruses do not cause latent or persistent infections; the virus is maintained in humans through direct contact during an acute infection (Brown et al., 1966). The minimal population required to sustain transmission is unknown. In the United States, influenza epidemics typically occur during the winter months when the virus can survive in aerosol under low relative humidity, characteristic of this season. The appearance of an epidemic during any given year is a result of the antigenic drift of the virus and the immunity in the particular population, given that immunity to influenza is long-lived and subtype specific.

According to CDC reports, in an average flu season in the United States, 114,000 people will be hospitalized and 36,000 will die of influenza infections.

Although transmission of avian influenza to mammals, particularly humans, has been repeatedly documented, adaptation, efficient transmission and establishment in the new host are rare events. In the case of adaptation to humans this process would lead to a pandemic situation. The direct transmission of avian H5N1 and H9N2 viruses to humans in southeastern China and Hong Kong, initially in 1997 and 1999, and in subsequent years 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 major, however not the only, barrier restricting the current H5N1 and H9N2 viruses 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 infection the chance exists for possible adaptation or reassortment between this avian virus and a co-circulating human strain, creating a new virus that can easily transmit to humans, leading to pandemic situations.

The host range of influenza 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. 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 SA $\alpha$ 2,3Gal, where human viruses prefer SA $\alpha$ 2,6Gal conformation (Rogers and Paulson, 1983). The binding preference appears to be associated with the amino acid located at position 226 in the HA, L226 for  $\alpha$ 2,6 and Q226 for  $\alpha$ 2,3. 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 possess SA $\alpha$ 2,3Gal and SA $\alpha$ 2,6Gal (Ito et al., 1998; Scholtissek et al., 1998) however, 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, which provide an environment similar to pigs (Gambaryan et al., 2002a; 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 directly to humans, are scenarios that seem to be increasing in familiarity (Lin et al., 2000; Peiris et al., 1999; Shortridge, 1999).

In 1997 in Hong Kong, H5N1 viruses, circulating in poultry were transmitted to humans, causing 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 an avian H9N2 was cocirculating with H5N1 and H6N1 avian viruses. Both the H6N1 and H9N2 viruses were 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/Hong Kong/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 cocirculating in poultry in 1997 and which caused fatal human infections. The H5N1 viruses were reassortants that derived their internal genes from a A/Quail/Hong Kong/G1/97-like virus (Lin et al., 2000; Makarova et al., 2003). Antigenic analysis of human and swine H9N2 isolates show that swine viruses were not intermediates in avian to human transmission implicating land-based poultry (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 requirement 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 genes of aquatic avian origin and more importantly creating a virus with pandemic potential.

Avian influenza (AI) is an infectious disease, which is both devastating to the poultry industry and of particular public health importance. Since 1997, many Asian countries have experienced frequent influenza outbreaks in chickens caused by H5N1 highly pathogenic avian influenza (HPAI) virus and the list of countries reporting the outbreaks has spread into Europe despite hundreds of millions of domestic poultry that have either died or been culled to control the spread of the disease. H9N2 influenza has created a similar situation and is now considered endemic in Asia, Europe and the Middle East. This subtype, albeit low pathogenic, carries with it human receptor specificity and the ability to infect species without causing alarming mortality, increasing its chances of adapting in host and spreading to other hosts. These characteristics allow H9N2 viruses to develop, unnoticed as a potential pandemic strain.

### 1.2 Research Objectives and Outline

The main goal of my dissertation research is to highlight the role domestic terrestrial birds play in the adaptation and host expansion of avian influenza not only to other domestic poultry species but also to mammals. Each influenza subtype has different properties that can be manipulated in the appropriate host, which allows it to adapt. In this study we focus on avian H2N2 and H9N2 influenza virus subtypes based on their past and future strengths as potential pandemic strains.

The research objectives of my dissertation are as follows:

I. Build on M.S. thesis results that support quail as an intermediate host of influenza A viruses by determining the minimal changes necessary for the quail-adapted A/mallard/Potsdam/178-4/83 (H2N2) –qa-mall/178- to replicate and transmit in both quail and chickens.

II. Further adapt qa-mall/178 H2N2 in white leghorn chickens –ch-qa/178- to determine if through adaptation it is possible to alter tissue tropism of qa-mall/178 in chickens from a fecal-oral to a respiratory infection.

III. Characterize, *in vivo*, the tissue tropism supported by the 27-amino acid deletion in the stalk region of the ch-qa/178 H2N2 NA.

IV. Determine the function of amino acid 226, in the receptor-binding site of HA, in avian H9N2 influenza A viruses and their transmissibility in the ferret model.

V. Identify the phenotype of an H9N2 avian-human reassortant in ferrets and conclude if through adaptation aerosol droplet transmission is possible.

VI. Highlight the minimal changes necessary for respiratory droplet transmission in the ferret-adapted H9N2 reassortant and its implication for pandemic preparedness.

### 1.3 Major Findings

I. Of the six amino acid changes that occurred in qa-mall/178, a single change in the HA (N170D) is crucial for transmission in quail; so much that given the other five adaptive changes, excluding the qa-mall/178 HA, quail transmit a virus with an adaptive change in the HA (K166E) indicating a change in this region is critical. This single change, rescued in the mallard backbone, can support transmission in quail. However, for efficient transmission in chickens all six amino acids must be present, implicating the importance of quail in expanding the host range of this mall/178 virus in other domestic poultry.

II. After only four passages of qa-mall/178 H2N2 in chickens the replication site of the virus changed from intestinal to respiratory following a 27 amino acid-deletion in the stalk region of the NA. This deletion was maintained through passage 7 and supported respiratory replication and transmission in chickens and quail.

III. *In vivo* studies suggest that the NA deletion changes viral tropism from intestinal to respiratory in the chicken, modulating the temperature phenotype of the virus with the deletion favoring replication at lower temperatures, similar to

the environment of the respiratory tract versus the higher temperature of the intestine.

IV. Leucine at amino acid 226 of the HA receptor binding site in avian H9N2 viruses supports efficient replication and, at times, direct contact transmission in ferrets.

V. An avian-human H9N2 reassortant virus, 2WF10:6M98, is more efficient at infection, leading to increased replication and transmission to direct contact ferrets as well as expanded tissue tropism and virulence in respiratory tissues, when compared to its parental avian H9N2 virus. Adaptation of 2WF10:6M98 in ferrets results in respiratory droplet transmission. This ferret-adapted virus, P10, contains only five amino acid changes compared to the 2WF10:6M98 virus.

VI. The adaptive changes on the surface proteins of the ferret-adapted P10 virus are sufficient to support respiratory droplet transmission. Antigenic profiles of the avian H9N2 and reassortant viruses indicate that the adaptation in ferrets, leading to respiratory droplet transmission, creates a virus with an altered HI profile, highlighting the potential discrepancies between antibodies produced against current avian H9N2 seed stocks and the H9N2 virus transmissible to humans.



## Chapter 2: Literature Review

### 2.1 Influenza Virus

#### **2.1.1 History and pandemics**

Influenza is an acute viral respiratory disease that affects millions each year. Sixteen serotypes of hemagglutinin (HA) (H1-H16) and nine neuraminidase (NA) (N1-N9) have been isolated from avian influenza A viruses. All possible combinations of these serotypes have the potential to circulate in the avian population, and therefore avian species are believed to be the main reservoir.

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, particularly migratory waterfowl. Fowl plague was described in Italy in 1878 but it was not until 1955 that the fowl plague virus identified as an influenza virus (Schafer, 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.

The history of influenza dates back to 412 BC when Hippocrates described an outbreak of flu-like illness that wiped out the Athenian army (Potter, 1998). 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 epidemic occurred in 1173-74 (Hirsch, 1883), although the first generally accepted pandemic occurred in 1580 (Pyle, 1986). Several pandemics were reported during the 14<sup>th</sup> to 17<sup>th</sup> centuries however data from 18<sup>th</sup> century onward is more informative (Creighton, 1894; Finkler, 1899; Potter, 2001; Thompson, 1890). The 18<sup>th</sup> century witnessed two major pandemics in 1729 and 1781 (Finkler, 1899; Pyle, 1986) while the 19<sup>th</sup> century experienced pandemics in 1830 and 1898 (Burnet, 1942; Jordan, 1972; Pyle, 1986). The 20th century contained three major pandemics in 1918, 1957 and 1968. The interval of time between pandemics varies from only a decade (1957-1968) to almost half a century (1729-1781) although a definite pattern does not exist.

The most serious pandemic of modern history occurred in 1918 and was responsible for roughly 20 millions deaths worldwide however, reports have even doubled the estimation (Crosby, 1976; Kaplan and Webster, 1977). This H1N1 pandemic, referred to as the “Spanish Flu”, is speculated to have originated in China. The virus became known as the “Spanish flu” because the country was the first to report an epidemic. The second and third waves of the pandemic were associated with a large

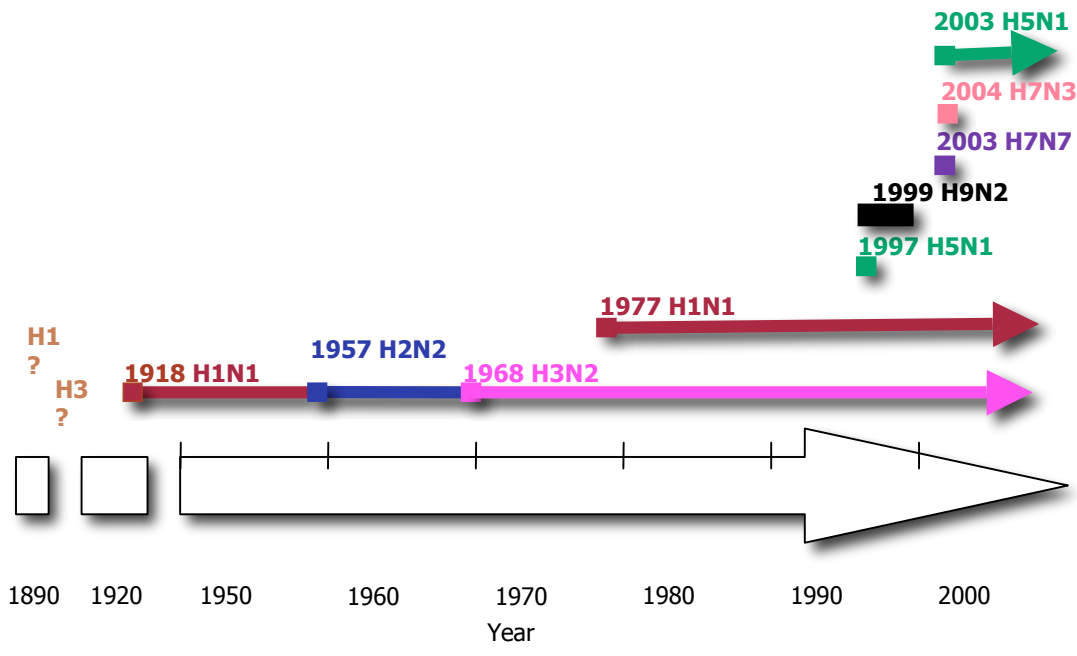
number of deaths, claiming roughly 50% mortality in the 20 to 30-year age group. In 1919 during the third wave the virus began to run its course and disappeared almost as rapidly as it began, replaced by a less pathogenic, endemic H1N1 strain, which circulated in the human population through the late 1950s (Crosby, 1976; Potter, 2001).

In 1957 an H2N2 influenza virus surfaced and was responsible for the “Asian flu” pandemic. This pandemic also originated in China and was a reassortant of the then circulating human 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 et al., 1989; Scholtissek et al., 1978). The emergence of H2N2 led to the disappearance of H1N1 viruses in the human population. There were two waves to the 1957 pandemic, with excess mortality occurring in each (Cox and Subbarao, 2000). This subtype circulated in the population for the next eleven years until the third influenza pandemic of the 20<sup>th</sup> century occurred in 1968. This pandemic, a H3N2 virus, later known as the “Hong Kong Flu”, a result of the reassortment between the HA and PB1 of an avian H3 strain and the remaining genes from the human H2N2. In 1977 the re-emergence of the H1N1 influenza virus caused a mild pandemic. The virus spread throughout the world mainly infecting those born after 1957. Since then mild influenza epidemics, also known as seasonal influenza, result from circulation of H1N1 and H3N2 viruses.

Since 1977, some might argue 1968, the human population has not experienced an influenza season of pandemic proportions (Potter, 1998). In May 1997, H5N1 emerged as an avian influenza strain that during the course of seven months infected 18 people and of those, killed 6 (Claas et al., 1998; 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 have affected its victims at alarming speed; roughly a 60% mortality rate is associated with this virus ([www.CDC.gov](http://www.CDC.gov)). The inability of this strain to transmit human-to-human is still a concern for virologist and influenza surveillance; once it has completed efficient human-to-human transmission this strain has all the makings of a pandemic. In addition to the H5N1 subtype, H9N2 influenza viruses have slowly and quietly created epidemic situations in domestic poultry throughout Eurasia. Although this subtype is not highly pathogenic, it can and has transmitted to humans causing flu-like illness and it carries with it human virus-like receptor specificity, increasing its chances of infecting and crossing the species barrier to humans. Please see Figure 1 for a comprehensive time-line of avian influenza transmission to humans.

Across the globe, approximately 20% of children and 5% of adults experience symptomatic influenza annually. During an average epidemic flu season in the United States, influenza cases account for over 30,000 deaths and more than 100,000 hospitalizations (Simonsen et al., 2000; Thompson et al., 2003). Influenza A infections cause excess mortality for the elderly with over 90% of seasonal deaths occurring in those 65 years and older (Hak et al., 2002). Although the risk of mortality from seasonal,

epidemic influenza is substantially lower in children than in the elderly, young children have a high rate of influenza-associated morbidity (Chiu et al., 2002; Thompson et al., 2004).



**Figure 1. Episodes of avian influenza transmission to humans.**

Three pandemics have affected in humans in the 20<sup>th</sup> century (1918, 1957, and 1968). Since 1997, bird-to-human transmission of avian influenza of the H5, H7, and H9 subtypes has highlighted our limited knowledge on the molecular mechanisms leading to interspecies transmission. H5 infections have continued to occur occasionally during the last five years.

Adapted from Perez et al., 2005 with permission

### **2.1.2 Discovery of influenza viruses**

The earliest and most regularly recorded date for the identification of avian influenza is 1878 when Perroncito described “fowl plague” as a virulent disease affecting chickens in Italy (Perroncito, 1878). This was not necessarily the first case of avian influenza however it was the first to differentiate fowl plague and fowl cholera. A few years later in 1901, fowl plague was shown to be caused by a filterable agent (Centanni, 1901).

Yet, it was not until 1930 that the first isolate was obtained in pigs (Shope, 1931) and then in 1933 Christopher Andrewes demonstrated that a filterable virus, influenza A, could be transmitted serially from ferret to ferret causing an immune response protecting them from re-infection (Smith, 1933). It was another 22 years until “fowl plague” was linked to influenza (Schafer, 1955). 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. The use of egg isolation led to the discovery of the third type of influenza virus in humans, type C (Smith 1935). 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 influenza A and B viruses are very similar. The main difference lies in the membrane channel lining the lipid envelope. In type A it is the M2 protein and in type B it is the NB protein and in type C it 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 genomic size is not due to protein size; rather it is due to 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 therefore 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 enzymatic activity of A and B's NA. This receptor destroying activity is not a neuraminidase but an esterase activity. The HEC protein has the same morphology of 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 (Hirst, 1941; McClelland L., 1941). 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 Madin Darby Canine Kidney (MDCK) cells is well established. Most human and avian influenza viruses can be grown



in eggs and detected through viral surface glycoproteins binding sialic acid receptors on erythrocytes causing (Reed, 1938), 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 influenza 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 that maps to the matrix protein, which has distinguishable properties between influenza types A, B and C (Hay, 1974; Klenk, 1974).

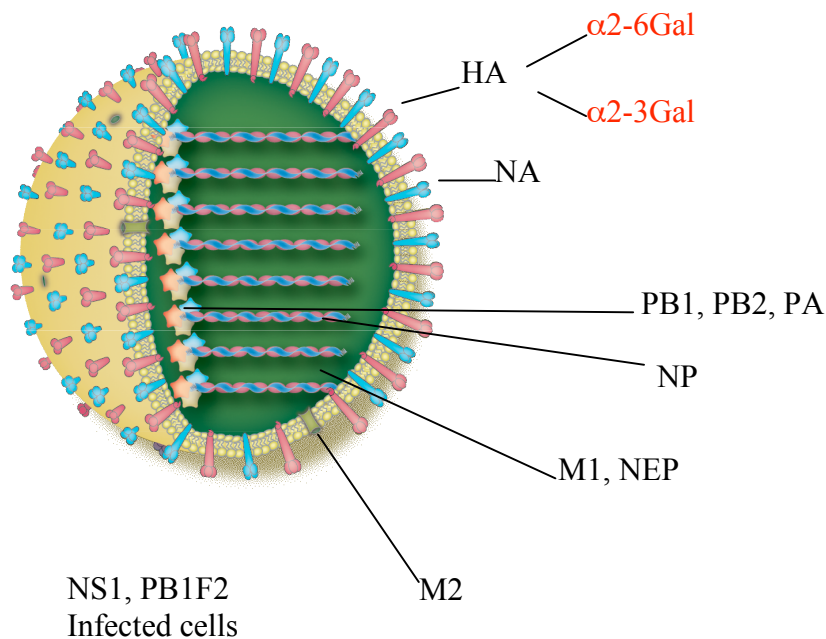
## 2.2 Influenza Virus Life Cycle

### **2.2.1 Orthomyxoviruses**

Influenza viruses are single stranded, negative-sense RNA viruses with a segmented genome. They belong to the family *Orthomyxoviridae*, which includes five genera, the influenza A, B, C viruses, *Thogotovirus*, and *Isavirus*. Type A viruses infect avian and mammalian species and are the most threatening genera of this family, responsible for human pandemics and millions of lives as well as seasonal influenza epidemics. Type B viruses are almost exclusively restricted to humans. The only other susceptible species appears to be the seal (Osterhaus et al., 2000). Mutation rates in type B are two to three times lower than influenza A making these viruses less genetically diverse and with only one serotype (Nobusawa and Sato, 2006). Immunity at a young age is achievable but not sufficient to counter the mutations in the serotype (Webster et al., 1992). Influenza C can affect humans leading to less severe illness and local epidemics. Type C is less common than A or B and appears to cause mild disease in children (Matsuzaki et al., 2006). The *Thogotovirus* affect vertebrates as well as invertebrates including ticks, mosquitoes and sea lice and contain the thogoto virus and Dhori virus. Members of the *Isavirus* genera cause infectious salmon anemia virus outbreaks in salmon.

Influenza A has eight segments, see Figure 2. They are independently encapsidated by the viral nucleoprotein (NP) and each segment is associated with a polymerase complex composed of the PB2, PB1 and PA 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 NEP (nuclear export protein) are found in the infected cell while NEP is found in small amounts in the virion (Pinto et al., 1992; Sugrue and Hay, 1991). Some

influenza A viruses express PB1-F2, a small protein derived from the second open reading frame of the PB1 protein. PB1-F2 has been implicated in apoptosis with human influenza viruses.



**Figure 2 Molecular structure of influenza A viruses.**

The virus contains a lipid bilayer derived from the host plasma membrane. Two surface glycoproteins, HA and NA, are the major antigenic determinants of the virus. The HA protein is responsible for binding sialic acid receptors. Human influenza viruses bind preferentially sialic acids in an alpha 2,6 conformation ( $\alpha 2-6\text{ gal}$ ), while those from avian species bind mostly to sialic acids in an alpha 2,3 conformation ( $\alpha 2-3\text{ gal}$ ). The virus also contains several copies of an ion channel proton pump (M2). Eight vRNA segments, each one of them associated to three polymerase subunits (PB2, PB1, and PA), and several copies of the nucleoprotein (NP) are located inside the virion protected by a protein mesh provided by the matrix protein (M1). In addition the virus carries few copies of the virus encoded Nuclear Export Protein (NEP). In infected cells, the virus expresses NS1, which interferes with the antiviral state mounted by the cell. Some influenza strains express PB1F2, a ~80 aa peptide, derived from the second open reading frame of segment 2. PB1F2 has been shown to modulate apoptosis in certain cell types infected with influenza.

Adapted from Sorrell et al., 2007

### 2.2.2 Virus entry

Upon infection, influenza attaches to host cells via the receptor –binding site in the distal tip of the HA molecule to sialic acid residues present on cell surface glycoproteins or glycolipids. Specificities for sialic acid linkage differs depending on specific residues in the HA receptor-binding pocket. The preference is between sialic acid bound to galactose by  $\alpha 2, 6$  or  $\alpha 2, 3$ -linkages (Matrosovich and Klenk, 2003). Receptor binding is followed by internalization of the viral particle by endocytosis. 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.

Upon exposure to low pH in the endosome, the HA undergoes a conformational change that results in fusion of the viral membrane with the endosomal membrane. When a virion particle has been endocytosed the low pH activated ion channel of the influenza M2 protein allows the flow of ions from the endosome to the virion interior disrupting protein-protein interactions and freeing the vRNPs 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 extracellular enzymes such as *tryptase Clara* present in lungs, whereas highly

pathogenic (HP) HAs containing multiple basic residues at the cleavage site are cleaved by intracellular proteases such as furin and subtilisin-type enzymes, present throughout the body, particularly on the endoplasmic reticulum and Golgi apparatus (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) where transcription and replication of vRNA occurs. The M1 protein, which is dissociated from the RNP enters via active diffusion.

Transcription of the RNPs takes place in the nucleus. Transcription and replication are both catalyzed by the viral RNA-dependent RNA polymerase complex. The PB1 subunit of the polymerase complex plays a major role in the catalytic activity of the viral polymerase and is responsible for elongation. 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 et al., 1996). It is responsible for the recognition and binding of host mRNAs while the PB1 binds to the terminal sequences of vRNA and cRNA and performs capped RNA endonuclease activities (Li et al., 2001; Ulmanen et al., 1981). PA is associated with the N-terminal of PB1 and replication activity of the polymerase (Mahy et al., 1983; Perales et al., 2000) (Perez and Donis, 2001). Optimal viral RNA

transcription and replication are dependent on the formation of the heterotrimer (Honda et al., 1990; Huang et al., 1990).

### **2.2.3 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. A 5'-methylated cap structure is required for priming influenza transcription. PB2 binds to methylated cap structures at the 5' terminal of the transcribed cellular mRNA 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 et al., 1983) and are 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, of 5 to 7 uridine (U) residues, reaches 15 to 22 nucleotides before the 5' ends of the vRNAs.

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 the case of the M and NS segments splicing is required for the synthesis of the M2 and NEP proteins, respectively, whereas



unspliced messengers are translated into the M1 and NS1 proteins. In infected cells the vRNAs are both transcribed into mRNAs and replicated.

#### **2.2.4 vRNA replication**

The trimeric polymerase complex also uses the vRNPs as templates for viral RNA (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. In this case initiation is primer independent and results in synthesis of co-linear complementary copies of vRNA, also known as cRNA. The cRNA serves as the template for synthesizing progeny vRNA. Newly synthesized vRNPs can serve as templates for new rounds of transcription or replication or they may exit the nucleus to be incorporated into progeny virions.

The mechanism that triggers the switch from primer-dependent mRNA synthesis to primer-independent cRNA synthesis is not well understood. Because the switch from transcription to replication calls for protein synthesis one or more newly synthesized virus-specific proteins are assumed to be necessary for the unprimed synthesis or anti-termination at the poly(A) site, or both. 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 et al., 1990). Therefore, 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, while the second type initiates synthesis of either template RNA or vRNA chains without a primer (Area et al., 2004). In 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 production of viral proteins is synchronized to when they are required during replication and 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 is 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 et al., 1987; Skehel, 1972). The production of NP and NS1 in early phase highlights their role in the regulation of transcription, replication of vRNAs and host immunity evasion.

The late phase focuses mainly on the production of virion structural components. The vRNAs are synthesized in equal amounts, necessary for the progeny genomes. Viral RNA and mRNA are no longer coupled during this second phase (Shapiro et al., 1987). Previously synthesized viral mRNAs direct the protein production with a change in production preference directed towards the HA and M1 proteins while decreasing NS1 production while vRNA synthesis remains high. The synthesis of all three virus-specific RNAs (mRNA, cRNA, vRNA) 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, synthesized late in infection, blocks transcription of vRNA 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, NEP, 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.

#### **2.2.5 Transport and assembly**

Exit of the vRNPs from the nucleus requires an association with M1, which in turn interacts through its C terminus with NEP. 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 PB2, PB1, 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 vRNAs in RNPs. The interaction with M1 prevents reentry of the vRNP into the nucleus and promotes their targeting to the virion assembly sites where the envelope proteins are inserted and budding occurs. 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. Processing within the ER include 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 protein folding and assembly 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 due their accumulation at the site of virus budding even when expressed alone (Hobman, 1993). However, 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 (Nayak and Hui, 2004). The mechanism behind packaging a complete set of eight vRNPs into a newly form virion is not fully understood, although packaging signals at the ends of each vRNA segment and organization of the eight

vRNPs in budding particles indicates that the vRNPs are incorporated according to a specific mechanism (Fujii et al., 2003; Noda et al., 2006).

Budding requires three 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 however, it is well accepted that interaction and involvement of both host and viral components is required. Bud formation and virion release are the last steps in viral replication and production of new infectious progeny.

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, in order for an influenza virus to be infectious, all eight RNA segments are required. Viruses must be released into the surrounding environment in order to infect other cells. Bud formation and pinching off of the virion may not be sufficient to release the virus into the external environment. The released virion may still be attached to the infected host cell by sialic acid therefore viral neuraminidase is necessary for the release of the effective virions. 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). Finally HA cleavage is required for infectivity of viral particles. 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 severe mortality (Liu et al., 1995; Mori et al., 1995; Subbarao and Katz, 2000).

### 2.3 Influenza Host Range

#### **2.3.1 Molecular determinants of host-cell restriction**

All known subtypes of influenza A viruses circulate in wild aquatic birds. Aquatic avian species can be infected by each of the 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes, in apparently any of the possible 144 subtype combinations (Fouchier et al., 2005; Webster et al., 1992) and thus represent the origin of all viral genes for both avian and mammalian strains. It is commonly accepted that migratory waterfowl including ducks, sea birds, or shorebirds, are responsible for introducing AI viruses into domestic poultry (Alexander, 2000a). Some of these viruses

can establish stable lineages in terrestrial birds (Order *Galliformes*) and a limited number of mammalian species including horses, pigs and dogs, among others (Crawford et al., 2005; Scholtissek, 1994; Scholtissek, 1995; Scholtissek, 1997; von Grotthuss and Rychlewski, 2006; Webster, 1997; Yoon et al., 2005).

Influenza A viruses have plenty of opportunities to cross the species barrier. The establishment of a stable lineage and the spread thereof is, however, a rare event (Hinshaw et al., 1980a; Hinshaw et al., 1980b; Webster et al., 1978). The likelihood of a virus becoming endemic in a new host depends on two major factors: at the macro scale level, the interaction between donor and recipient animal species, and at the molecular level, the intricate interactions between the new recipient host and virus components (Kuiken et al., 2006). Influenza A viruses appear well adapted to the wild bird reservoir in which infections are rarely accompanied by signs of disease (Perkins and Swayne, 2001). Due to complex interactions of the virus in various wild bird species, the mechanisms involved in the genesis of novel influenza strains and the epidemiological factors implicated in the emergence of pandemic outbreaks are poorly understood. Some HA and NA subtypes appear more prevalent in certain aquatic bird species than in other (Kawaoka et al., 1988a). In addition, these viruses appear to undergo cycles of prevalence that may last few months to several years.

Host range is a polygenic trait (Ito and Kawaoka, 2000), however the HA subtype plays a major role in infectivity, virulence and transmissibility. Influenza A virus host specificity is in part mediated by the HA which binds to receptors containing glycans

with terminal sialic acids. The majority of avian influenza viruses bind to receptors with sialic acids having an  $\alpha$ 2,3 linkage to the penultimate galactose (SA $\alpha$ 2,3-gal), while human viruses prefer receptors that are present with an  $\alpha$ 2,6 linkage (SA $\alpha$ 2,6-gal). The switch from  $\alpha$ 2-3 to  $\alpha$ 2-6 receptor specificity is thought to be critical in the adaptation of AI viruses to humans and appears to limit most AI viruses from directly crossing the species barrier (Beare and Webster, 1991). 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).

The role of the internal proteins in host range and pathogenicity of influenza viruses is less well understood. The involvement of polymerase gene products in host range and/or pathogenicity has been demonstrated in a number of studies (Reid et al., 2000; Nobusawa et al., 2006; Matsuzki et al., 2006). It is generally accepted that interactions exist between viral proteins and host factors and these interactions play key roles in viral fitness and pathogenicity. Adaptive mutations, particularly within the RNP would lead to optimal interactions between virus and host. The viral NS1 protein has pleiotropic functions that counteract cellular antiviral activity (Scholtissek et al., 1998). NS1 inhibits NF- $\kappa$ B and IRF3 transcription factors, both of which are involved in the synthesis of IFN  $\alpha$ / $\beta$  (Talon et al., 2000; Wang et al., 2000). In addition, NS1 inhibits the dsRNA-activated kinase or PKR, which is also a mediator and inducer of IFN  $\alpha$ / $\beta$  (Bergmann et al., 2000; Kido et al., 1999). Two NS alleles circulate in the avian



reservoir, A and B; in contrast only allele A is found in humans, suggesting that host restriction might play a role in determining which allele is able to cross to mammals.

Likewise, the lethality of the 1997 H5N1 viruses in mice is correlated with specific amino acid residues in M1, PB1, and PB2 (Chin et al., 2002). Amino acid 627 in PB2 is crucial for the pathogenesis of these viruses. Avian viruses have glutamic acid at 627 and human viruses have lysine (Scholtissek, 1998). Nevertheless, avian influenza viruses with a typical avian PB2 at position 627 can also be highly pathogenic in mammals further supporting the idea that multiple changes and/or gene constellations modulate the host range and virulence of influenza viruses. We know much more about the role of viral factors in pathogenesis than about the molecular steps that enable an aquatic avian influenza virus to transmit to other species. The putative regions in the internal genes that influence interspecies transmission have not been identified.

### **2.3.2 Receptor specificity in host range**

The key to influenza infection is the binding of the HA antigenic surface protein to the glycan cell surface receptor on epithelial cells of either the intestine (avian) or respiratory tract (avian and mammalian). Receptor binding occurs through recognition of the terminal sialic acid and its linkage to the second to last galactose of carbohydrates on the target tissues (Stevens et al., 2006). Avian viruses preferentially bind receptors with an  $\alpha$ 2-3 linkage while mammalian-adapted viruses favor an  $\alpha$ 2-6 linkage (Connor et al.,

1994; Rogers and D'Souza, 1989). The switch from  $\alpha$ 2-3 to  $\alpha$ 2-6 receptor specificity appears to be the key determinant in avian influenza viruses gaining the ability to cross the species barrier, particularly to humans, and appears to limit most of the avian viruses from directly infecting humans. It was initially thought that in order for an avian influenza virus to cross into a human host an intermediate host was necessary. This intermediate host would need to possess both  $\alpha$ 2-3 and  $\alpha$ 2-6 surface receptors and so the swine, meeting the criteria was pegged the “mixing vessel”. Therefore, the pig, able to become infected with both avian and human viruses, could act as the ideal host for viral replication, possible adaptation and genetic reassortment (Ito et al., 1998; Oxford et al., 2005). However, only a limited number of avian and human influenza viruses have established stable lineages in pigs (Makarova et al., 2003). Recent evidence introduces certain terrestrial poultry, such as chickens and quail, which provide an environment similar to pigs by displaying both SA $\alpha$ 2,3-gal and SA $\alpha$ 2,6-gal receptors (Gambaryan et al., 2002a; Guan et al., 1999; Wan and Perez, 2006). In addition, within the last decade there have been numerous accounts of human infection with fully avian viruses of the H5, H7 and H9 subtypes (Claas et al., 1998; Fouchier et al., 2004; Guan et al., 2004; Li et al., 2004; Peiris et al., 1999; Tweed et al., 2004). Such instances in 1997 with H5N1 and in 1999 with H9N2, where avian viruses circulating in poultry in Hong Kong were transmitted directly to humans, are scenarios that seem to be increasing in familiarity (Lin et al., 2000; Peiris et al., 1999; Shortridge, 1999). Sialic acids with the  $\alpha$ 2-3 linkage have been found in human airway epithelium (Matrosovich et al., 2004) indicating that given the appropriate circumstances, including but not limited to: contact, viral dose and immune status, humans can become infected and shed avian influenza strains. However

it is not well understood whether the change in receptor specificity of avian influenza A viruses is an obligatory step to initiate sustained human-to-human transmission.

Receptor specificity is an important determinant of host range restriction of influenza A viruses (Ito et al., 1998; Ito and Kawaoka, 2000; Matrosovich et al., 2004; Wan and Perez, 2006). At least two mechanisms exist for the host range variation of influenza A viruses, (1) selection due to host cell receptors, availability and presence of sialylsugar chains (Ito et al., 1997; Suzuki et al., 2000) and (2) selection due to host anti-influenza antibodies (Suzuki et al., 1989). Analysis of H3 serotypes linked HA1 residues 226 and 228 within the receptor-binding site (RBS) to receptor specificity (Skehel and Wiley, 2000; Wan and Perez, 2007). Leucine (L) 226 and serine (S) 288 were found in human H3 viruses, whereas glutamine (Q) 226 and glutamine 228 were found in avian viruses. However more recent work from our lab has shown that a single amino acid change from Q226L in the receptor binding site of the HA (H3 numbering) causes a dramatic change from 2-3 to 2-6 binding (Wan and Perez, 2007; Wan et al., 2008). This single amino acid difference at 226 defined the location of the receptor binding site (Rogers and Paulson, 1983). Residue 226 does not directly contact the sialosides however the Q266L mutation alters the conformation of the RBS (Weis et al., 1988). Amino acid 226 is located on the left edge of the receptor-binding pocket of the H3 HA, which is close to the glycosidic linkage of the receptor sialyl linkage, linking this single change to receptor specificity (Suzuki, 2005). This mutation appears to be a key step in host adaptation of avian influenza in mammals. Other amino acids that may be relevant to receptor specificity include residues 183 and 190 (Wan and Perez, 2007).

### 2.3.3 Antigenic shift and drift

The segmented nature of the influenza genome allows for reassortment between influenza A viruses when two or more strains infect the same cell and exchange gene segments, also known as antigenic shifts. This is an important mechanism allowing rapid diversity and potentially expanding host range and transmission when a naïve population is infected by a novel strain carrying a completely different HA subtype. Although antigenic shift could result in the emergence of a new NA subtype, the NA surface protein seems to play a less important role than the HA in terms of significance in disease outbreaks or pandemics. In the past century there have been at least three pandemics, the Spanish (1918, H1N1), the Asian (H2N2, 1957) and the Hong Kong (H3N2, 1968) pandemics, caused by reassortment between human and avian strains (Kawaoka et al., 1989; Scholtissek, 1997; Scholtissek et al., 1978).

In contrast antigenic drift is the accumulation of viral strains with minor genetic changes, mainly amino acid changes in the HA and NA proteins that does not lead to a new subtype. Antigenic drift drives a particular subtype to evade the host immune response and can expand the host range into other animal species. Influenza A replication by the virus-encoded RNA-dependent RNA polymerase is relatively error-prone. The lack of proofreading among RNA polymerases accounts for replication errors to the order of 1 in 10,000 bases (Holland et al., 1982; Steinhauer and Holland, 1987) per replication cycle, responsible for a major source of antigenic drift (Zambon, 2001). Therefore, each

round of replication produces a mixed population of virus variants, a majority of which are nonviable, however some carry advantageous mutations that can become dominant under selective pressure i.e.) adaptation to a new host. Viral evolution and selection favors human influenza strains with antigenic drift and shift involving the HA and NA proteins because these viruses are able to evade neutralizing antibodies from prior infections and/or vaccinations. This type of selection permits re-infection with the same subtype accounting for seasonal influenza epidemics.

#### **2.3.4 Interspecies transmission**

Influenza A viruses from aquatic avian species can readily infect terrestrial, domestic poultry as well as mammals. Successful establishment of avian influenza viruses in new hosts, particularly humans, is a multi-step process that includes transmission from the avian reservoir to the new host, efficient replication in the new host and an ability to establish a sustained chain of transmission and efficiently compete with related viruses previously circulating in that particular host (Naffakh et al., 2008). The probability of all the above factors occurring is influenced by multiple other factors and relies on the host. Efficient replication and transmission in the new host depends on the ability of the virus to interact with host components at each step of viral replication and to counteract the immune response. Mutations, including substitutions, insertions and deletions are some of the mechanisms by which variant influenza strains are produced. For successful introduction and stability 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. 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).

As previously noted, swine and some domestic poultry species contain receptors in both the SA $\alpha$ 2, 6Gal and SA $\alpha$ 2, 3Gal conformation (Kida et al., 1994). Therefore these hosts can facilitate reassortment producing new subtypes to which the human population is immunonaive. In areas of Asia where backyard pigs co-mingle with chickens, and even migratory ducks, the potential for reassortment is ever-present. The infrequent transmission of avian influenza viruses to pigs may be the limiting factor in their role as intermediate hosts. In the last century only three of the HA subtypes and two of the NA subtypes have successfully established lineages in the human population, leading to three pandemics; H1N1 in 1918, H2N2 in 1957 and H3N2 in 1968. These pandemics arose from reassortment of avian and human viruses via infection in still unidentified intermediate host(s) (Ito et al., 1998).

### **2.3.5 Implications of domestic poultry in the spread of influenza**

The initial human cases of H5N1 (1997) and H9N2 (1999) in Hong Kong and H7N7 (2003) in the Netherlands and H7N3 (2004, 2006) in British Columbia and the United Kingdom, in addition to the more recent outbreaks of H5 and H9 in the last few years, is proof that AI viruses can transmit directly to humans. There is growing evidence that influenza viruses in poultry evolve at rates similar to that of mammals (Suarez, 2000), suggesting that poultry species are not natural, but potential intermediate hosts in zoonotic transmission.

Avian influenza viruses may infect several domestic poultry species; the progression and outcome of the infection, however varies within each species. For example, a particular isolate may transmit in quail and turkeys, only produce severe disease in turkeys, but not replicate in chickens or any other poultry. Morbidity and mortality can be as high as 100%, particularly in gallinaceous species. Avian influenza viruses from aquatic birds undergo significant selective pressure when adapting to domestic poultry leading to definite changes in both the surface and internal genes (Banks et al., 2001; Hossain et al., 2008; Matrosovich et al., 1999; Sorrell and Perez, 2007).

The accumulation of basic amino acids at the cleavage site of the HA protein is a hallmark of the generation of avian influenza viruses with high pathogenic potential. For the HPAI viruses, the cleavability of the HA protein plays a critical role in the

pathogenicity of AI viruses because it restricts tissue tropism (Horimoto and Kawaoka, 1994; Horimoto and Kawaoka, 1997; Kawaoka et al., 1987; Kawaoka and Webster, 1988; Rott et al., 1995; Steinhauer, 1999; Walker and Kawaoka, 1993). Only AI viruses of the H5 and H7 subtypes have shown the potential to become highly pathogenic. An H5 or H7 virus can present itself as a low pathogenic virus but mutate without warning to become highly pathogenic, usually once they are introduced in domestic poultry, particularly chickens and turkeys (Banks et al., 2001; Ito et al., 2001; Kawaoka et al., 1984; Senne et al., 2006). Influenza subtypes, other than H5 and H7 that establish lineages in domestic poultry produce a mild disease, which can be exacerbated by secondary infections.

How do avian influenza viruses transfer from the wild bird reservoir into domestic poultry? A major factor is agricultural and commercial practices that promote interspecies transmission and emergence of influenza viruses through co-mingling of natural and non-natural hosts (Alexander, 1982). Live bird markets, backyard flocks, and free-range raised poultry are a few examples of such practices where natural and non-natural hosts of influenza can come in contact (Bulaga et al., 2003; Liu et al., 2003b; Mullaney, 2003), favoring the circulation and transmission from natural to intermediate hosts. These same conditions have also favored the transmission of avian influenza from poultry to humans.

The great variety of potentially susceptible domestic avian species increases the complexity and diversity of influenza strains in nature. Quail (*Coturnix coturnix*), for



example, have been shown to provide an environment where influenza viruses from the wild bird reservoir can increase their host range and infect other avian species (Makarova et al., 2003; Perez et al., 2003a; Perez et al., 2003b; Sorrell and Perez, 2007), they appear more susceptible than chickens to avian influenza viruses from wild birds, typically harboring asymptomatic infections (Makarova et al., 2003; Perez et al., 2003a).

Compared to chickens and turkeys, quail are usually delayed in displaying clinical symptoms from HPAI, while shedding substantial amount of virus, increasing the chances for the spread of the virus (Webster et al., 2002). Interestingly, the respiratory tract of quail possesses a pattern of expression of sialic acid receptors similar to the one observed in humans; i.e. ciliated cells that express abundant SA $\alpha$ 2,6Gal receptors and mucin-producing cells that express SA $\alpha$ 2,3Gal receptors (Wan and Perez, 2006). Other studies suggest that pheasants could possess similar characteristics to those observed in quail (Humberd et al., 2006). Furthermore, domestic bird species that are infected with LPAI often do not show signs of disease during the period when the virus is effectively transmitted, whether in live bird markets, back yard flocks, or commercial poultry operations.

From an epidemiological perspective, however, the prevention and control of avian influenza in aquatic and domestic poultry is crucial (Hulse-Post et al., 2005). Several studies have pointed to free-grazing ducks as major determinants in the spread of H5N1 among domestic poultry in South East Asia (Gilbert et al., 2006) and most likely responsible for the spill over of H5N1 into wild aquatic birds and the consequent massive geographic spread (Martin et al., 2006). There is no precedent for the presence of a

HPAI virus that can infect so many wild bird species as well as mammalian species (cats, humans, mice and ferrets) and with such lethal outcome. Previous reports of HPAI in wild birds have been characterized by either limited mortality or limited spread. The current H5N1 strains have undergone cycles of increased virulence and mortality for wild birds, domestic ducks and other aquatic birds, while maintaining a typical HPAI phenotype for terrestrial poultry. The potentially devastating ecological consequences of such events remain to be seen.

## **Chapter 3: Adaptation and Expanded Host Range of A/mallard/Potsdam/178-4/83 (H2N2) in Land-based Birds**

### 3.1 Abstract

The events and mechanisms that lead to interspecies transmission and host adaptation are unknown, however both surface and internal proteins have been identified as having a role. In my MS thesis I assessed the potential of quail as an intermediate host of avian influenza, using A/Mallard/Potsdam/178-4/83(H2N2) –mall/178- virus to determine if through adaptation in quail, a mallard strain can increase its host range and transmissibility to other domestic poultry. After five serial passages of lung homogenate a virus arose, which replicated and transmitted directly to contact quail. To test whether this adaptation in quail lead to inter-species transmission white leghorn chickens were infected with the wild type (mall/178) and quail-adapted mall/178 (qa-mall/178) H2N2 viruses. The results ed that mall/178 H2N2 does not establish an infection in chickens nor does it transmit, while qa-mall/178 H2N2 infects and transmits to contact chickens causing clinical signs like depression and diarrhea. Completed sequences identified six amino acid changes spanning four genes: PB2, PB1, HA, NP, suggesting that the internal genes play a role in host adaptation (Sorrell and Perez, 2007).

My dissertation research began with the further adaptation of qa-mall/178 in white leghorn chickens to create a virus that replicated more efficiently in the upper and lower respiratory tract leading to a change in tissue tropism. Sequence analysis of the chicken-adapted virus, after only four passages, pointed to a 27-amino acid deletion in the neuraminidase stalk region, the largest known N2 deletion to date (Sorrell and Perez, 2007). A repeat adaptation of mall/178 in quail confirmed their role as intermediate hosts with the reproducibility of transmission in quail after 5 lung passages.

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. In attempts to elucidate the mechanism of adaptation and to determine the minimal changes necessary for transmission of qa-mall/178 in quail and chickens we created reassortant viruses between the wild type mall/178 and the qa-mall/178 viruses. The single change on the HA of qa-mall/178, N170D, is essential for transmission in quail however, all six amino acid changes found in the qa-mall/178 virus are necessary for transmission in chickens, providing further evidence that quail are a necessary intermediate in the transmission of mall/178 to chickens. Investigation of the 27-amino acid deletion located in the ch-qa-mall/178 virus NA stalk region found that the deletion supported viral replication at lower temperatures supporting the change in tissue tropism seen during the chicken adaptation.

## 3.2 Introduction

### **3.2.1 H2N2 influenza A viruses**

Of the 16 known HA subtypes, only 3 (H1, H2 and H3) have established lineages in humans. H2 viruses caused a pandemic in 1957 and circulated in the human population until the reassortment of the H2N2 with an avian H3 resulting in the H3N2 pandemic of 1968 (Schafer et al., 1993). Since then, H2N2 influenza has been absent from the human population, however it is still isolated in wild bird surveillance (Liu et al., 2004; Schafer et al., 1993). The first avian H2 influenza virus isolated was reported in 1972 and has continued to circulate in wild and domestic birds (Schafer et al., 1993). Avian H2N2s isolated in the 1990s show low divergence from the '57 pandemic sequence (Schafer et al., 1993) and there is evidence of interregional transmission of avian H2N2 viruses between North American and Eurasian lineages (Liu et al., 2004).

More recent isolations include those from ducks in Japan (2001), The Netherlands (1999), Minnesota (1998); chickens in Pennsylvania (2004), New York and New Jersey (1995), guinea fowl in New York and New Jersey (1995) and in turkeys throughout the United States (1993-2000s) and it has most recently been isolated in a H2N3 avian-swine reassortant which caused disease and transmitted in swine and ferrets (Ma et al., 2007). No reports of H2N2 influenza have been reported in swine, however direct transmission of H2N2 from birds to humans, although a rarity has occurred (Claas et al., 1998;

Subbarao et al., 1998; van Kolfshoeten, 2003). Therefore, the chance for a H2N2 avian virus to come into contact with susceptible humans still exists, with those born after 1968 being extremely susceptible to H2 virus.

### **3.2.2 Quail as an intermediate host**

Quail (*Coturnix japonica* or *Coturnix coturnix*), among other terrestrial birds, are extensively farmed around the world. The first reported influenza A outbreak in quail was the late 1960s (Nardelli et al., 1970). Many influenza subtypes have been isolated from quail in North America, Europe and Asia including H5N2, H7N2, H7N3, H9N2 and H10N8 (Guan et al., 1999; Guo et al., 2000; Saito et al., 1993; Suarez et al., 1999) however, minimal research has been done to study quail as a potential intermediate host. Extensive surveillance efforts resulting from the initial bird-to-human transmission of H5N1 viruses in Hong Kong in 1997 revealed a high incidence of influenza viruses in quail in live bird markets (Matrosovich et al., 1999). More importantly, 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, namely the H9 and H5 subtypes (Chin et al., 2002; Guan et al., 2000; Lin et al., 2000). As a result, quail were banned from live bird markets in Hong Kong in 2000, under the suspicion that they were a contributing factor in the perpetuation of avian influenza viruses in the markets. However, quail remain a usual member in other live bird markets around the world. Further laboratory studies suggested that quail are highly susceptible to avian influenza

viruses isolated from live bird markets and are, in general, more susceptible than chickens to influenza viruses from aquatic birds, although these latter group of viruses do not necessarily transmit very efficiently among quail (Alexander, 1986; Liu et al., 2003a; Liu et al., 2003b; Makarova et al., 2003; Perez et al., 2003a). Influenza infection in quail 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 (Makarova et al., 2003). This location of replication and shedding promotes a change in the tissue tropism of avian strains, permitting the emergence of variants transmitted by aerosol (Perez et al., 2003a). 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. Our group and others have highlighted quail as an intermediate reservoir for influenza viruses, suggesting that replication and transmission are two distinguishable biological properties of influenza viruses, determined by the host and by molecular changes within the viral genome (Hossain et al., 2008; Slemons and Easterday, 1972; Sorrell and Perez, 2007; Tashiro et al., 1987).

### **3.2.3 NA deletions in adaptation to terrestrial birds**

NA releases sialic acid both from host cell receptors and from the sialic acids expressed on the surface of the virion's HA. The NA enzymatic domain is distanced from the virus envelope by a polypeptide stalk of variable length (Blok and Air, 1982). Using NA-deficient viruses, studies have shown that NA is important for release of infectious

progeny virus particles, which would remain aggregated at the cell surface due to virus-virus or virus-cell HA and sialic acid interactions (Liu and Air, 1993; Liu et al., 1995). Stalk length affects the NA's ability to cleave sialic acid, the shorter the stalk the closer it is to the active site and the less efficient it is at releasing and disaggregating progeny (Els et al., 1985). In vivo studies showed that stalk length contributes to the host range of the virus with a deleted stalk supporting replication in tissue culture but not in embryonated eggs or mice (Castrucci and Kawaoka, 1993). There have been claims of associations between increases in glycosylation sites on the HA and the shortening of the NA stalk, as a balance of HA and NA activity (Baigent and McCauley, 2001; Wagner et al., 2000). However it has been noted that NA deletions appear associated with the adaptation of mallard viruses to chickens and other land-based birds and that some of these viruses do not contain additional glycosylation sites, indicating that lack of an additional glycosylation of HA or a shortened NA stalk are not restrictive factors for replication and/or pathogenicity in poultry (Baigent and McCauley, 2001; Banks et al., 2001; Giannecchini et al., 2006; Hossain et al., 2008; Matrosovich et al., 1999; Sorrell and Perez, 2007).

It is noteworthy that early human H1N1 viruses, including the 1918 pandemic strain, which is believed to have originated from a fully-avian H1N1 virus, as well as the highly pathogenic H5N1 virus that transmitted to humans in 1997, contained deletions in their NA stalks (Baigent and McCauley, 2001; Banks et al., 2001).



### 3.3 Materials and Methods

#### **3.3.1 Virus isolation and propagation**

The repository at St. Jude Children's Research Hospital, Memphis Tennessee, provided the influenza virus, A/Mallard/Potsdam/178-4/83 (H2N2). The virus was isolated from mallards and propagated in 10-day-old embryonated SPF chicken eggs and stored at  $-70^{\circ}\text{C}$ . The virus was received as an E1 (egg 1) passage and we used the E2 passage in this study. The qa-mall/178 virus was created after five serial passages of lung homogenate in quail, as described previously (Sorrell and Perez, 2007). Stock virus was grown in embryonated chicken eggs as E1 passage. The viruses were titrated to determine the median egg infectious dose 50 (EID<sub>50</sub>) by the Reed and Muench method (Reed, 1938).

#### **3.3.2 Viral sequencing**

Swab samples and lung homogenate were directly used for RNA extraction to confirm the presence of mutations in the adapted viruses. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni12 primer (5'-AGCAAAGCAAGG-3') and AMV reverse transcriptase (Promega, Madison, WI, USA) (Hoffmann et al.,

2001). PCR amplification was performed using universal primers described by Hoffmann et al., (Hoffmann et al., 2001) as well as specific primers designed for the particular viruses. The PCR products were sequenced using the BigDye-Terminator protocol V3.1 and a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Full-length sequences were performed for all genes of the viruses used in these studies.

### **3.3.3 Animals and experimental infections**

Four to six-week old Japanese quail (*Coturnix coturnix*, Department of Animal and Avian Sciences, University of Maryland, College Park / M<sup>C</sup>Murray Hatchery, Webster City, IA) and three to four-week White Leghorn chickens (Charles River Laboratories, Wilmington, MA) were used. Groups of three birds were inoculated intranasally, intratracheally and at times intracloacally (chickens only) with  $5 \times 10^6$  EID<sub>50</sub> of virus/ml. One ml of virus inoculum was used for chickens while 0.6 ml was used for quail. Tracheal and cloacal swabs were collected at days 1, 3, 5, 7, 9 post-infection (p.i.) and stored in glass vials in 1 ml freezing medium (50% glycerol in phosphate buffered saline (PBS) containing 1% antibiotics) and titrated for activity in 10-day-old embryonated chicken eggs by the method of Reed and Muench (Reed, 1938). Birds were observed and scored daily for clinical signs of infection and general well being. Experiments were carried out under BSL2 conditions with investigators wearing appropriate protective equipment and compliant with all Institutional Animal Care and

Use Committee (IACUC) approved protocols and under Animal Welfare Act (AWA) regulations.

#### **3.3.4 Replication, transmission and tropism of influenza A viruses in quail and chickens**

For the replication experiments, birds were infected with either 1 ml or 0.6 ml doses and tracheal and cloacal swabs were collected as described above. For transmission experiments three contact birds were introduced one day post-infection to the cage where the infected birds were kept. Water and food bowls, as well as cage liners, were changed in order to prevent transmission of virus via contaminated water or food. Tissue samples were collected 3 days p.i. and homogenized to determine viral titers as described previously (Sorrell and Perez, 2007).

#### **3.3.5 Adaptation studies in chickens and quail**

Adaptation experiments for the qa-mall/178 H2N2 virus in chickens comprised of seven passages. The dose for passage 1 was viral stock ( $5 \times 10^6$  EID<sub>50</sub>/ml) grown once in embryonated chicken eggs, as described above. Passages 2-4 consisted of a 1:10 dilution of allantoic fluid from cloacal swabs grown once in embryonated chicken eggs while

passages 5-7 were of pooled lung homogenate. Lungs were collected on day 3 or 5 pi and prepared as a 10% homogenate, pooled from three chickens/passage, to become the viral dose for the next passage. Tracheal and cloacal swabs were collected and tested for virus presence by analyzing the infectivity in embryonated chicken eggs. Transmission of the chicken-adapted virus –ch-qa-/178- was tested at passage 7, described above. The repeat adaptation of mall/178 in quail was carried out as describe previously (Sorrell and Perez, 2007) with five passages of 10% pooled lung homogenate as passage doses and transmission was tested at passage 6.

### **3.3.6 Cloning and generation of viruses by reverse genetics**

The genes of mall/178, qa-mall/178 and ch-qa/178 (H2N2) were cloned using a set of universal primers as described (Hoffmann et al., 2001). Cloned genes were sequenced and compared to the corresponding viral sequences to determine that the clones did not carry spurious mutations. Sequences were generated as described above.

Viruses were rescued as described (Hoffmann et al., 2002; Neumann et al., 1999). Briefly, the day before transfection, confluent 293T and MDCK cells in a 75 cm<sup>2</sup> flask were trypsinized, and 10% of each cell line was mixed in 18 ml OptiMEM I; 3 ml of this cell suspension was seeded into one well of a six-well plate. The next day, 1 µg of each plasmid (~8 µg) was mixed with 18 µl of TransIT LT-1 (Mirus, Madison, WI). After 45 min incubation at room temperature, the mixture was added to the cells. Six hours later,

the DNA-transfection mixture was replaced by Opti-MEM I. Thirty hours after transfection, 1 ml of Opti-MEM I containing 1 µg/ml TPCK-trypsin was added to the cells. Viruses were propagated in 10-day-old embryonated chicken eggs, and titrated by EID<sub>50</sub>. The recovery of recombinant viruses was verified by sequencing using specific primers.

### **3.3.7 Plaque assays**

To validate the results of the tissue tropism study in chickens the temperature phenotype of the recombinant viruses was examined by plaque assay in primary chicken embryo kidney (CEK) cells at 35°C and 42°C as described previously (Song et al., 2007; Wan and Perez, 2007). Briefly, confluent cell monolayers in 6-well plates were infected with 10-fold dilutions of virus in a total volume of 0.4 ml of PBS for 1 h at 37°C. Cells were washed twice with PBS and covered with an overlay of modified Eagle's medium containing 1.8% agar, 0.02% BSA, 1% glutamine, and 1µg/ml TPCK trypsin. The plates were then incubated at 35°C and 42°C under 5% CO<sub>2</sub>. After 3 days of incubation the overlays were removed and the cells were stained with crystal violet.

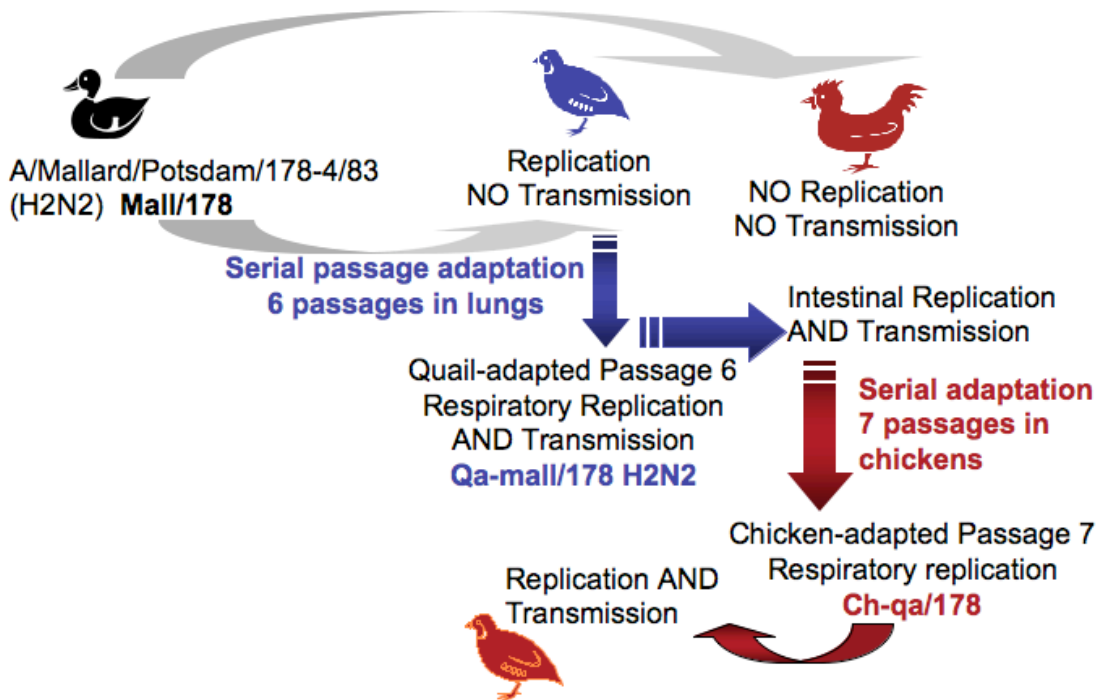
### 3.4 Results

#### **3.4.1 Further adaptation of qa-mall/178 (H2N2) in chickens leads to a virus with expanded tissue tropism**

Once we confirmed that the qa-mall/178 virus infected and transmitted in chickens we wanted to determine whether a transition in shedding from fecal-oral to respiratory was possible through adaptation, Figure 3. We adapted the virus using swab samples, as indicated in the materials and methods, and by the fourth passage we detected virus in the lung homogenate. Adaptation of ch-qa/178 continued through passage 7 with positive titers in the lungs of each group (passages 4-7). Titers at passage 4 indicate that shedding of the chicken-adapted virus (ch-qa/178) is similar in the lungs and the trachea, Table 1. It is interesting to note that the change from intestinal to respiratory shedding changed clinical signs. With each passage, the diarrhea decreased and by passage 5 we noted excess mucus in the trachea of infected chickens.

### 3.4.2 Sequence analysis of ch-qa/178

Sequences from lung homogenate and swab medium at passage 4 and passage 7 were analyzed and compared to the qa-mall/178 virus from which it originated, Table 2. All amino acid changes that arose during the quail adaptation were maintained in the chicken adaptation, indicating their importance in establishing infection and transmission in the chicken. One additional change was found on HA at position L318Q (immature HA). However, the major change occurred in the NA protein in which a 27 amino acid deletion was found in passage 4 and maintained through passage 7, corresponding to the virus isolation in lung homogenate. The deletion is located in the stalk region of the neuraminidase, covering amino acids 54-81, the largest known N2 subtype NA deletion.



**Figure 3. Adaptation Scheme for A/Mallard/Potsdam/178-4/83 (H2N2) in Quail and Chickens.**

When tested in domestic poultry, A/Mallard/Potsdam/178-4/83 (H2N2) replicated but did not transmit in quail and could not establish an infection in chickens. Therefore steps were taken to adapt mall/178 in quail using lung homogenate. After six passages a quail-adapted virus, qa-mall/178, was able to replicate and transmit in quail and in chickens. This qa-mall/178 was further adapted in chickens in attempts to change viral tropism from an intestinal infection to an upper respiratory infection. After seven passages, ch-qa/178 was established which could replicate and transmit in both quail and chickens and was isolated almost solely in the upper respiratory tract of chickens.

Adapted from Sorrell and Perez, 2007



### **3.4.3 Adaptation of mall/178 in quail is reproducible**

Repeat adaptation of mall/178 in quail led to successful transmission at passage six. The adaptation scheme was followed as in the initial adaptation by passing pooled lung homogenate in quail. Interestingly, sequence analysis of lung homogenate from this second adaptation, qa-mall/178<sub>2</sub>, revealed a total of ten amino acid changes, a majority of which were found on the surface proteins. The only consistent change in the quail adaptations occurred at position N170D (immature protein, 160 with H3 numbering), Table 2.

This virus was then tested in chickens to determine if these 10 amino acid changes supported transmission. This qa-mall/178<sub>2</sub> was able to infect chickens however transmission to contact cage mates was not observed, implicating the role of changes in the internal genes on transmission to chickens.

**Table 1. Transition of fecal-oral to respiratory shedding of qa-mall/178 and ch-qa/178 H2N2 viruses in chickens**

Species	Virus	Titer (EID <sub>50</sub> /ml)		
		Tracheal (swab)	Cloacal (swab)	Lung
Chicken	qa-mall/178	< 1*	2.0 x 10 <sup>5</sup> ± 1.0	< 1*
Chicken	ch-qa/178 <sup>+</sup>	1.6 x 10 <sup>3</sup> ± 1.4	9.0 x 10 <sup>1</sup> ± 0.5	3.9 x 10 <sup>3</sup> ± 0.5

\* < 1: below level of detection

+ Passage 4 in adaptation

**Table 2. Comparison of amino acid changes in qa-mall/178 (H2N2) and ch-qa/178 to the wild type mallard H2N2 virus.**

Gene	Amino acid changes (compared to mall/178)		
	<u>Qa-mall/178</u>	Ch-qa/178 <sup>a</sup>	<u>Qa-mall/178<sub>2</sub></u> <sup>b</sup>
PB2	A588V	A588V	R318K, S688F
PB1	Q268R, D398E, S654I	Q268R, D398E, S654I	
HA <sup>d</sup>	N170D	N170D, <b><i>K318Q</i></b>	<u><i>N170D</i></u> <sup>c</sup> , T203K, F403L, M424V
NP	A234T	A234T	V242A
NA		<b><i>deletion 54-81</i></b>	T171A, V231I, G235R

<sup>a</sup> All changed in quail adaptation maintained in chicken adaptation. Additional changes are italicized and bold

<sup>b</sup> 2<sup>nd</sup> adaptation was a completely independent adaptation of mall/178 H2N2 in quail

<sup>c</sup> Common changes between quail adaptations are italicized and underlined

<sup>d</sup> Based on immature protein

#### **3.4.4 Minimal changes necessary for transmission of mall/178 in quail and chickens**

The adaptation of mall/178 in quail resulted in the creation of a virus with transmissibility and extended host range, Figure 3. Sequence analysis indicated that only six amino acid changes were present in four gene segments, reviewed in Table 2. To fully understand the role each amino acid played in the ability for this qa-mall/178 virus to transmit in quail and to replicate and transmit in chickens we created viruses using reverse genetics that contain single gene reassortants in a 7:1 ratio to determine if the transmissibility in quail was multigenic. Viruses created are listed in Table 3. The PB2, PB1, HA and NP from the qa-mall/178 viruses were exchanged one at a time for the mall/178 gene in order to determine if a single gene from the quail-adapted virus was responsible for the transmission phenotype in quail. The reverse was also done to determine if exchanging out one gene in the qa-mall/178 backbone for wild type, mall/178, would eliminate irrelevant amino-acid mutations and highlight the minimal amino acids out of the six that are needed for transmission in quail. Note that the PA, NA, M and NS genes did not experience amino acid mutations during the adaptation of mall/178 H2N2 in quail.

**Table 3. List of reassortant viruses created for replication and transmission studies in quail and chickens.**

	PB2	PB1	PA*	HA	NP	NA*	M*	NS*
mall/178								
qa-mall/178								
Mall:Qa <sub>PB2</sub>								
Mall:Qa <sub>PB1</sub>								
Mall:Qa <sub>HA</sub>								
Mall:Qa <sub>NP</sub>								
Qa:Mall <sub>PB2</sub>								
Qa:Mall <sub>PB1</sub>								
Qa:Mall <sub>HA</sub>								
Qa:Mall <sub>NP</sub>								

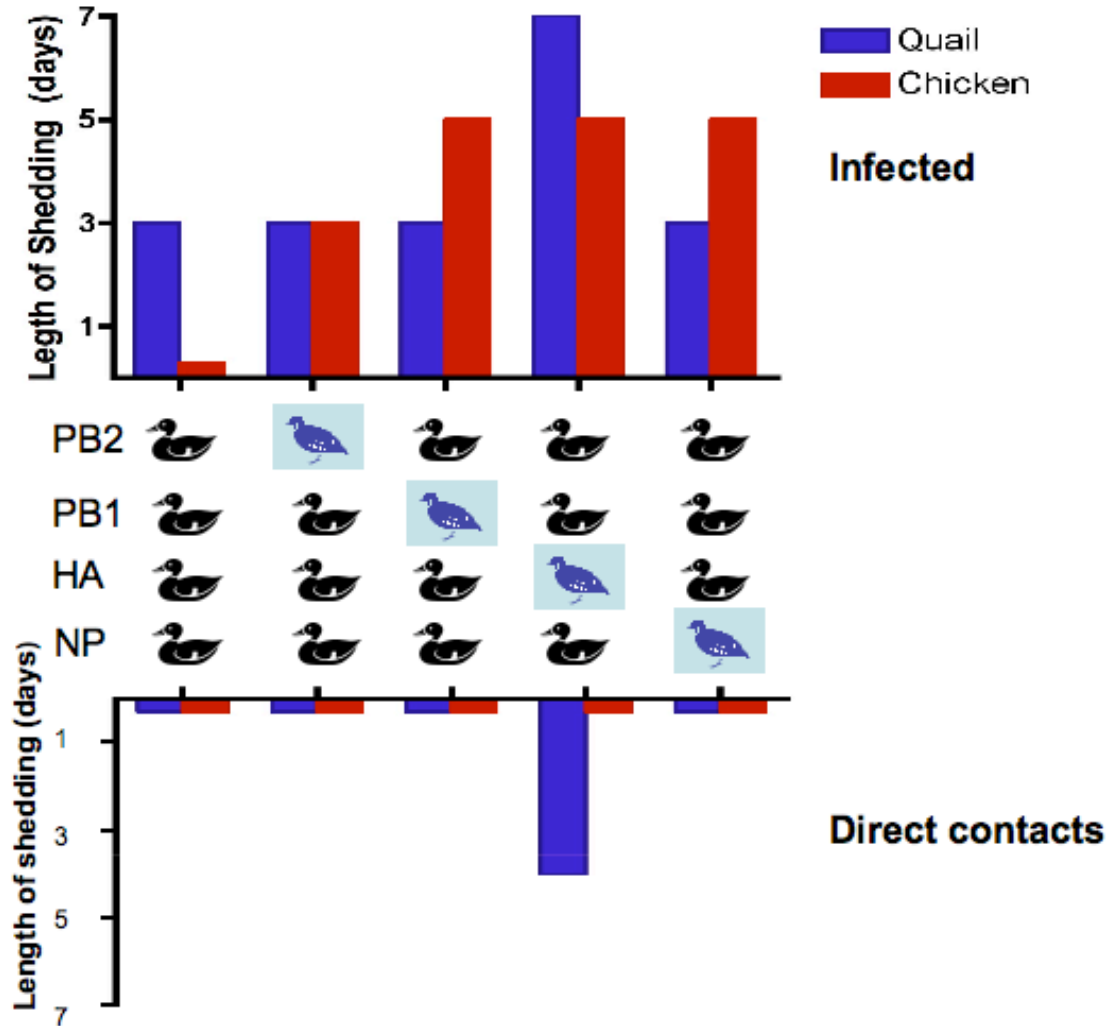
\* PA, NA, M and NS did not experience any amino acid changes during the adaptation.

Quail were infected as described above and sentinel contacts introduced one day post-infection. As shown in Figure 4, when given the mall/178 rescued virus only quail were infected and this virus did not transmit, similar to the wild type mall/178. Virus infection was supported in chickens and quail for 3 days when infected with Mall:Qa<sub>PB2</sub>. When given either the Mall:Qa<sub>PB1</sub> or Mall:Qa<sub>NP</sub> viruses replication and shedding were supported in quail and chickens for 3-5 days respectively, still no transmission was observed in either species for either viruses. However, when given Mall:Qa<sub>HA</sub> transmission was observed in quail. The contacts began shedding virus by day 3 p.i. and shed for at least 4 days, as indicated by the blue bar in the bottom graph. This same virus was unable to transmit in chicken indicating that all 6 amino acids are required for transmission in chickens, fully supporting the role quail played in this adaptation and host expansion in chickens.

When the reverse study was performed and genes from the qa-mall/178 were exchanged in a 7:1 ratio with the mall/178 virus we confirmed our finding that all 6 amino acid changes from the qa-mall/178 virus are necessary to support transmission in chickens, Figure 5. Many conclusions can be drawn from these studies however we feel confident in drawing 2 major conclusions. First, three amino acid changes, which occur on PB1 during adaptation, are not critical for transmission in quail when given the qa-mall/178 backbone. This virus, Qa:Mall<sub>PB1</sub>, is able to replicate and transmit in quail, however not to the extent of the full qa-mall/178. Sequence analysis of tracheal swabs collected from the positive contacts indicated no adaptive changes in the virus, confirming the ability of this reassortant to replicate and transmit

in quail without the three amino acid changes in the quail-adapted PB1. Second, when given Qa:Mall<sub>HA</sub> the virus is able to replicate and transmit in quail to the level of the full qa-mall/178 virus. Analysis of tracheal swabs from the contact quail indicated that a change from K166E instead of the observed N170D (156,160 respectively, in H3 numbering) change in adaptation. This same virus was inefficient in establishing an infection in chickens, compared to 3 or 5 days of shedding with other 7:1 viruses, indicating that the N170D change on the HA is crucial for establishing an infection in the chicken. Given the other five amino acids, the chicken cannot support the necessary mutation at or around 170, further confirming the need for quail in establishing this virus in chickens.

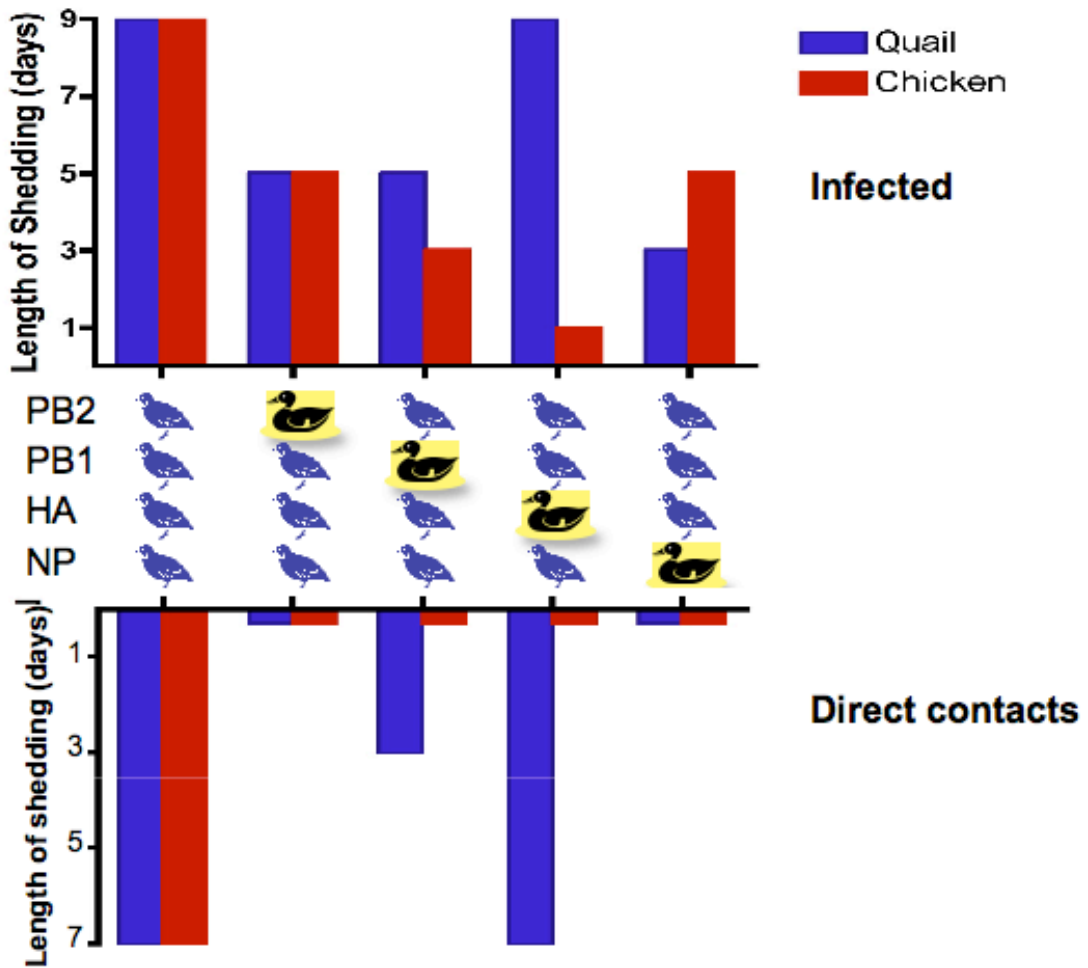
Interestingly the only change that was consistent in the two quail adaptations of mall/178, was the change at HA (N170D) Table 2. This second qa-adapted virus was able to replicate and transmit in quail, as efficiently and to the same extent as the original qa-mall/178 yet it was unable to transmit in chickens. Thus, the N170D change on HA is imperative for replication and transmission of the mall/178 virus in chickens and quail however it works in concert with additional changes to allow for the transmission of the qa-mall/178 in quail and in chickens.



**Figure 4. Minimal changes necessary for replication and/or transmission of mall/178 in quail and in chickens.**

Three birds were infected with virus and tracheal and cloacal swabs were collected days 1, 3, 5 and 7 pi. Swabs were grown in embryonated chicken eggs to determine virus presence. Blue bars represent viral shedding in quail (tracheal swabs) while red bars represent viral shedding in chickens (cloacal swabs).

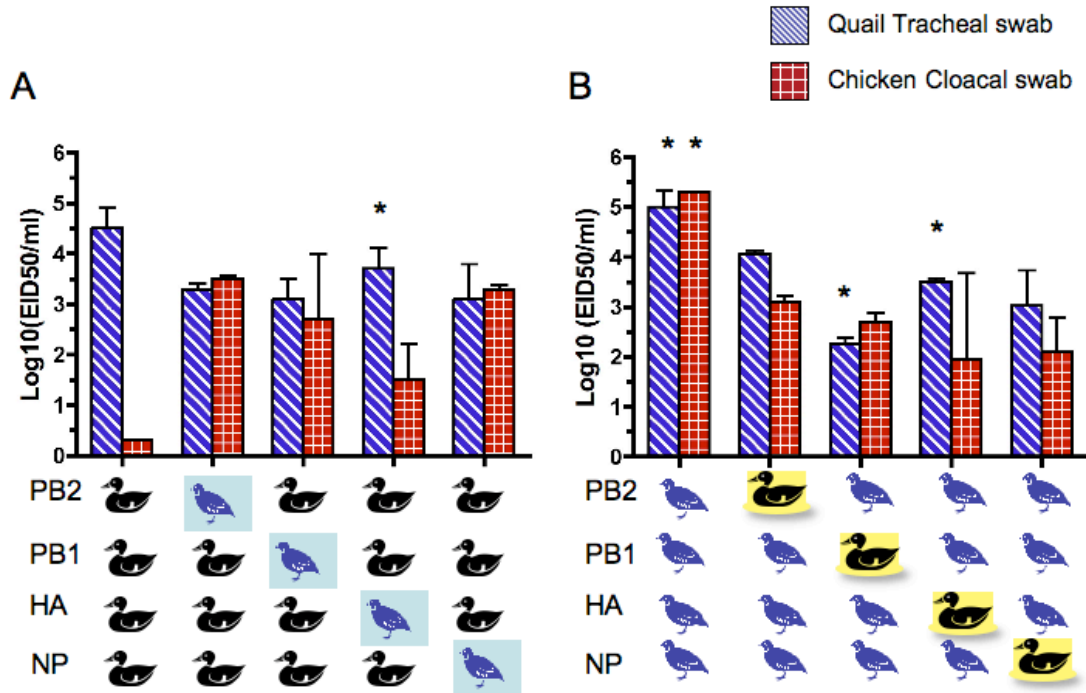




**Figure 5. Minimal changes necessary for replication and/or transmission of qa-mall/178 in quail and in chickens.**

Three birds were infected with virus and tracheal and cloacal swabs were collected days 1, 3, 5, 7 and 9 pi. Swabs were grown in embryonated chicken eggs to determine virus presence. Blue bars represent viral shedding in quail (tracheal swabs) while red bars represent viral shedding in chickens (cloacal swabs).

In order to determine if transmission of the reassortant viruses is dependent on the amount of virus shed from infected birds we collected tracheal (quail) and cloacal (chicken) swabs day 3 p.i., shown to be peak shedding for these viruses. As indicated in Figure 6A, the Mall:Qa<sub>HA</sub> which was able to transmit to contact quail shed peak titers at least 1 log lower than the rescued mall/178 virus, that did not transmit, and was similar to the other Mall:Qa 7:1 reassortants highlighting that the amount of virus shed is not necessarily a determining factor on whether a virus will transmit, at least for quail. Interestingly cloacal titers from chickens infected with the same viruses showed similar levels of viral shedding (exception Mall:Qa<sub>HA</sub>) with no transmission. The same is true for Qa:Mall 7:1 reassortant viruses, Figure 6B. Viruses that were able to transmit in quail (Qa:Mall<sub>PB1</sub> and Qa:Mall<sub>HA</sub>) were shed to peak titers less than the non-transmissible Qa:Mall<sub>PB2</sub>. Interestingly Qa:Mall<sub>PB1</sub> was the lowest virus shed in quail however still able to transmit to sentinels day 3 p.i. We cannot conclude whether viral titers shed are important for transmission in chickens since the only virus able to transmit was the full qa-mall/178. However, we can conclude with some certainty that given these viruses, transmission in quail appears to be more dependent on key combinations of genes rather than the amount of virus shed.



**Figure 6. Viral titers shed in quail and chickens with mall/178 and qa-mall/178 rescued viruses.**

Tracheal (quail) and cloacal (chicken) swabs collected from Mall:Qa 7:1 (A) and Qa:Mall 7:1 reassortant viruses (B) on day 3 p.i. Asterisks indicate transmission of the virus to sentinel cage mates.

### 3.4.5 Changes in tissue tropism, role of NA deletion

Qa-mall/178 replicates and transmits efficiently in a fecal-oral route in chickens. To determine whether this virus could further adapt within this host and replicate in the upper respiratory tract of chickens we continued adapting the qa-mall/178 in chickens. After only 4 passages we isolated virus in the lung and sequence analysis identified a 27 amino-acid deletion in the stalk region of the NA, listed in Table 2. Three additional passages confirmed the stability of this stalk deletion, as no additional changes were observed through passage 7, ch-qa/178.









































The ability of this deletion to support viral replication in the lung was investigated. We infected chickens and tested for transmission and viral titers in the lungs at day 3 pi. Mall/178 was not able to transmit, as previously reported, and was not isolated in the lungs. Qa-mall/178 was able to be isolated from 1 of 3 infected chickens' lungs, in a relatively low titer and was transmitted to contact chickens via fecal-oral. Ch-qa/178 replicated and transmitted and virus was isolated in all lungs collected at a 6-fold increase to the qa-mall/178 virus, Table 4A. The ch-qa/178 virus transitioned from a fecal-oral to a respiratory-transmitted virus as supported by a 3 fold increase of viral titers in tracheal swabs and a 4 fold decrease in cloacal swabs, Table 1 (Sorrell and Perez, 2007). To clearly assign the NA deletion responsibility for the observed change in viral replication, we created 7:1 viruses, rescuing the ch-qa/178 NA in the mall/178 and qa-mall/178 backbones. Virus was isolated in the lungs of chickens infected with the mall:ChNA virus, three logs higher than that of

the mall/178, however the NA alone was not able to support transmission in chickens, Table 4A. When rescued in the qa-mall/178 backbone the chicken NA increases virus load in the lungs by roughly 5 fold, as compared to the qa-mall/178 virus, indicating that the chicken-adapted NA deletion alone accounts for at least 5 of the 6 fold increase in virus presence and replication in the chicken lung.

The change in tissue tropism correlates to a change in replication temperature. The intestinal shedding and replication of typical avian viruses is supported at a range of 40-42<sup>0</sup>C compared to that of 35-37<sup>0</sup>C in a respiratory infection. In order to determine the role of the stalk deletion in temperature phenotype we used the rescued ch-qa/178 virus in CEK plaque assays and compared them to mall/178, qa-mall/178 viruses (not shown) at temperatures 35<sup>0</sup>C and 42<sup>0</sup>C. Plaques in Table 4B indicate that the chicken-adapted virus at 42<sup>0</sup>C diminishes replication and plaque formation at this higher temperature, whereas there is an increase in plaque size and number at 35<sup>0</sup>C, corresponding to virus isolation in lungs rather than the intestine.

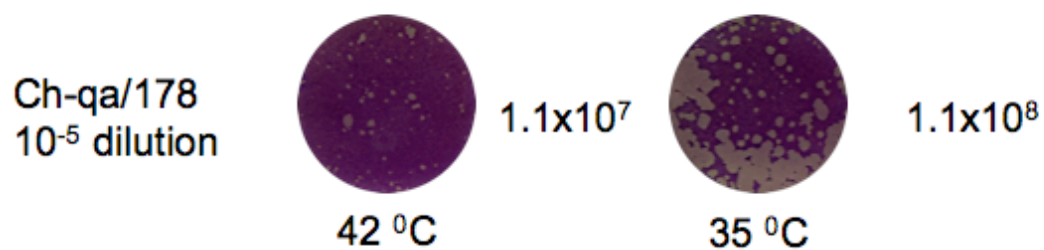
Table 4. Ability of the N2 deletion to favor replication in the respiratory tract of chickens.

**A**

	PB2	PB1	PA*	HA	NP	NA	M*	NS*	Transmission (in Chickens)	Lung Titer Log <sub>10</sub> (EID <sub>50</sub> /ml)
mall/178									No	< 1
qa-mall/178									Yes	1.1 ± 0 <sup>+</sup>
ch-qa/178									Yes	7.15 ± 0.18
mall: ch <sub>NA</sub>									No	3.3 ± 1.3
qa: ch <sub>NA</sub>									Yes	5.9 ± 0.76

\* PA, M and NS no amino acid changes during adaptation  
 + only one positive lung sample

**B**



### 3.5 Discussion

These findings support the role of quail in the adaptation of mallard influenza viruses. The adaptation did not increase the pathogenicity of the virus in quail however the virus gained the ability to transmit very efficiently in quail and chickens. Thus, our results suggest that the adaptation of a mallard virus in quail could potentially lead to the emergence of influenza strains that can cross the species barrier a second time and that these viruses can cause disease symptoms in other animal species. Additional studies revealed that the qa-mall/178 and ch-qa/178 viruses did not lose their ability to transmit in mallard ducks quail, respectively; a finding that is consistent with observations that adaptation of influenza viruses from waterfowl in terrestrial birds lead to viruses with broader host ranges (Perez et al., 2003a).

It is also important to note that the changes which, occurred during adaptation in quail, were maintained in 7 passages of the virus in chickens. This supports the notion that these changes are biologically relevant in the adaptation of aquatic avian influenza in terrestrial bird species. The results from reassortant studies are consistent with the notion that changes on the surface genes alone are not sufficient for transmission of avian influenza in chickens (Makarova et al., 2003; Perez et al., 2003a; Perez et al., 2003b). Transmission in the chicken was only supported when all 4 quail-adapted genes were present, proving the adaptation in quail was necessary to gain replication and transmission in chickens. Amino acid 170 (160, H3 numbering)

appears to be crucial in transmissibility of the mall/178 in quail, enough so that when given all other adaptive changes the quail are able to foster a change at 166 (156 in H3 numbering), three amino acids upstream from the original mutation. This amino acid is located close to the receptor-binding site. The adaptive mutations in PB2 and NP appear necessary for transmission in quail however, they alone cannot support transmission. The change in PB2 at 588 has been reported as prone to adaptive changes in other species and it is located in a domain that interacts with NP. The change in NP is located in a NP-NP interaction domain, 20 amino acids away from the NP-PB2 interaction domain (Naffakh et al., 2008).

A second adaptation of A/mallard/Potsdam/178-4/83 (H2N2) virus in quail led to a virus that after five passages of lung homogenate, replicated longer and transmitted efficiently in quail. This confirms the initial adaptation experiments were valid and that adaptation of mall/178 in quail is reproducible. The resulting qa-mall/178<sub>2</sub> however had a different set of adapted mutations, except for the N170D change in the HA protein, and was not able to support efficient transmission in chickens, indicating the complexity of adaptation and establishment of lineages in non-natural hosts. We show that even within the same strain adaptive mutations can vary drastically and that there is no categorical way in which mallard viruses adapt in intermediate hosts to expand host range. This is not to say that this qa-mall/178<sub>2</sub> cannot extend its host range to other terrestrial avian species, however it was not investigated in this study. Regardless, this repeat adaptation does point to critical



adaptive mutations within the internal proteins, which occurred during the initial adaptation that support transmission in chickens.

Many papers have investigated the biological importance of the neuraminidase (NA) stalk in influenza infection and release. The antigenic structure and structure-function relationship of the NA head have been studied (Air et al., 1987; Webster et al., 1982) however information regarding the stalk region is limited. Reports that stalk length plays a role in replication and viral release have been published (Baigent and McCauley, 2001; Castrucci and Kawaoka, 1993) however there is little information of what role the deletion plays in pathogenesis, transmission and host range. Stalk deletions have been tied to host adaptation of mallard viruses to domestic poultry, including chickens and quail (Banks et al., 2001; Hossain et al., 2008) however, variation in the stalk region is not correlated to host immune pressure (Laver, 1978). The number and sequence of the stalk regions vary, even within subtypes (Colman, 1989). *In vitro* and *in vivo* studies have linked stalk length to the ability to release erythrocyte-bound viruses and growth in eggs (Castrucci and Kawaoka, 1993; Els et al., 1985) however a mutant lacking the entire NA stalk was viable and replicated to titers comparable to the parent virus in tissue culture (Castrucci and Kawaoka, 1993) still the effect of stalk deletion in an animal model remains undefined. Viruses carrying NA deletions without additional glycosylation sites are typically new introductions into poultry and evolve into lineages when mutations are fixed (Fitch et al., 1997) ie) when the viruses have acquired glycosylation sites as previously described . Our NA deletion supports the theory of

viral adaptation in chickens and indicates a mechanism, still undefined, allows changes in tropism and temperature phenotypes.

In summary, minor changes are responsible for the modification in the transmission phenotype of the mallard H2N2 virus in quail and chickens, however these minor changes supported through adaptation in quail are critical for replication and transmission of this strain in chickens. Further adaptation of this virus in chickens leads to the establishment of an upper respiratory infection, NA deletion and virus stability.

To avert the emergence of pandemic avian influenza viruses a better understanding of the role of potential intermediate hosts is crucial. There is no question as to whether terrestrial birds can become intermediate hosts for influenza strains that have the potential to cross to humans. In the past few years, numerous outbreaks in Asia and Europe resulting in direct transmission of avian influenza viruses from domestic poultry to humans highlight the need to better understand the role that terrestrial birds play in the ecology of avian influenza viruses. Poultry production is rapidly increasing worldwide, although this increase is not necessarily accompanied by improvements in biosecurity or quality of poultry farming. Previous studies and those presented above portray a picture for quail as an important intermediate host for influenza (Makarova et al., 2003; Perez et al., 2003a). Quail are, in general, more susceptible to infection with avian influenza, yet more resistant to disease than other bird species, which places them as an ideal candidate for the

spread of avian influenza viruses from the aquatic reservoir to other avian and mammalian species. The role of this bird species in the ecology of influenza needs to be better understood.

# **Chapter 4: Replication and Transmission of Avian H9N2 Influenza A viruses in Ferrets: Evaluation of Pandemic Threat**

## 4.1 Abstract

H9N2 avian influenza A viruses are endemic in poultry of many Eurasian countries and have caused repeated human infections in Asia since 1998. To evaluate the potential threat of H9N2 viruses to humans, we investigated the replication and transmission efficiency of H9N2 viruses in the ferret model. Five wild-type (WT) H9N2 viruses, isolated from different avian species from 1988 through 2003, were found to replicate in ferrets; however these viruses achieved mild peak viral titers in nasal washes when compared to those observed with a human H3N2 virus. Two of these H9N2 viruses transmitted to direct contact ferrets, however no aerosol transmission was detected in the virus displaying the most efficient direct contact transmission. A leucine (L) residue at amino acid position 226 in the hemagglutinin (HA) receptor-binding site (RBS), responsible for human virus-like receptor specificity, was found to be important for the transmission of the H9N2 viruses in ferrets. In addition, an H9N2 avian-human reassortant virus, which contains the

surface glycoprotein genes from an H9N2 virus and the six internal genes of a human H3N2 virus, showed enhanced replication and efficient transmission to direct contacts. Although no aerosol transmission was observed, the virus replicated in multiple respiratory tissues and induced clinical signs similar to those observed with the parental human H3N2 virus. These findings suggest that the establishment and prevalence of H9N2 viruses in poultry pose a significant threat for humans.

## 4.2 Introduction

### **4.2.1 H9N2 influenza A viruses, a pandemic threat**

H9N2 avian influenza viruses were first isolated in the United States in 1966 (Homme and Easterday, 1970). Since then, H9N2 influenza viruses have been isolated from wild ducks and have caused outbreaks in turkeys, however there have been no reports of H9N2 in chickens in the U.S. (Halvorson, 1997; Kawaoka et al., 1988b; Sharp et al., 1997; Sharp et al., 1993). In Asia, H9N2 influenza viruses were only isolated in apparently health ducks in the few surveillance studies performed during 1975-1985 in live poultry markets and farms in Hong Kong, however, this subtype gained prevalence in domestic poultry by the early 1990s (Shortridge, 1992). Surveillance studies performed in live poultry markets in Hong Kong in December of 1997 indicated that H9N2 viruses were the second most commonly isolated AI virus,

accounting for 4% of infected poultry, mainly chickens (Shortridge, 1999).

Concurrent with this observation, an H9N2 endemic developed with cases reported in Korea, Asia, Saudi Arabia, Pakistan, India, South Africa, Germany, Italy and Ireland (Alexander, 2003; Cameron et al., 2000; Lee et al., 2000; Naeem et al., 1999; Nili and Asasi, 2002; Perk et al., 2006). Two lineages had established themselves in domestic poultry in Asia, A/Duck/Hong Kong/Y280/97 and A/Quail/Hong Kong/G1/97 (Guan et al., 1999; Li et al., 2003; Peiris et al., 2001). In particular, A/Quail/HK/G1/97 is thought to have been involved in the generation of the highly pathogenic H5N1 virus of 1997. 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 indicated that swine were not intermediates in avian to human transmission indicating land-based poultry as the source of H9N2 internal genes that transmitted to humans (Makarova et al., 2003; Shortridge et al., 1998; Webby and Webster, 2001). The H9N2 Y280-like and G1-like lineages, established and dominant in terrestrial poultry, are predicted to have an affinity for human receptor binding; providing evidence of a stable avian lineage with human virus-like receptor specificity. These viruses have the ability to infect different poultry species including quail, chicken and pheasant (Matrosovich et al., 2001), indicating 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). This virus was reportedly transmitted via aerosol in the poultry markets (Guan et al., 2000).

The threat of human infection was concerning but had not been reported. In August of 1998, H9N2 influenza had been isolated in swine, again raising the concern of possible transmission to humans. By July and August that same year, H9N2 had been isolated from five patients with influenza-like illness in Guangdong province of China (Guo et al., 1999). By March 1999, a new pandemic threat arose when H9N2 influenza infected two children in Hong Kong. The isolates were similar to a Hong Kong quail isolate (Lin et al., 2000) and no known link between the humans cases concluded that the infection most likely resulted from direct transmission from infected birds.

Phylogenetic analysis of the H9N2 influenza viruses isolated in domestic ducks in southern China during the 2000-2001 season indicated that this H9N2 lineage transmitted from domestic poultry back to the ducks, creating double and sometimes triple resident reassortants in the ducks (Li et al., 2003). This created 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 viruses with pandemic potential. In a Hong Kong surveillance study carried out between 2001-2003, the H9N2 subtype was found to be the most prevalent subtype regularly isolated from different types of poultry (Choi et al., 2004; Li et al., 2003; Liu et al., 2003b). In November 2003, an avian H9N2 influenza virus was isolated from a child in Hong Kong, closely related to Y280-like genotype. Previous studies performed in southern China highlighted that at least 2% of the human population was seropositive for H9 (Guo et al., 1999; Peiris et al., 1999). In

addition, during a random screening test 2.5% of serum samples from voluntary donors tested positive for neutralizing antibodies to Dk/HK/Y280/97 virus (Butt et al., 2005). These direct infections with avian H9N2 confirm that interspecies transmission of H9N2 influenza from avian species to mammalian hosts occurs and it is not uncommon. Because H9N2 viruses are not pathogenic for poultry or humans, the extent of infection is likely to remain underappreciated and go unrecognized and unreported.

Reassortment between the current human epidemic strain and an avian virus of a different subtype is postulated for the generation of the next pandemic strain. Given the receptor specificity of H9N2 viruses, they appear to have the potential to cross the species barrier more easily and often than H5N1 strains and their repeated introduction in humans increases their opportunity for reassortment and/or adaptation for human-to-human transmission. Two previous pandemic strains, H2N2 in 1957 and H3N2 in 1968, were derived from low pathogenic avian strains reassorting with the circulating human strain (Horimoto and Kawaoka, 2001). However the question remains, with human receptor specificity and direct infections possible what is missing for this virus to transmit human-to-human and possibly lead to the next pandemic.



#### 4.2.2 Ferrets: A model for human influenza

The impact of influenza viral infections on public health is widely recognized (Meltzer et al., 1999). Influenza epidemics differ from year to year and transmission patterns are poorly understood. The rate and behavior of a virus during its evolution to human transmission is not well known.

Use of a ferret model to study influenza virus transmission creates an environment to observe controlled serial transmission, impossible to do in a complex human epidemic where persons infected in the same community shed viruses with sequence diversity (Herlocher et al., 2001). Ferrets provide a population to study the interplay of transmission with infection, illness and sequence variability. The ferret also displays physical characteristics that allow easy identification and characterization of clinical symptoms. Although a mouse model has been used in the past to study influenza infections (Schulman, 1968; Schulman and Kilbourne, 1962), the mouse is not a natural host and therefore limits the information available. Nonhuman primate models have also been successfully used to study human influenza infections (Renegar, 1992; Rimmelzwaan et al., 2001) however their size, regulations, cost and housing needs provide obstacles in their regular use.

Ferrets (*Mustela putorius furo*) became a model of influenza in 1933 when influenza-induced rhinitis was observed (Smith and Sweet, 1988). Since then, ferrets have been extensively used for studying various effects of human influenza (Barber

and Small, 1978; Fenton et al., 1981; Smith and Sweet, 1988; Yetter et al., 1980). Ferrets are naturally susceptible to human influenza virus types A and B and their disease response to infection widely resembles that of humans. The relative virulence of certain influenza A viruses was shown to be similar in human and in ferret infections (Campbell et al., 1979; Maassab et al., 1982). Ferrets are also susceptible to avian, equine and swine influenza A viruses, although only swine and human A viruses induce febrile illnesses in ferrets (Hinshaw et al., 1981; Marois et al., 1971). The ferret is also a good model for studying influenza tissue tropism because the swine, equine and avian influenza viruses replicate in the intestinal wall as well as the respiratory tract (Renegar, 1992).

Ferrets and humans display similar clinical symptoms of infection. The clinical symptoms that typically appear, usually restricted to the upper respiratory tract, are rapid onset of sneezing, nasal discharge, malaise and pyrexia (Herlocher et al., 2001; Renegar, 1992; Smith and Sweet, 1988). Variability of symptoms, like in humans, is a result of the strain of the virus, environmental conditions, the presence and degree of secondary infections and age of the ferret. During infection, transmission to other ferrets and humans is possible with aerosol droplets being the main mode of transmission (Haff et al., 1966; Herlocher et al., 2001; Renegar, 1992; Smith and Sweet, 1988).

The virus selectively infects ciliated respiratory epithelium like it does in humans. Influenza attaches to the sialic acid via an  $\alpha$ 2,6-glycosidid linkage

(Herlocher et al., 2001) and preferentially attach to ciliated cells in ferret tracheal epithelium. Regardless of ferret age, virus attaches to ciliated cells 77-87% of the time while only 1-9% binds to nonciliated cells (Piazza et al., 1991). Ferrets are also susceptible to influenza viruses that preferentially bind in the  $\alpha$ 2,3 conformation indicating that ferrets can be susceptible hosts for a wide range of avian influenza viruses.

#### **4.2.3 The role of amino acid 226 in host receptor specificity**

The HA residue at position 226 has been implicated in determining the receptor specificities of H1N1, H3N2 and H9N2 viruses (Connor et al., 1994; Matrosovich et al., 2001; Matrosovich et al., 2004; Rogers and Paulson, 1983; Vines et al., 1998). This residue at 226 does not directly contact the sialosides but a glutamine to leucine mutation has been shown to alter RBS conformation. Amino acids 183 and 190 in the HA receptor binding pocket also interact with cell surface receptors and are usually found in two combinations; H/E or N/A respectively. The H/E combination has been identified as important for adaptation to humans and is found in many 2,6-binding avian H9N2 viruses. Numerous avian H9N2 influenza isolates contain L-226 in the RBS of their HAs (Choi et al., 2004; Li et al., 2005; Li et al., 2003; Peiris et al., 2001) and preferentially binding receptors with sialic acid (SA) linked to a galactose by  $\alpha$ 2,6 linkage (SA $\alpha$ 2,6Gal) (Matrosovich et al., 2001); traits of human H3 viruses and elements that make these particular viruses steps closer to

efficient human-to-human transmission. The acquisition of L226 in avian H9N2 viruses is thought to have occurred in avian hosts preceding human infections (Peiris et al., 2001). There is however limited support for the enabling role of L226 in human infections of H9N2 viruses. Previous work in our lab has shown the effects of L226 in cell tropism and growth of avian H9N2 influenza A viruses in human airway epithelial (HAE) cultures, regardless of the amino acid combination at 183 and 190 (Wan and Perez, 2007). Therefore this key, preliminary *in vitro* work demonstrated that L226 causes H9N2 viruses to preferentially bind to and infect nonciliated cells ( $\alpha$ 2,6-containing cells) and grow more efficiently in HAE cultures, than those strains containing G226, highlighting the possible implications of this amino acid for infection and sustainability in humans. Additional *in vivo* studies in ferrets showed that a mutation in the Q226-containing viruses to L226 increased replication efficiency and supported partial transmission to direct contact ferrets. The reverse change from L226Q brought shedding down to minimal detection levels and completely abolished transmission to direct contact ferrets highlighting that this single amino acid change in avian H9N2 viruses alters transmission phenotype. Glycan microarray studies complimented the ferret work indicating a major preference for  $\alpha$ 2,6 sialosides in L226-containing viruses (Wan et al., 2008).

### 4.3 Materials and Methods

#### **4.3.1 Virus isolation and propagation**

The wild-type (WT) viruses used in this study, including five avian H9N2 viruses, one avian H2N2 virus and one human H3N2 virus, are listed in Table 5. The viruses were kindly provided by Robert G. Webster from St. Jude Children's Research Hospital, Memphis, TN, and by Ilaria Capua from the OIE, FAO and National Reference Laboratory for Avian Influenza and Newcastle Disease, Padova, Italy. The recombinant A/Guinea fowl/Hong Kong/WF10/99 (H9N2) (RGWF10), A/Quail/Hong Kong/A28945/88 (H9N2) (RGQa88) and A/Memphis/14/98 (H3N2) (RGM98) viruses, were recovered using reverse genetics as previously described in Chapter 3, section 3, subsection 6. In addition, an H9N2 avian-human reassortant virus, 2WF10:6M98, which contains the hemagglutinin (HA) and neuraminidase (NA) genes of RGWF10 and the six internal genes of RGM98, was also recovered. Cloned genes were sequenced and compared to the corresponding viral sequences to confirm that the clones did not carry spurious mutations, as previously described in Chapter 3, section 3, subsection 6. Recovery of the virus was verified by sequencing the reassortant's full-genome. All of the work and handling of this virus was performed in a USDA-approved biosafety level 3+ containment facility. Viruses were propagated in 10-day-old embryonated chicken eggs or Madin-Darby canine kidney (MDCK) cells. The median tissue culture infectious dose (TCID<sub>50</sub>) of each virus was determined in MDCK cells. Primary chicken kidney cells were used for the RGQa88

and mQa88 due to increased susceptibility of these cells for these two viruses (Wan and Perez, 2007).

#### **4.3.2 Plaque assays**

Viruses were examined by plaque assay in MDCK cells as described previously in chapter 3, section 3, subsection 7 (Wan and Perez, 2007).

**Table 5. Wild-type influenza viruses used for replication and transmission studies in ferrets**

<u>Virus</u>	<u>Subtype</u>	<u>Abbreviated Name</u>	<u>Residue at 226</u>
A/Quail/Hong Kong/A28945/88	H9N2	RGQa88 <sup>a</sup>	Q
A/Duck/Hong Kong/Y280/97	H9N2	Dk/HK/Y280/97	L
A/Chicken/Hong Kong/SF3/99	H9N2	Ck/HK/SF3/99	L
A/Guinea fowl/Hong Kong/WF10/99	H9N2	RGWF10 <sup>a</sup>	L
A/Chicken/Jordan/554/03	H9N2	Ck/Jordan/554/03	Q
A/Mallard/Potsdam/178-4/83	H2N2	Mall/178	Q
<u>A/Memphis/14/98</u>	<u>H3N2</u>	<u>RGM98<sup>a</sup></u>	<u>L</u>

<sup>a</sup> Viruses generated by reverse genetics

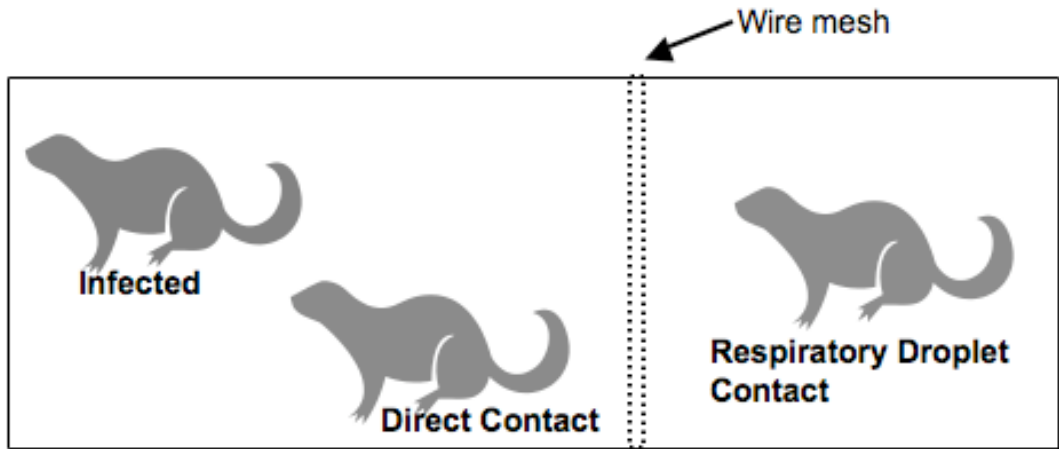
#### 4.3.3 Infection and transmission studies in ferrets

Female Fitch ferrets, 3 to 6-months old, were purchased from Triple F Farms (Sayre, PA). Prior to infection, ferrets were housed in a BSL2 facility and monitored for 5 to 7 days to measure body weight and establish baseline body temperatures. A subcutaneous implantable temperature transponder (Bio Medic Data Systems, Seaford, DE) was placed in each ferret for identification and temperature readings. Temperatures were recorded daily and fevers were defined as 3 standard deviations above the baseline reading. Three days prior to infection, blood was collected and serum tested for antibodies using the hemagglutination inhibition (HI) assay. Ferrets with HI titers at or lower than 10 were considered “influenza A-free” and were used in the study.

Ferret studies were performed in a BSL3+ facility in HEPA-filtered isolators. Studies were conducted under guidelines approved by the Animal Care and Use Committees of the University of Maryland and the Centers for Disease Control and Prevention. The basic set-up consisted of three ferrets: one infected, one direct contact and one aerosol contact. Ferrets were housed in wire cages placed inside isolators. Ferrets were lightly anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg) via an intramuscular injection and inoculated intranasally (i.n.) with  $10^6$  TCID<sub>50</sub> of virus in PBS, 250 µl per nostril. Twenty-four hours later, two naïve ferrets were introduced into the isolator. One (direct contact) was introduced into the same



cage as the infected ferret while the other (aerosol contact) was placed in a cage separated from the infected ferret by a wire mesh. The wire mesh prevented physical contact between the aerosol and infected/direct contact, allowing only air to be shared between the ferrets, Figure 7. All materials inside the cage of the inoculated ferrets were removed and replaced before introducing the direct contacts in order to ensure transmission occurred through contact with the inoculated ferrets and not infected/contaminated materials in the cage. Individual body temperatures, weights and clinical symptoms were measured daily and ferrets were scored. To monitor viral shedding, nasal washes were collected daily for up to two weeks. Briefly, ferrets were anesthetized as described above, and 1ml of PBS was used to induce sneezing. The nasal washes were collected into Petri dishes and brought to a total volume of 1ml with PBS. The nasal washes were immediately tested for virus using the FLU DETECT™ Antigen Capture Test Strip (Synbiotics Corp., San Diego, CA) and additional aliquots were stored at  $-80^{\circ}\text{C}$  before performing  $\text{TCID}_{50}$  titration in MDCK cells. At day 14 post-infection (pi) and post-contact (pc), blood was collected and seroconversion was determined by HI assay.



**Figure 7. Cage system for direct and respiratory droplet transmission studies in ferrets.**

One ferret is infected with  $10^6$  TCID<sub>50</sub>/ml of virus, twenty-four hours later bedding, food and water are replaced and a direct contact is placed in the same cage as the infected ferret allowing direct, physical contact while a second, respiratory droplet contact ferret, is placed in the same isolator but different cage. The only item shared between the respiratory droplet contact ferret and infected and direct ferrets is the air in the isolator, a wire mesh dividing the cages prevents physical contact for the respiratory droplet contact ferret.

#### **4.3.4 HI assays**

Serum samples were treated with receptor-destroying enzyme (Accurate Chemical and Scientific Corp., Westbury, NY) to remove nonspecific receptors and the anti-viral antibody titers were evaluated using the HI assay system outlined by the WHO Animal Influenza Training Manual (WHO/CDS/CSR/NCS/2002.5). HI assays were performed using homologous viruses as shown in the results section.

#### **4.3.5 Histopathology and tissue tropism**

Groups of two ferrets were inoculated with  $10^6$  TCID<sub>50</sub> of each virus, as described above. Ferrets were euthanized on day 4 pi. Brain, olfactory bulb, nasal turbinate, trachea, lung, heart and liver were collected and samples were both fixed with buffered neutral formalin for histological evaluation and stored at  $-80$  °C for virus titration. For histopathology, paraffin-embedded sections of 5- $\mu$ m thickness were cut and stained with H&E (Histoserv, Inc., Germantown, MD). Representative microscopic photos were taken with the SPOT ADVANCED software (Version 4.0.8, Diagnostic Instruments, Inc., Sterling Heights, MI). To determine the tissue distribution of the virus, 10% (w/v) of tissue homogenate was prepared with PBS and the viral titers were determined in MDCK cells.

## 4.4 Results

### **4.4.1 Replication and direct contact transmission of H9N2 viruses in ferrets**

Five WT H9N2 viruses isolated during the period of 1977 to 2003 were used in this study, Table 5. Three of these viruses, Dk/HK/Y280/97, Ck/HK/SF3/99, and Ck/Jordan/554/03 were field isolates. The remaining two, RGWF10 and RGQa88, were WT viruses rescued using reverse genetics. Sequencing analysis revealed that the HA of RGWF10, Dk/HK/Y280/97 and Ck/HK/SF3/99 contain L226 at the RBS, whereas RGQa88 and Ck/Jordan/554/03 contain Q226 (Wan and Perez, 2007).

To evaluate the replication and transmission of avian H9N2 viruses, we first determined whether H9N2 viruses could establish significant infections in the ferret model and whether these viruses could be transmitted to direct contact ferrets. For each virus tested, two ferrets were directly infected with  $10^6$  TCID<sub>50</sub> of virus (in the case of RGWF10, 3 ferrets were inoculated). At 24 h pi, a direct contact ferret was introduced into the same cage as each infected ferret. Ferrets were monitored as described in section 3 subsection 3. No overt signs of disease, including sneezing, were observed in any of the inoculated ferrets with any of the five WT H9N2 viruses used. However, lethargy and anorexia were noted in some cases, usually lasting 2 to 3 days. The inoculated ferrets experienced slight body weight loss (average <3%) Table 6. Transient elevation of body temperature (maximum elevation of 2.1°C) was detected in all RGWF10-infected ferrets and in at least one ferret from each of the

remaining H9N2 viruses. Temperatures were highest between 2 to 3 days pi, when a majority of the viruses were at peak shedding in nasal washes.

Virus was detected in the nasal washes from all of the inoculated ferrets, with peak titers ranging from 2.7 to 5.2 log<sub>10</sub>TCID<sub>50</sub>/ml, Figure 8, A-E. The highest peak titer was achieved in the Dk/HK/Y280/97 group while the lowest was in the RGQa88 group. For the RGWF10 group, virus was detected in all the inoculated animals from 1 to 5 days pi and was transmitted to all direct contact ferrets, as demonstrated by the detection of virus in nasal washes using FLU DETECT™ Antigen Capture Test Strip and viral titration in MDCKs. The direct contact animals began to shed detectable levels of virus by day 4 and 5 pc, and each shed virus for 4 to 6 days, with peak titers comparable to those found in the inoculated ferrets, Figure. 8A. Anti-H9 antibodies were detected in all ferrets, with hemagglutination inhibition (HI) titers of 1280 in the inoculated ferrets, and 320 to 640 in the contact ferrets, Tables 6 and 7. Virus was detected in both Dk/HK/Y280/97-inoculated ferrets for 6 days and transmitted to 1 of the 2 direct contacts, which shed virus for 5 days, Figure 8B. In the RGWF10 and Dk/HK/Y280/97 groups, the viral positive contacts exhibited lethargy, anorexia, weight loss and elevated temperature similar to the inoculated ferrets. The RGQa88, Ck/HK/SF3/99 and Ck/Jordan/554/03 groups shed viruses for up to 7 days and developed high titers (640-1280) of H9 antibodies, Table 6. However, neither virus shedding nor seroconversion was detected in any of the contact ferret, Figures 8C, D, E and Table 7, reflecting the lack of direct contact transmission. These results suggest that the ferret model is able to recapitulate the infection of H9N2 viruses as

observed in humans and pigs and that the ferret represents a good animal model to study the potential changes that could lead to efficient transmission of avian H9N2 viruses in humans.

**Table 6. Clinical signs, virus replication and seroconversion associated with wild type viruses in infected ferrets**

Virus	Infected Ferrets		
	Weight Loss (%) <sup>a</sup>	Sneezing (day of onset)	Serum (HI titer) <sup>b</sup>
RGWF10 <sup>c</sup>	1.8 ± 0.57	0/3	1280, 1280, 1280
RGWF10	2.3 <sup>d</sup>	0/2	2560, 2560
RGQa88	2.8 <sup>d</sup>	0/2	1280, 1280
Dk/Hk/Y280/97	1.55 ± 0.35	0/2	1280,1280
Ck/HK/SF3/99	1.55 ± 0.05	0/2	1280, 1280
Ck/Jordan/554/03	1.9 ± 2.1	0/2	640, 640
RGM98	6.54 ± 0.13	2/2 (3,3)	5120, 2560
2WF10:6M98	5.1 ± 0.85	2/2 (2,2)	2560, 2560

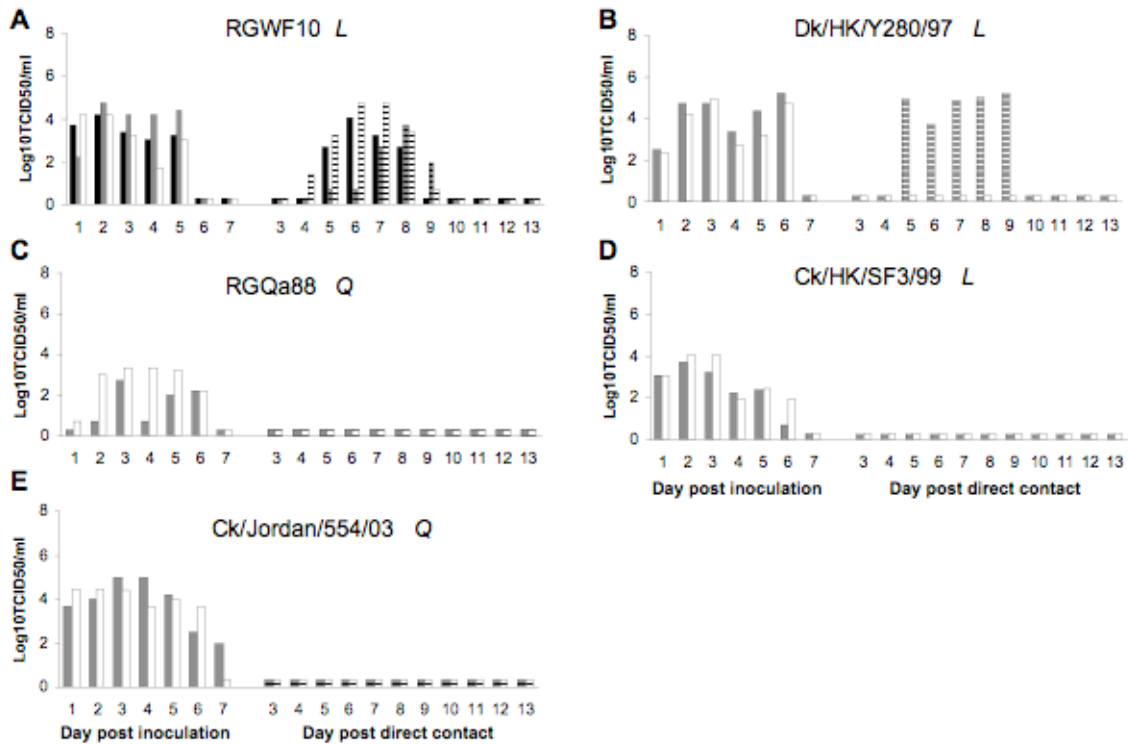
<sup>a</sup> Average body weight loss is shown ± standard error mean. <sup>b</sup> Homologous virus was used in HI assays to detect anti-H9 antibodies. <sup>c</sup> Two independent experiments were performed. <sup>d</sup> Only one ferret lost weight

**Table 7. Clinical signs, virus replication and seroconversion associated with wild type viruses in direct and respiratory droplet contact ferrets**

Virus	Direct Contact Ferrets			Respiratory Droplet Contact Ferrets		
	Virus in nasal wash <sup>a</sup>	Sneezing (day of onset)	Serum (HI titer) <sup>b</sup>	Virus in nasal wash <sup>a</sup>	Sneezing (day of onset)	Serum (HI titer) <sup>b</sup>
RGWF10 <sup>c</sup>	3/3	0/3	640, 320, 320	ND <sup>d</sup>	ND	ND
RGWF10	2/2	0/2	640, 1280	0/2	0/2	<10, <10
RGQa88	0/2	0/2	<10, <10	ND	ND	ND
Dk/HK/Y280/97	1/2	0/2	640, <10	ND	ND	ND
Ck/HK/SF3/99	0/2	0/2	<10, <10	ND	ND	ND
Ck/Jordan/554/03	0/2	0/2	<10, <10	ND	ND	ND
RGM98	2/2	2/2 (4,4)	5120, 5120	2/2	2/2 (8,10)	2560, 5120
2WF10:6M98	2/2	2/2 (4,5)	1280, 1280	0/2	0/2	<10, <10

<sup>a</sup> Virus in nasal wash analyzed by FLU DETECT™ Antigen Capture Test strip (Synbiotics Corp.) and titrated by TCID<sub>50</sub>. <sup>b</sup> Homologous virus was used in HI assays to detect anti-H9 antibodies. <sup>c</sup> Two independent experiments were performed. <sup>d</sup> ND, not done





**Figure 8. Replication and direct contact transmission of H9N2 viruses.**

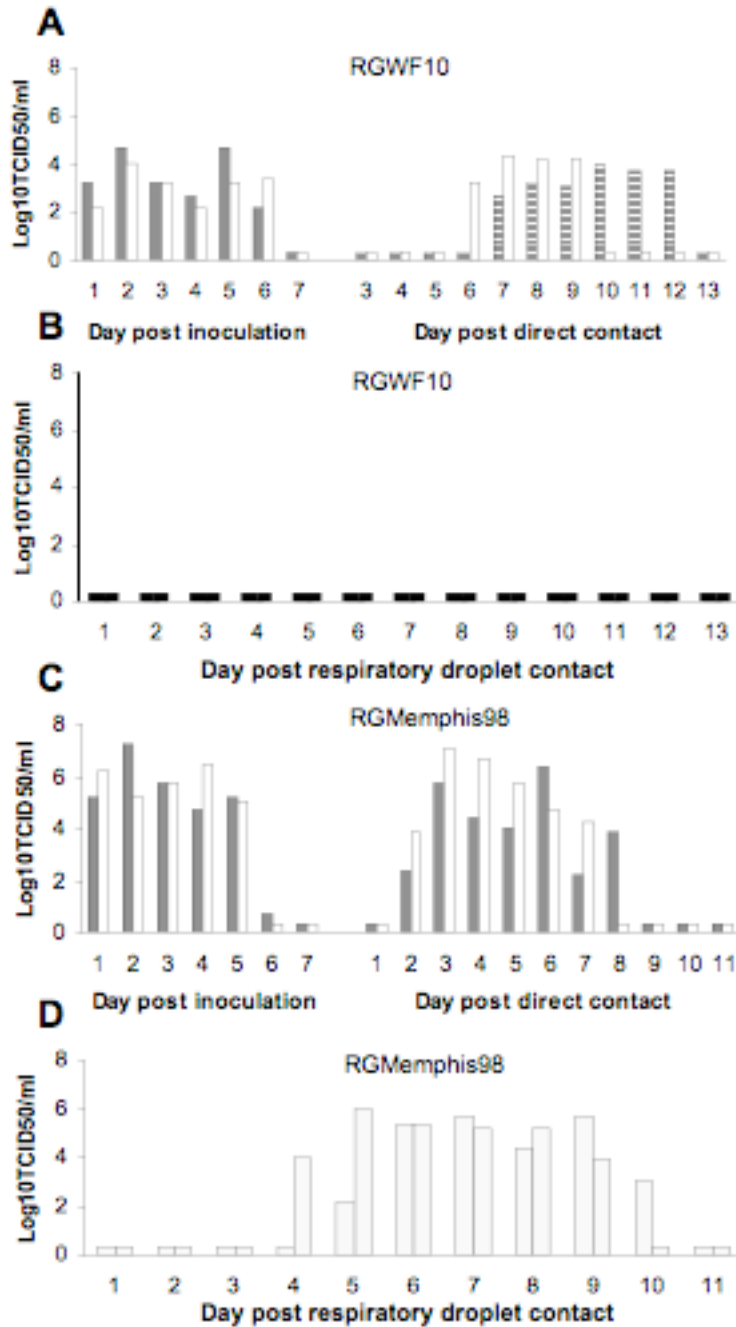
Ferrets were inoculated intranasally with  $10^6$  TCID<sub>50</sub> of H9N2 viruses RGWF10 (A), Dk/HK/Y280/97 (B), RGQa88 (C), Ck/HK/SF3/99 (D), and Ck/Jordan/554/03 (E). Twenty-four hours later, one naïve ferret (direct contact) was added to the same cage as each of the infected ferrets. Nasal washes were collected daily and were titrated in MDCK cells. Black, white and gray bars represent individual ferrets sampled and the amount of viral shedding at different days pi. The titers are expressed as log<sub>10</sub> values of TCID<sub>50</sub>/ml with the limit of detection at 0.699 log<sub>10</sub>TCID<sub>50</sub>/ml. The dotted line was arbitrarily set at  $< 0.3$  log<sub>10</sub>TCID<sub>50</sub>/ml in order to represent samples below the detection limit. *L* and *Q* correspond to L226 and Q226, respectively in the HA RBS.

#### **4.4.2 Lack of respiratory droplet transmission in the ferret model**

To test respiratory droplet transmissibility in ferrets we chose the RGWF10 virus due to its lineage and efficient transmission to direct contacts. This strain belongs to the A/Quail/Hong Kong/G1/97-like viruses, which closely resembles the virus isolated from the first human index case of H9N2 infection in 1999 (Lin et al., 2000; Peiris et al., 1999). Two ferrets were inoculated with RGWF10 virus and 24 h later, the direct contact and respiratory droplet contact ferrets were introduced, as previously described. No overt clinical signs of disease were observed in the inoculated ferrets; however, they displayed slight weight loss and transient elevation of body temperature, as noted with the initial study, Table 6. As shown in Figure 9A, viral shedding was detected in both inoculated and direct contact ferrets. By day 14 pi or pc, both the inoculated and direct contacts developed high titers of anti-H9 antibodies, however no viral shedding or seroconversion was detected in the respiratory droplet contacts, Figure 9B and Table 7, indicating the lack of respiratory droplet transmission.

To validate our system of detecting respiratory droplet transmission, we performed transmission studies with prototypic human and avian viruses, A/Memphis/14/98 (H3N2) (RGM98), and A/Mallard/Potsdam/178-4/83 (H2N2) (Mall/178). High titers of virus were detected in all ferrets in the RGMemphis98 group: inoculated, direct- and respiratory droplet-contacts, Figures 9C and D. The

respiratory droplet contacts began shedding virus by day 4 and 5 pc, respectively, and both shed virus for up to 6 days. All ferrets showed clinical signs including sneezing and developed high antibody titers against RGM98, Tables 6 and 7. For the Mall/178 group, the virus was shed from inoculated ferrets for 3 to 4 days. No viral shedding or seroconversion was detected in any of the direct or respiratory contact ferrets. Taken together, this experiment validated our transmission scheme and indicated that although some avian H9N2 viruses can infect and transmit to direct contact ferrets, they lack the properties to support respiratory droplet transmission.



**Figure 9. Respiratory droplet transmission of H9N2 and H3N2 viruses in ferrets.** Ferrets were inoculated i.n. with  $10^6$  TCID<sub>50</sub> of RGWF10 or RGMemphis98 virus. Twenty-four hours later, one naïve ferret was added to each infected ferret to serve as direct contact, and another ferret was placed into an adjacent cage separated by a wire mesh to serve as respiratory droplet contact. Nasal washes were collected daily and were titrated in MDCK cells. (A, C) infected and direct contacts. (B, D) respiratory droplet contacts.

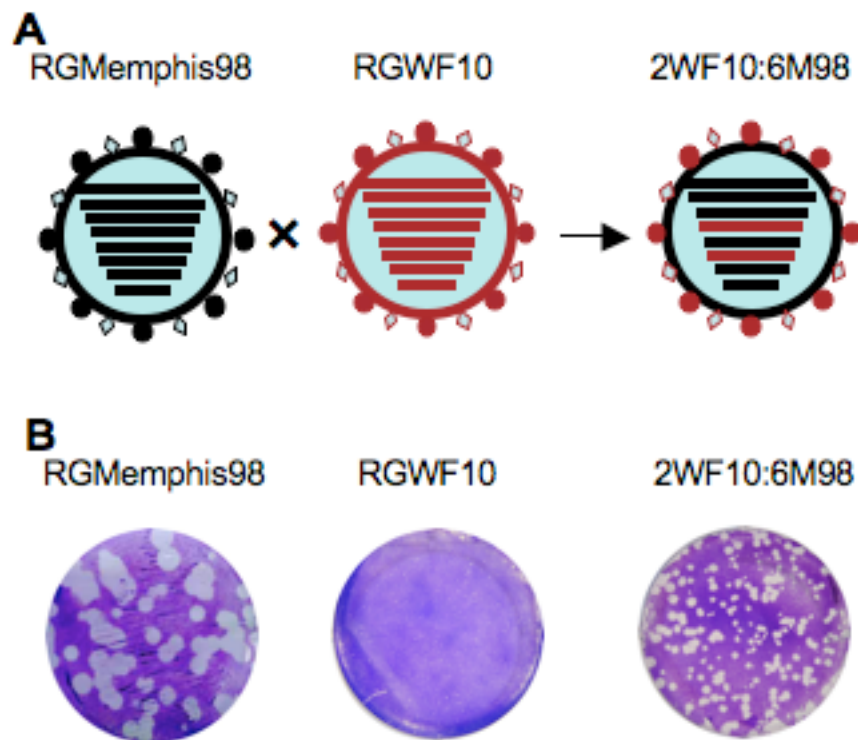
#### **4.4.3 Enhanced replication and transmission of an H9N2 avian-human reassortant virus in ferrets**

Two previous human influenza pandemics, in 1957 (H2N2) and in 1968 (H3N2), were the result of reassortment between low pathogenic avian influenza viruses and circulating human viruses of that time (Horimoto and Kawaoka, 2001; Kawaoka et al., 1989). Due to the multiple introductions of avian H9N2 viruses carrying human virus-like receptor specificity, L226, into the human population in the last decade, we wanted to determine whether an H9N2 avian-human reassortment would enhance the transmissibility of L226-containing H9N2 strains. Our previous reports have proven the L226 at the HA RBS directs cell tropism of avian H9N2 viruses in HAE cells *in vitro* as well as *in vivo* with studies highlighting the crucial role L226 plays for avian H9N2 viruses in conferring an  $\alpha$ 2,6 sialoside preference in glycan arrays and subsequent direct transmission in ferrets (Wan and Perez, 2007; Wan et al., 2008).

We recovered an H9N2 avian-human reassortant virus, 2WF10:6M98, containing the HA and NA genes of A/Guinea fowl/Hong Kong/WF10/99 (H9N2) and the six internal genes from A/Memphis/14/98 (H3N2), using reverse genetics, Figure 10A. The reassortant virus grew efficiently in MDCK cells, reaching a titer of  $8.7 \log_{10} \text{TCID}_{50}/\text{ml}$ , comparable to the titer of the parental H3N2 virus ( $8.4 \log_{10} \text{TCID}_{50}/\text{ml}$ ) and higher than the parental H9N2 virus ( $7.2 \log_{10} \text{TCID}_{50}/\text{ml}$ ),

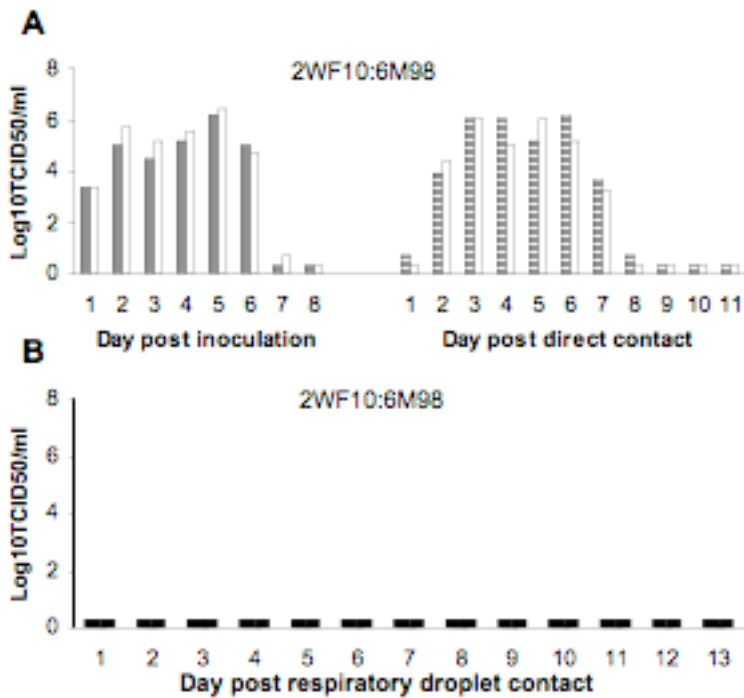
indicating good compatibility of the gene constellation for this reassortant virus. We also analyzed the growth phenotype of these viruses by plaque assay. We observed that the reassortant 2WF10:6M98 virus and the WT RGMemphis98 virus formed large, clear plaques, while the WT RGWF10 virus only produced pinpoint, less defined plaques, Figure 10B.

The replication and transmission of the 2WF10:6M98 reassortant virus was then investigated. Ferrets were inoculated with  $10^6$  TCID<sub>50</sub> of 2WF10:6M98 and the direct and respiratory droplet contacts were introduced at 24 h pi. As shown in Figure 11, viral shedding was detected in both inoculated and direct contact ferrets, with contacts shedding virus on day 2 pc, similar to that observed for RGM98 virus. The peak viral titers ( $>6.0 \log_{10}$ TCID<sub>50</sub>/ml) in nasal washes from both inoculated and direct contacts were higher than those from the RGWF10 group ( $<5.0 \log_{10}$ TCID<sub>50</sub>/ml). However, no viral shedding was detected in the respiratory droplet contacts. In addition, the infected and direct contact ferrets developed signs of disease, characterized by lethargy, anorexia and sneezing, similar to those found in RGM98-infected ferrets. The 2WF10:6M98-infected ferrets showed body weight loss, close to 3% more than that found in RGWF10-infected animals, Table 6. High antibody titers against H9 were detected in both inoculated and direct contact ferrets, but not in the respiratory droplet contacts, Tables 6 and 7. These results demonstrate that reassortment with a human H3N2 virus enhanced viral shedding and transmission of the H9N2 virus to direct contacts however, the reassortant virus still lacked the ability to transmit efficiently in respiratory droplets.



**Figure 10. Recovery and plaque assay of an H9N2 avian-human reassortant.**

(A) Diagram outlining gene segment exchange to create the reassortant virus. (B) Plaque morphology of the parental H3N2 virus RGM98 (left), the parental H9N2 virus RGWF10 (center) and the 2WF10:6M98 reassortant virus (right).



**Figure 11. Replication and transmission of the H9N2 avian-human reassortant virus.**

Ferrets were inoculated i.n. with  $10^6$  TCID<sub>50</sub> of 2WF10:6M98 virus. Twenty-four hours later, the direct and respiratory droplet contacts were placed in the cages as described above. Nasal washes were collected daily and were titrated by TCID<sub>50</sub>. (A) 2WF10:6M98 virus inoculated and direct contacts. (B) 2WF10:6M98 virus respiratory droplet contacts.

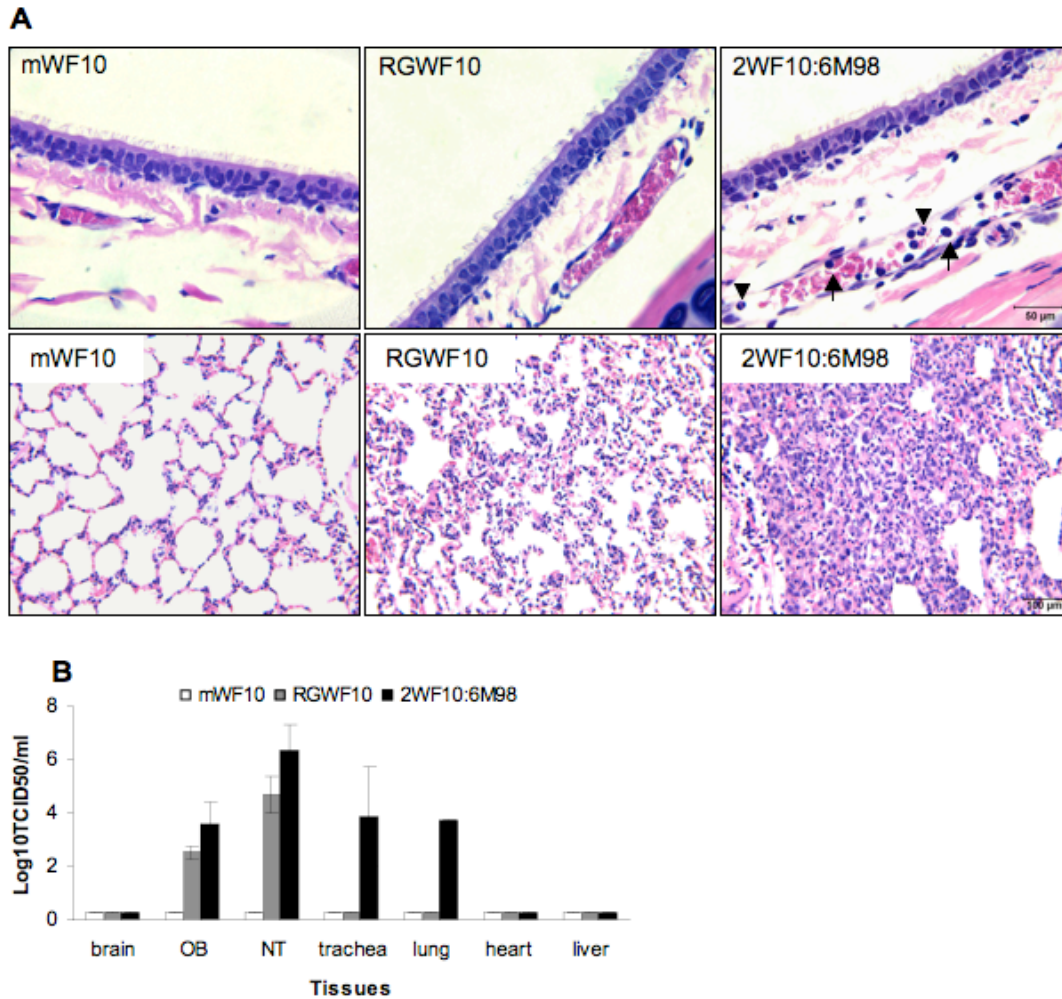


#### **4.4.4 Increased pathology and tissue tropism of the H9N2 avian-human reassortant virus**

We further compared the 2WF10:6M98 virus to its parental RGWF10 for histopathology, focusing mainly on the respiratory tissues. We included the replication defective mWF10 (Wan et al., 2008) as a Q226-containing virus for comparison. Histological examination of the tissues collected at 4 days pi revealed that compared to the parental RGWF10, the 2WF10:6M98 reassortant virus induced more severe lesions in the lungs. Evident alveolar edema and severe infiltration of inflammatory cells, including mononuclear cells, lymphocytes and neutrophils, were observed, Figure 12A. The lungs from RGWF10-inoculated ferrets showed only mild lesions, characterized by slight thickening of alveolar septi. Focal alveolar edema was also noted in the lungs. However, the lungs from mWF10-infected ferrets did not show significant pathological changes. In general, the pathology of the tracheas for each virus was less severe than the lungs. Marked margination of neutrophils and mononuclear cells was observed in small blood vessels in the *lamina propria* of the tracheas from the 2WF10:6M98-infected ferrets, while no lesions were observed in tracheas from either the RGWF10 or mWF10 virus-infected animals.

The tissue tropism of the reassortant 2WF10:6M98 virus was also examined and compared to those of RGWF10 and mWF10. On day 4 pi, virus was recovered in multiple tissues from ferrets infected with 2WF10:6M98, including the olfactory

bulb, nasal turbinate, trachea and lung. RGWF10 was detected only in the olfactory bulb and nasal turbinate, albeit to lower titers than 2WF10:6M98, while replicative defective mWF10 virus was not recovered in any of the tissues examined, Figure 12B. These results indicate that the H9N2 avian-human reassortant is more virulent for ferrets and has a broader tissue tropism than the parental WT H9N2 virus.



**Figure 12. Histopathology and virus tropism of H9N2 viruses in ferrets.**

Two ferrets were inoculated i.n. with  $10^6$  TCID<sub>50</sub> for each virus: mWF10, RGWF10 or 2WF10:6M98. At day 4 p.i, ferrets were euthanized and the tracheas and lungs were harvested for histological analysis. (A) Histopathological findings in the respiratory tract. Upper panel, tracheas: note the margination of neutrophils (▼) and mononuclear cells (↑) in a small vein in the 2WF10:6M98-infected trachea. Lower panel, lungs: note the severe inflammatory infiltration in the 2WF10:6M98-infected lung. (B) Tissue tropism in organs collected from ferrets inoculated with mWF10, RGWF10, or 2WF10:6M98 virus. OB, olfactory bulb. NT, nasal turbinate.

#### 4.5 Discussion

H9N2 viruses are prevalent in avian species in various parts of the world (Alexander, 2000b; Xu et al., 2007a; Xu et al., 2007b) and several human cases of H9N2 infection have been recorded since 1998 (Butt et al., 2005; Guo et al., 1999; Lin et al., 2000; Peiris et al., 1999). The recurring presence of H9N2 infections in humans has raised concerns about the possibility of H9N2 viruses evolving into pandemic strains. Therefore, it is crucial to evaluate the potential pandemic threat posed by H9N2 viruses using appropriate *in vitro* and *in vivo* models. In this study, the replication and transmission of a panel of avian H9N2 viruses, isolated during the last two decades (from 1988 to 2003), were evaluated in the ferret model. Ferrets inoculated with these H9N2 strains shed moderate levels of virus in the nasal washes when compared to a typical human H3N2 strain. Some of the inoculated ferrets showed transient lethargy and temporary body temperature elevation, yet no overt signs of disease (sneezing and/or nasal discharge) were observed. These observations are consistent with the benign nature of H9N2 infection in humans. In contrast to the clinically mild H9N2 infections, ferrets infected with a human H3N2 virus developed clinical signs including sneezing and body weight loss (>6%). By placing two naïve ferrets, one into the same cage as the inoculated ferret and a second into an adjacent cage separated by a wire mesh, we created direct and respiratory droplet transmission models to mimic potential, natural routes of transmission. Our data indicated that 2 out of the 5 WT H9N2 viruses tested were able to transmit from the inoculated to the

direct contact ferrets. The infected contacts showed similar levels of viral shedding, body weight loss and serum antibody titers as their inoculated counterparts. Our results suggest that ferrets, which have been used in the studies of influenza viruses of other subtypes, can serve as a useful model for the studies of avian H9N2 viruses, particularly for the evaluation of H9N2 replication and transmission in mammals.

To date, the H9N2 viruses isolated from humans have been considered to be of avian origin (Butt et al., 2005), although the exact mechanism of transmission of H9N2 viruses from poultry to humans remains to be fully elucidated. It is notable that the human virus-like receptor specificity, related to the presence of L226 in the HA appears to be critical for transmissibility of H9N2 viruses to mammals, as revealed by our site-directed mutagenesis studies (Wagner et al., 2000). Our study revealed that a L226Q mutation of amino acid 226 in the HA RBS severely impaired replication and completely abolished transmission of an H9N2 virus to direct contact ferrets.

Restoring a L226 into the RBS transformed a nontransmissible virus into one that transmits by direct contact. It is likely that the L226-containing viruses are more efficient than Q226-containing viruses in binding and replicating in ferret airway epithelium because of the rich presence of SA $\alpha$ 2-6 receptors. Our glycan data clearly showed a dramatic contrast in the glycan binding pattern of L226 vs Q226 viruses (Wan et al., 2008), which could in part explain the transmissibility phenotype of the former. Speculation as to why RGQa88 was able to establish some replication compared to mWF10 may be due to the observation of dual receptor specificity among Q-containing avian H9N2 strains. The presence of glycine at residue 225 in

the HA has been linked to this trait in H1N1 viruses (Glaser et al., 2005), however it has not been observed for these avian H9N2s and can only be speculated. Consistent with this concept, we and others have previously shown that the presence of L226 in the HA correlated with enhanced replication of influenza viruses in an *in vitro* HAE model, especially in HAE cells that express mainly SA $\alpha$ 2-6 receptors (Matrosovich et al., 2007; Wan and Perez, 2007). These observations provide valuable clues for understanding why avian H9N2 viruses carrying the L226 signature in the HA can cross the species barrier and cause infections in humans.

Unlike the H5N1 avian-human reassortant viruses that lack transmissibility in the ferret model (Maines et al., 2006), our H9N2 avian-human reassortant virus replicated in ferrets more efficiently than the parental H9N2 virus ( $\sim 2\text{-log}_{10}$  higher peak viral titers in nasal washes), transmitted efficiently among direct contact ferrets, and induced clinical signs of disease. Our *in vitro* study also showed that the reassortant virus induced larger and more defined plaques in MDCK cells than WT H9N2 viruses. Furthermore the reassortant H9N2 avian-human virus showed broader tissue tropism, including the ability to replicate in the upper and lower respiratory tract. This ability of H9N2 viruses to replicate in multiple compartments of the respiratory tract provides more opportunities for the virus to select transmissibility traits. These data further support the notion that virulence of influenza viruses is a polygenic trait.

It is important to note that the avian virus, RGWF10, and the avian-human reassortant virus, 2WF10:6M98, which transmitted efficiently to the direct contact ferrets, failed to transmit to the respiratory droplet contacts. Surprisingly, the ferrets infected with the reassortant virus displayed clinical signs, including sneezing, and shed virus titers similar to those observed for the parental H3N2 virus, indicating that the inability to transmit by respiratory droplet cannot be attributed to lack of sufficient viral shedding or sneezing, characteristics typically associated with respiratory droplet transmission. It appears that avian H9N2 viruses, including those that have acquired SA $\alpha$ 2-6 receptor specificity, still lack a key component necessary for efficient respiratory droplet transmission among mammals and, perhaps, humans. It would be reasonable to speculate that the molecular restriction lies within the surface glycoproteins, particularly the HA.

Despite this restriction in respiratory droplet transmission, three key factors in avian H9N2 viruses should be noted. First, a number of studies have demonstrated that H9N2 viruses are undergoing extensive evolution and reassortment (Li et al., 2005; Li et al., 2003; Xu et al., 2007a; Xu et al., 2007b) fueling their pandemic potential. Second, there have been several lines of evidence that H9N2 viruses have transmitted to pigs (Cong et al., 2007; Peiris et al., 2001; Shi et al., 2008; Xu et al., 2004), the proposed intermediate host that is permissive to both avian and human influenza viruses. The pig could therefore serve as an ideal environment for avian H9N2 viruses to acquire alterations, either through mutations or reassortment,

favoring human infection and possibly human-to-human transmission. Third, serological data from separate studies suggest that there may be more human cases of H9N2 infection than previously anticipated (Guo et al., 1999; Peiris et al., 1999), and that the possibility of a limited level of human-to-human transmission cannot be absolutely excluded (Butt et al., 2005). Therefore, avian H9N2 viruses are in an ideal position to undergo further adaptation for more efficient transmission among mammals, most critically, humans.

In summary this data, in addition to previous reports from our lab, demonstrate that avian H9N2 viruses are able to replicate in the respiratory tract of ferrets and those viruses with L226 in the HA RBS have propensity to transmit relatively efficiently to direct contacts. The transmission and replication phenotype can be further improved by providing the virus with a gene constellation more adapted for replication in ferrets. Efficient respiratory droplet transmission, a prerequisite for a human pandemic, was not observed. However, considering the widespread prevalence of H9N2 viruses in poultry, the human virus-like receptor specificity of some avian and swine H9N2 isolates, co-circulation of H9N2 with H3N2 viruses in Asian swine, and the repeated direct transmission to humans, the public health threat of H9N2 viruses cannot be overemphasized.



## **Chapter 5: Respiratory Droplet Transmission of H9N2 Influenza in Ferrets**

### 5.1 Abstract

The generation of an influenza pandemic requires interspecies transmission of a novel strain, which can adapt to its new host through either reassortment or point mutations. It is poorly understood what subset of internal genes, if any, are needed in combination for an avian-human reassortant to transmit to humans, however we do know that in order to cause a pandemic the HA must be novel. Given that two previous pandemics were the result of reassortment between low pathogenic avian viruses and human subtypes of that period, and given the endemic situation of avian H9N2 viruses in Eurasia, we wanted to determine if adaptation of an avian-human H9N2 reassortant in ferrets could support mammalian respiratory droplet transmission. Here we show for the first time that, after ten serial passages in ferrets, a reassortant virus carrying the HA and NA of an avian H9N2 virus can transmit in

respiratory droplets. The adapted virus contains only five amino acid changes in the entire genome, three of which are in the surface proteins. Mutations in HA account for the transmissibility, antigenicity and hemagglutination inhibition profile of the virus. This is the first report of respiratory droplet transmission of H9N2 influenza, which carries profound implications for pandemic preparedness. The amino acid changes on the HA might identify critical, adaptive mutations necessary for respiratory transmission in subsequent pandemic avian influenza strains. More importantly, we demonstrate that an aerosol droplet phenotype is not exclusive to current human influenza subtypes. Overall, these studies have profound implications for influenza pandemic preparedness.

## 5.2 Introduction

### **5.2.1 The role of aerosol in influenza transmission**

For the transmission of any respiratory pathogen three simple components are required: 1) the source (infected individual), 2) the surrounding environment, and 3) the recipient (non-infected individual). However, the transmission of any agent depends largely on other factors such as the number of infectious particles, volume of space, ventilation of the enclosure, length of exposure and the immune response of the affected individual (Zayas et al., 2005). Transmission occurs if two criteria are

met; first infectious aerosols are inhaled by the susceptible animal in sufficient number and secondly, that the infectivity of the inhaled aerosols is maintained.

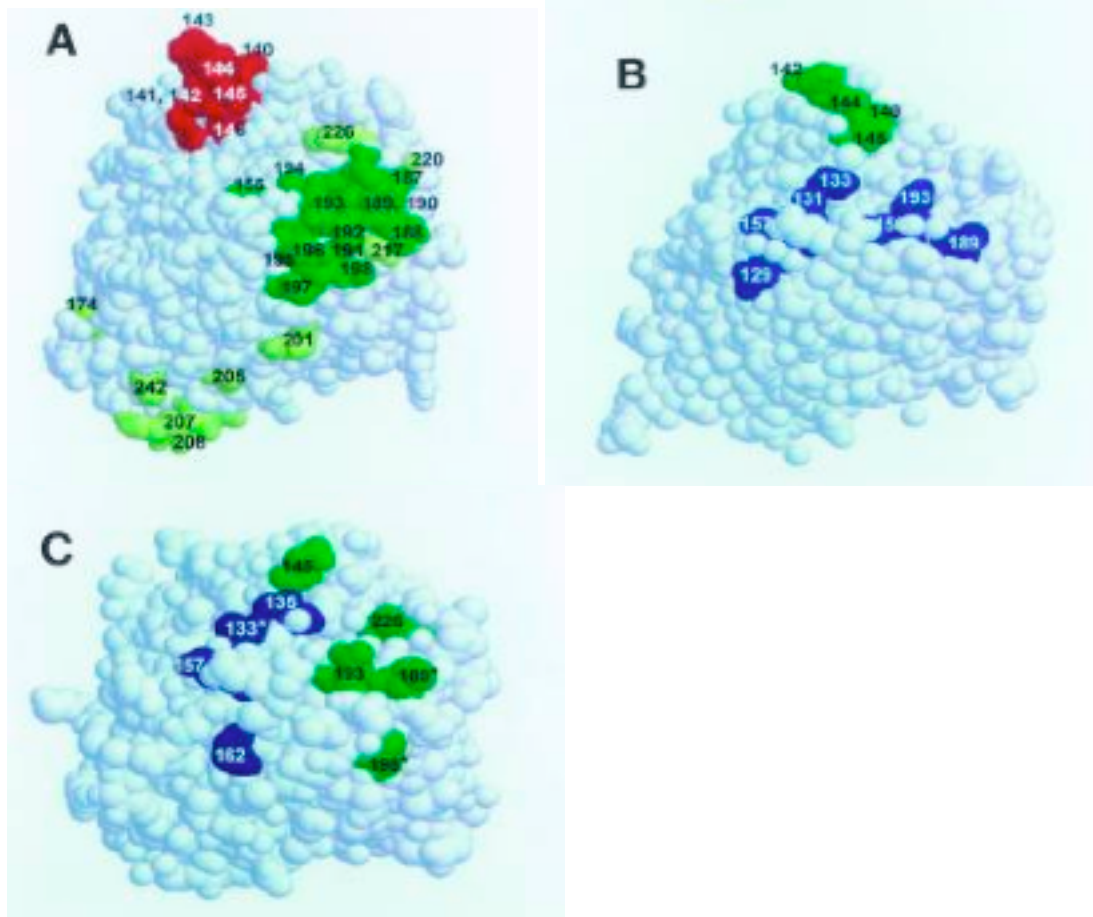
The postulated transmission modes for natural influenza A infections include large droplets, aerosols and direct contact with secretions and/or fomites. Influenza can remain on dry, nonporous surfaces for up to 48 hours (Bean et al., 1982). In practice, ruling out one or more of these modes to identify a single mode of transmission is complex thus; establishing the relative contribution of each is difficult. However, experimental transmission studies can provide clear separations between routes of transmission.

Aerosol transmission is an important mode of transmission one which has obvious implications for pandemic influenza planning. Aerosols, by definition, are suspensions in air (or in gas) of solid or liquid particles, small enough that they remain airborne for prolonged periods of time due to their low settling velocity (Tellier, 2006). Coughing and sneezing produces a considerable amount of particles which rapidly shrink in size due to evaporation, increasing the number of particles that behave as aerosols. Infected animals can generate aerosols very efficiently by sneezing and coughing. Knight (1973) observed that a human sneeze produces approximately  $2 \times 10^6$  droplet particles with more than 75% smaller than  $2 \mu\text{m}$ . Coughing is less efficient with approximately  $9 \times 10^5$  droplet particles with 95% smaller than  $2 \mu\text{m}$ . The particle size is relevant because it influences the time it takes the particle to settle and therefore the depth of penetration in the respiratory tract

upon inhalation (Stark, 1999). Disease animals through their activities are a source of infectious aerosol. Aerosol is positively correlated with the level of animal activity. The concentration of infectious agents in aerosols is also directly proportional to the strength of the source, indicated by the number and concentration of infected individuals (stocking density) for example animals on a farm, flock, or humans in a closed environment (Stark, 1999).

### **5.2.2 Antigenic variation**

Anti-HA antibodies neutralize influenza virus. Therefore, changes in HA structure that prevent antibody binding are necessary for the creation of seasonal, epidemic viruses. This type of antigenic drift has been occurring in the H3N2 subtype since 1968; research has shown this subtype has accumulated roughly 3.5 residue changes each year (Bean et al., 1992). Studies performed with antigenic variants of multiple subtypes indicate that all antigenic sites are located on the surface of the membrane distal HA1 domain, predominantly surrounding the RBS, Figure 13 A-C (Kaverin et al., 2004; Skehel and Wiley, 2000). The primary receptor binding site contacts sialic acids through 3 HA<sub>1</sub> secondary structural groups, the 190 helix (residues 190-198), the 130 loop (residues 135-138) and the 220 loop (residues 221-228) (Skehel and Wiley, 2000).



**Figure 13. Antigenic sites of H3, H5 and H9 subtypes.**

Antigenic sites on the globular head of the HAs from H3 (A), H5 (B) and H9 (C) subtypes are shown. Red, green and yellow indicate the antigenic sites A, B and D respectively of H3 (A). Blue and green indicate the antigenic sites of H5 and H9 with an asterisk representing overlapping epitopes. Adapted from Kaverin et al., 2004.

### 5.3 Materials and Methods

#### **5.3.1 Virus isolation and propagation**

Viruses were grown and titrated in MDCK cells, as described in chapter 4, section 3, subsection 1. All experiments with live virus were performed in a biosafety level-3<sup>+</sup> containment facility. Reassortant viruses were rescued using a set of universal primers, as described previously (Hoffmann et al., 2002; Hoffmann et al., 2001) and summarized in Chapter 3, section 3, subsection 6. The recovery of recombinant viruses was verified by sequencing using specific primers. Single amino acid mutations were introduced into the P10 HA using a QuickChange II site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) according to the manufacturer's protocols. The mutant viruses were recovered and grown in MDCK cells and sequenced to verify the presence of desired and the absence of unwanted mutations.

#### **5.3.2 Adaptation of 2WF10:6M98 in ferrets**

We inoculated one ferret with the 2WF10:6M98 virus at  $10^6$  TCID<sub>50</sub>/ml. On day 3 pi, nasal wash was collected, brought to 1 ml total volume in PBS and used as inoculum for the next ferret (passage 1). The adaptation continued to passage 10

where nasal wash was grown in MDCKs, titered and  $10^6$  TCID<sub>50</sub> of passage 9 was used as the dose for infection of two ferrets in a transmission study, passage 10. Transmission experiments were set up and followed as noted in chapter 4, section 3, subsection 3. HI assays and tissue tropism studies were conducted and followed the descriptions included in chapter 4, sections 3, subsections 4 and 5 respectively.

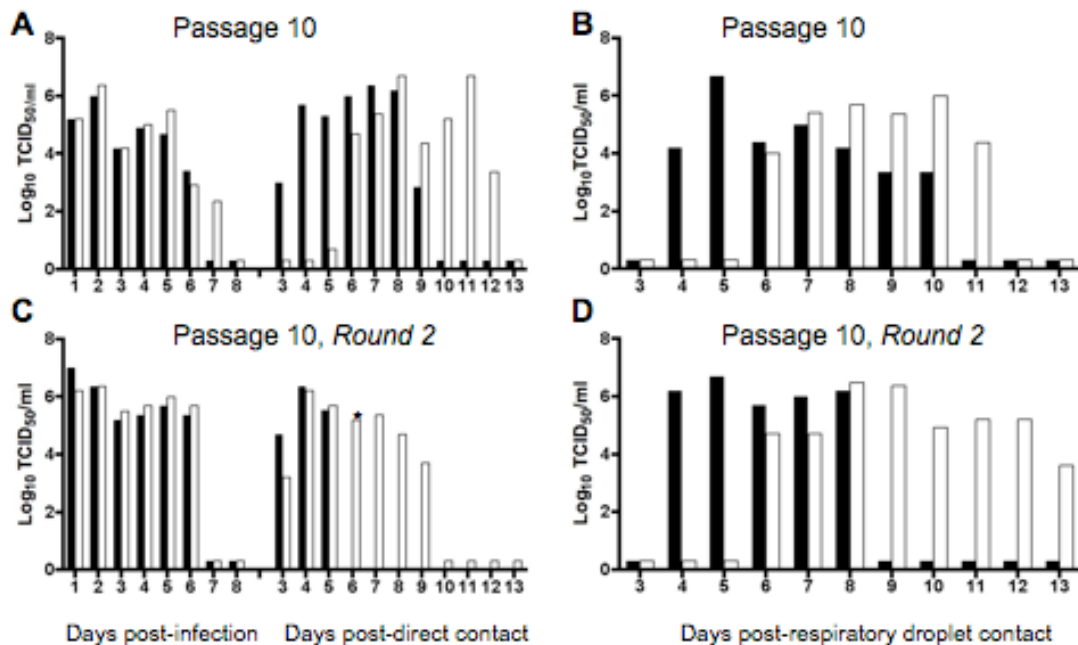
## 5.4 Results

### **5.4.1 Adaptation of 2WF10:6M98 in ferrets leads to respiratory droplet transmission**

A transition from  $\alpha$ 2,3 linked sialic acid (SA $\alpha$ 2,3) to  $\alpha$ 2,6 linked sialic acid (SA $\alpha$ 2,6) receptors appears to be a crucial step for avian H9N2 viruses to replicate efficiently and transmit in humans. Recent avian H9N2 viruses carrying leucine at position 226 in the HA receptor-binding site, confer SA $\alpha$ 2,6-preference and thus are able to replicate and spread in human epithelial cells as well as in the ferret model (Matrosovich et al., 2001; Wan and Perez, 2007; Wan et al., 2008). In addition to possessing human virus-like receptor specificity, avian H9N2 viruses induce typical human flu-like illness, which can easily be overlooked, and therefore have the opportunity to circulate, reassort and improve transmissibility in humans. Creation of the H9N2 avian-human reassortant, 2WF10:6M98, led to increased replication, direct transmission and expanded tissue tropism in ferrets when compared to the parental

RGWF10 avian H9N2 (Wan et al., 2008). This reassortant however lacked the ability to transmit via respiratory droplet. With the knowledge that early human H2N2 and H3N2 pandemic strains required adaptation in their new host before gaining their full pandemic potential, we began adapting this avian-human reassortant, 2WF10:6M98, in ferrets. After ten passages of nasal washes collected, 3 days p.i., we tested the transmissibility of our virus, herein referred as P10 virus. Within two days p.c., direct contacts were shedding virus and were able to transmit to respiratory droplet contacts by day 3 and 5 p.c., Figure 14A, B. All ferrets, including respiratory droplet contacts, shed virus up to 6-7 days and displayed clinical symptoms, similar to that of a human virus infection (Wan et al., 2008), including sneezing, Table 8 and 9, respectively.





**Figure 14. Respiratory droplet transmission of H9N2 avian-human reassortant virus after adaptation in ferrets.**

Ferrets were inoculated intranasally (i.n.) with  $10^6$  TCID<sub>50</sub> of either ferret-adapted P10 virus (A, B) and a second independent study with the P10 virus (C, D). Twenty-four hours later, one naïve ferret (direct contact) was placed in the same cage as the infected ferret (A, C) and a respiratory droplet contact was placed in an adjacent cage separated by a wire mesh (B, D). Nasal washes were collected daily and titrated in MCDK cells. Black and grey bars represent individual ferrets, each color consisting of a group of 3 ferrets. Each bar represents a ferret sampled and the amount of viral shedding at that sample. In 1C, day 6p.c., the direct contact from the group represented by the black bars died, as noted by a star in the bar graph. Titers are expressed as log<sub>10</sub> values of TCID<sub>50</sub>/ml with the limit of detection at 0.699 log<sub>10</sub>TCID<sub>50</sub>/ml.

**Table 8. Clinical signs, virus replication and seroconversion associated with H9N2 reassortant viruses in infected ferrets**

Virus	Infected Ferrets		
	Weight Loss (%) <sup>a</sup>	Sneezing (day of onset)	Serum (HI titer) <sup>b</sup>
2WF10:6M98	5.1 ± 0.85	2/2 (2,2)	2560, 2560
P10 <sup>c</sup>	4.01 ± 1.2	4/4 (3,5,7)	2560, 2560, 2560, 2560
2RCP10:6M98 <sup>c</sup>	4.67 ± 1.7	4/4 (5,6)	2560, 2560, 2560, 2560
RCP10(A189,G192) <sup>c</sup>	5.0 ± 2.48	4/4 (5,5)	2560, 2560, 1280, 2560
RCP10(T189, R192) <sup>c</sup>	3.69 ± 1.43	4/4 (5,6)	2560, 1280, 2560, 1280

<sup>a</sup> Average body weight loss is shown ± standard error mean. <sup>b</sup> Homologous virus was used in HI assays to detect anti-H9 antibodies. <sup>c</sup> Two independent experiments with 2 infected, 2 direct and 2 respiratory droplet ferrets each.

**Table 9. Clinical signs, virus replication and seroconversion associated with H9N2 reassortant viruses in direct and respiratory droplet contact ferrets**

Virus	Direct Contact Ferrets			Respiratory Droplet Contact Ferrets		
	Weight Loss (%) <sup>a</sup>	Sneezing (day of onset)	Serum (HI titer) <sup>b</sup>	Weight Loss (%) <sup>a</sup>	Sneezing (day of onset)	Serum (HI titer) <sup>b</sup>
2WF10:6M98	1.65 ± 0.50	2/2 (4,5)	1280, 2560	ND	0/2	<10, <10
P10 <sup>c</sup>	5.36 ± 0.1	4/4 (5,7)	2560, 2560, 2560, 2560	7.91 ± 1.98	4/4(7,8,9)	2560, 1280, 2560, 2560
2RCP10:6M98 <sup>c</sup>	2.79 ± 1.43	4/4 (7,9)	1280, 1280, 1280, 1280	2.07 ± 0.59	4/4	1280, 1280, 640, 640
RCP10(A189,G192) <sup>c</sup>	1.67 ± 0.82	4/4 (5,7)	1280, 2560, 2560, 1280	ND	0/4	<10, <10, <10, <10
RCP10(T189, R192) <sup>c</sup>	8.65 ± 5.16	4/4 (6,7)	2560, 2560, 1280, 1280	ND	0/4	<10, <10, <10, <10

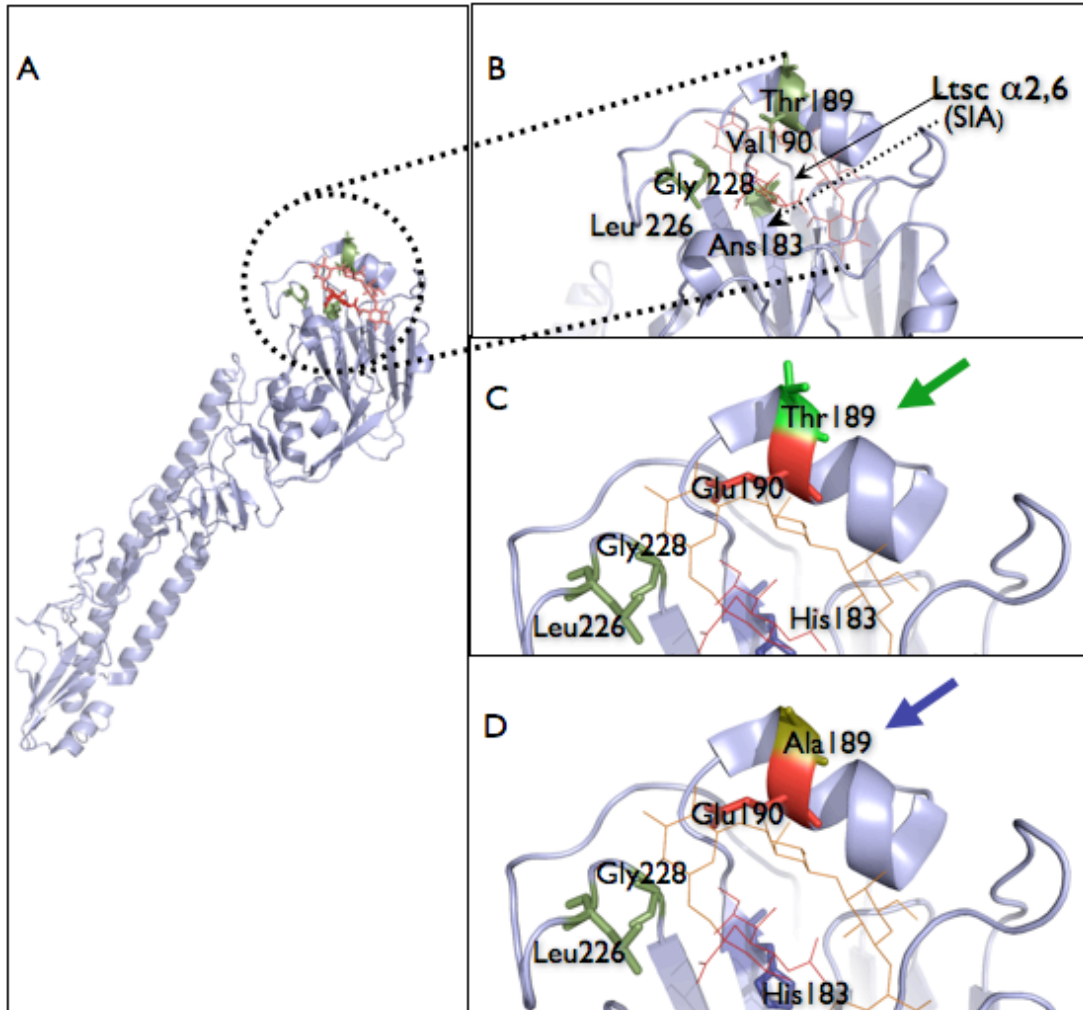
<sup>a</sup> Average body weight loss is shown ± standard error mean. <sup>b</sup> Homologous virus was used in HI assays to detect anti-H9 antibodies. <sup>c</sup> Two independent experiments with 2 infected, 2 direct and 2 respiratory droplet ferrets each. ND, Not determined because no viral replication occurred in ferrets

#### 5.4.2 Sequence analysis of the ferret-adapted virus

The viruses collected from the nasal washes of respiratory droplet contacts, defined as RCP10 [A/ferret/MD/P10-UMD/08 (H9N2)], were sequenced to determine the molecular changes supporting respiratory droplet transmission. Viruses were sequenced directly from the nasal wash without any passages in embryonated chicken eggs or cell culture. Only five amino acid changes occurred from 2WF10:6M98, to nasal washes from RCP10. In addition, sequence analysis from four separate respiratory droplet contact nasal washes yielded the same five amino acid changes, indicating their selection in respiratory droplet transmission. Three amino acid changes are located on the surface proteins while two are located on the internal proteins. Two changes occurred on the HA, one on the HA<sub>1</sub> portion of the molecule at position 189 (H3 numbering) in the antigenic site of the HA (site B) and within the RBS, Figure 13 and Figure 15. This amino acid change from T to A has been documented before (Kaverin et al., 2004) and is also found in naturally occurring isolates (Ha et al., 2002). However, the combination of key amino acid residues at the RBS found in our P10 ferret-adapted virus; i.e. H183, A189, E190 and L226 has not yet been identified in nature, Figure 15D.

The available human H9N2 sequences from the NCBI database that have yet to show sustained human-to-human transmission, show H183, T189, E190 and L226. The only major difference in these viruses and the RCP10 virus is at position 189.

The second change is located on the HA<sub>2</sub> at position 192 (H3 numbering), a change from G to R, three amino acids away from the transmembrane region of the HA<sub>2</sub>. Unfortunately, this amino acid change lies within a region that has not yet been resolved by crystallography and therefore cannot be mapped structurally. The change in the NA at position 28 appears located in the transmembrane domain. This domain has been reported to participate in virus assembly and/or shedding (Barman et al., 2004; Barman and Nayak, 2000; Bos et al., 1984; Kundu et al., 1996). The two remaining changes L374I in PB2 and H110Y in M1 map to regions of unassigned functions within these two proteins.



**Figure 15. Adaptive mutations in the H9 HA surface protein necessary for respiratory droplet transmission.**

A) Cartoon representation of the H9 HA monomer as presented by Ha et al. 2002 binding the Ltsc  $\alpha 2,6$ sialic acid analog (structure represented in orange and red lines) in the RBS. B) Magnification of the globular head of the HA showing stick representations (in green) of key amino acids in the RBS binding to  $\alpha 2,6$ sialic acid (SIA, red lines). Numbers correspond to amino acid positions based on the H3 HA numbering system. A pink cloud on the tip of the molecule indicates the overlap with the antigenic B site. C) Cartoon representation of the H9 HA RBS with amino acids corresponding to the WF10 HA wild type sequence, which differs from the published crystal structure at two positions: histidine at position 183 (His, dark blue stick) and glutamic acid at position 190 (Glu, red stick). Thr at position 189 is represented as a bright green stick. D) Cartoon representation of the H9 HA RBS with amino acids corresponding to the RCP10 HA sequence, which differs from the WF10 HA sequence at a single position: Alanine (Ala) at position 189 represented as an olive green stick. Cartoon and predicted amino acid structures were generated using MacPymol (DeLano Scientific, LLC).

We additionally sequenced the P10 inoculum [P9] (nasal wash from passage 9 ferrets used to infect passage 10) and P9 inoculum [P8] (nasal wash from passage 8 ferrets used to infect passage 9) to ensure the amino acid changes necessary for aerosol droplet transmission occurred during the tenth passage. The virus from the nasal wash and the stock prepared from MDCKs used to infect passage 10 was identical at the sequence level. We also repeated the transmission study to confirm respiratory droplet transmission and to determine whether the changes observed in respiratory droplet contact nasal washes were reproducible. In our repeat experiment [P10<sub>2</sub>], ferrets were infected with the same virus, nasal wash collected from passage 9, and transmission to direct as well as respiratory droplet contacts was again observed, Figure 14C, D. Sequence analysis of the nasal washes collected from respiratory droplet contacts (RCP10<sub>2</sub>) yielded the same five amino acid changes, most notably the two changes in the HA gene, implying these changes are selected during adaptation and are necessary for respiratory droplet transmission. Sequence analysis is presented in Table 10.

**Table 10. Sequence analysis of 2WF10:6M98 ferret-adapted passages.**

Gene	Origin	Amino Acid Position		Parent	P8 <sup>a</sup>	P9 <sup>b</sup>	RCP10 <sup>c</sup>	RCP10 <sub>2</sub> <sup>d</sup>
PB2	Human	374		L	I	I	I	I
PB1	Human	No changes <sup>f</sup>			ND <sup>g</sup>	ND		
PA	Human	No changes			ND	ND		
HA	Avian	HA1	189	T	T	T	A	A
		HA2	192	G	G	<b>G/R</b> <sup>e</sup>	R	R
NP	Human	No changes			ND	ND		
NA	Avian	28		I	V	V	V	V
M	Human	M1	110	H	H/Y <sup>e</sup>	Y	Y	Y
		M2	No changes		ND	ND		
NS	Human	NS1	No changes		ND	ND		
		NEP	No changes		ND	ND		

<sup>a</sup> P8 is the inoculum (nasal wash from passage 8) used to infect passage 9

<sup>b</sup> P9 is the inoculum (nasal wash from passage 9) used to infect passage 10

<sup>c</sup> RCP10 are the nasal washes collected from the aerosol droplet contacts during passage 10

<sup>d</sup> Represents a completely independent infection of P10 virus, and is the nasal washes collected from aerosol droplet contacts during the repeat of passage 10.

<sup>e</sup> Bold and italicized letter denotes more prominent residue at particular amino acid position

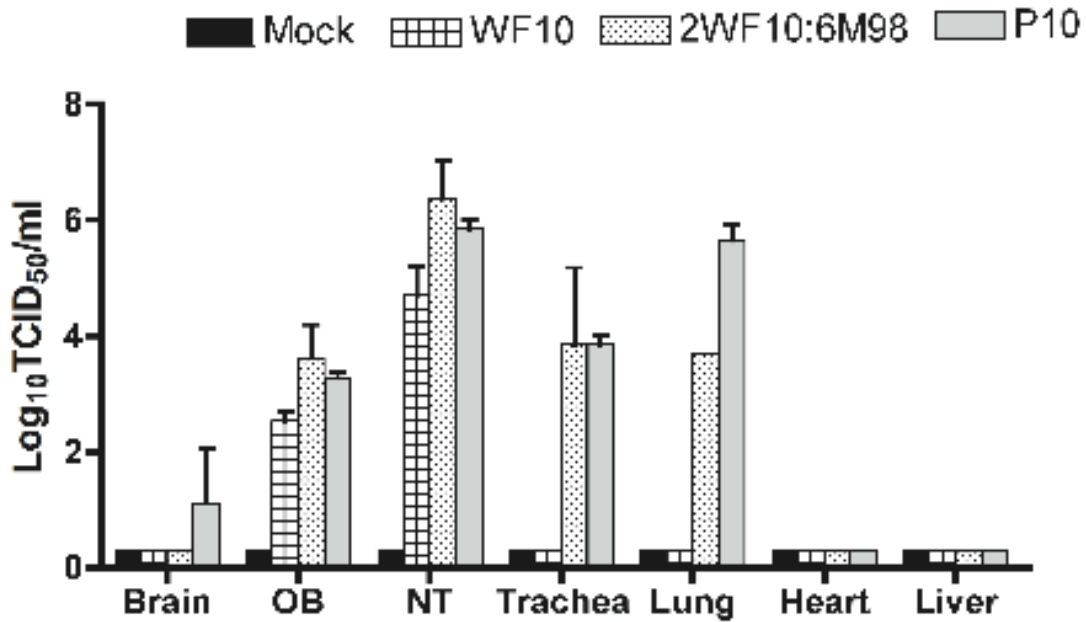
<sup>f</sup> No amino acid change detected between parent and either of the RCP10, RCP10<sub>2</sub> viruses

<sup>g</sup> ND, sequence not done



### 5.4.3 Tissue tropism

The consistency of obtaining respiratory droplet transmission and identical amino acid changes in both rounds of transmission led us to investigate and compare tissue tropism of the P10 virus to the parental 2WF10:6M98 and wild type WF10 viruses. Ferrets were also mock-infected with PBS as a negative control. Tissues were collected day 5 p.i., homogenized and virus titrations performed as previously described in chapter 4, section 3, subsection 5. While 2WF10:6M98 was able to replicate and expand tissue tropism compared to WF10, the RCP10 virus shows more consistent isolation in lung, with all ferrets positive and over 1.5 log more virus compared to 2WF10:6M98, Figure 16, indicating that the changes occurring during adaptation allow for expanded tissue tropism and higher virus replication. We also isolated virus from the brain suggesting that the virus not only changes its transmission phenotype but that it also has the potential to become more virulent.



**Figure 16. Improved tissue tropism of ferret-adapted P10 virus.**

Two ferrets were infected intranasally (i.n.) with  $10^6$  TCID<sub>50</sub> of ferret-adapted RCP10 virus, 2WF10:6M98 (P1) virus, RGWF10 and mock (PBS). At day 5 p.i., ferrets were euthanized and organs were collected and homogenized. \* Note only 1 of 2 ferret lungs were positive for virus in the P1 group. Titers are expressed as log<sub>10</sub> values of TCID<sub>50</sub>/ml with the limit of detection at 0.699 log<sub>10</sub>TCID<sub>50</sub>/ml. OB, olfactory bulb. NT, nasal turbinate.

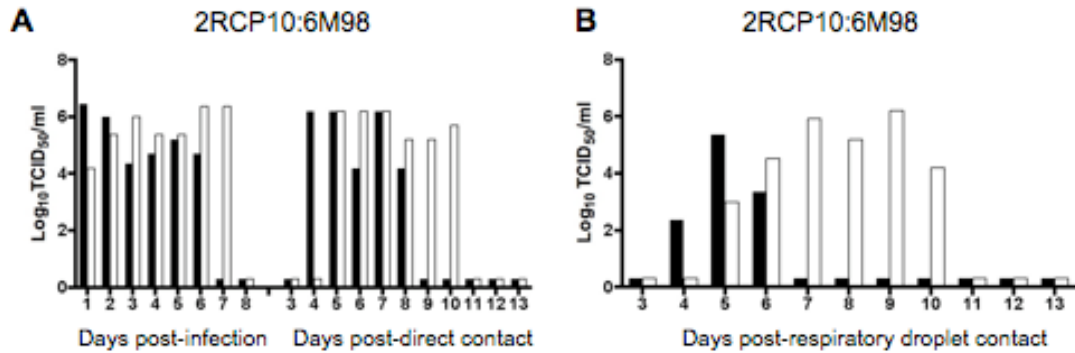
#### 5.4.4 Minimal changes necessary for respiratory droplet transmission

A majority of the adaptive amino acid changes occurred prior to passage 10, with the exception of the changes found on the HA. We therefore wanted to determine whether the surface proteins alone are sufficient for respiratory droplet transmission. Using reverse genetics, we created a reassortant virus, 2RCP10:6M98, which contains the HA and NA from the ferret-adapted RCP10 virus and the internal genes from the human RGM98 (H3N2) virus. We found that the changes in the surface proteins alone are indeed sufficient for respiratory droplet transmission, Figure 17A, B. Respiratory droplet transmission of 2RCP10:6M98 was confirmed in a second, independent study with similar results.

Since the HA is the major determinant in the transmission of pandemic influenza, we took advantage of site-directed mutagenesis to create RCP10 HA-mutant viruses that carry only one of the two HA changes. RCP10(A189,G192) contains the adaptive change of alanine at HA<sub>1</sub> 189 and the avian glycine at HA<sub>2</sub> 192 while RCP10(T189, R192) contains the avian threonine at HA<sub>1</sub> 189 and the adaptive arginine at HA<sub>2</sub> 192. Our transmission studies suggest that both mutations in HA are necessary for respiratory droplet transmission of our avian-human H9N2 reassortant virus, Figure 18 A-D. These results are somehow unexpected. We could predict that a change in HA<sub>1</sub>, within the RBS, would be necessary for respiratory droplet transmission; however, we could not anticipate that a change in the HA<sub>2</sub> portion of the molecule would have an impact on transmission. These studies highlight the

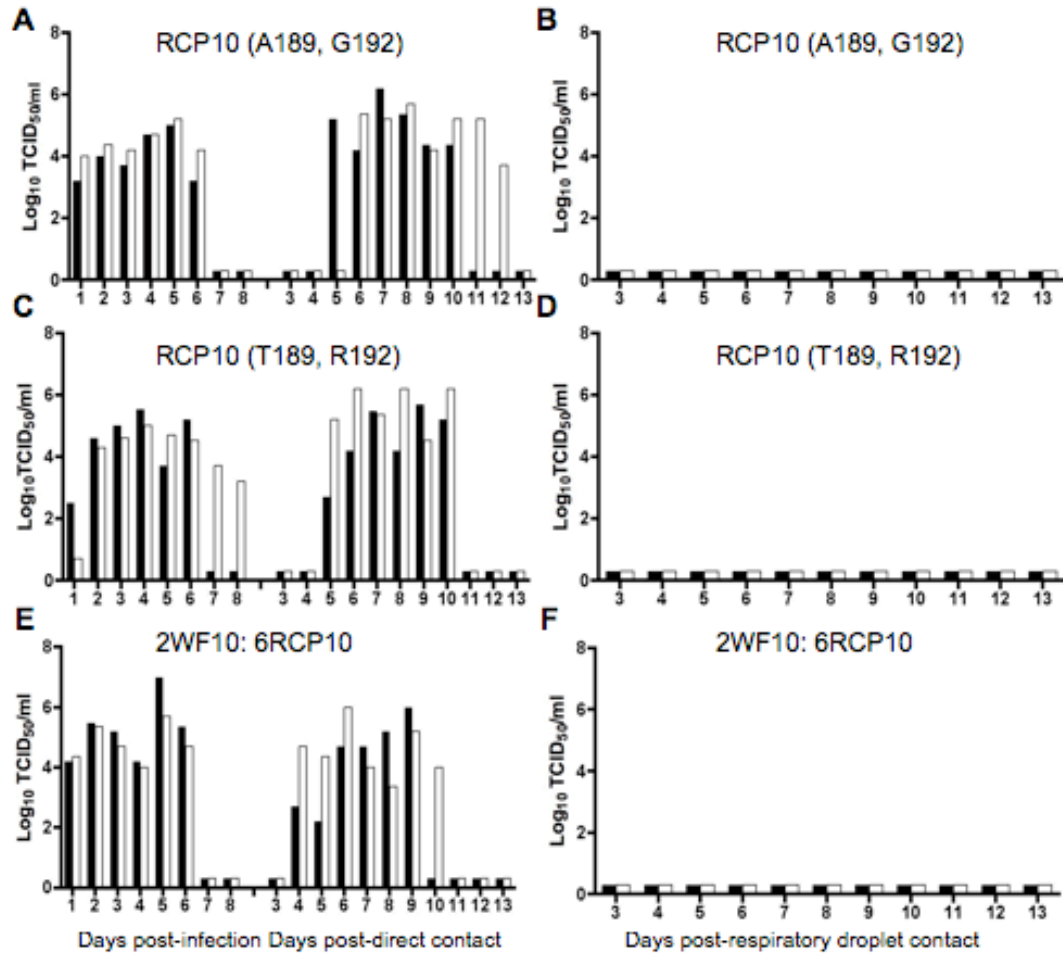
complexities associated with transmissibility of influenza viruses and emphasize the role that *in vivo* studies have to better understand such complexities.

Efficient respiratory droplet transmission was supported through the surface proteins of the RCP10 virus in the M98 backbone, we therefore wanted to determine if the two changes in internal proteins could support respiratory droplet transmission in the context of the un-adapted avian surface proteins, the HA and NA of WF10. Thus, a virus was rescued encoding the RCP10 backbone and the HA and NA of WF10, 2WF10:6RCP10. We found that the virus was able to replicate and transmit efficiently to direct contact ferrets however no respiratory droplet transmission was observed, Fig 18E and F. This result is consistent with notion that adaptive mutations on the surface proteins are essential for respiratory droplet transmission of the avian-human H9N2 reassortant virus. A complete set of viruses tested and their transmission phenotype are listed in Supplemental Table 11.



**Figure 17. Respiratory droplet transmission supported through adaptive changes on the P10 virus surface proteins.**

Ferrets were inoculated intranasally with  $10^6$  TCID<sub>50</sub> 2P10:6M98 virus. Twenty-four hours later, one naïve ferret (direct contact) was placed in the same cage as the infected ferret (A) and a respiratory droplet contact was placed in an adjacent cage separated by a wire mesh (B). Nasal washes were collected daily and titrated in MCDK cells. Black and grey bars represent individual ferrets, each color consisting of a group of 3 ferrets. Each bar represents a ferret sampled and the amount of viral shedding at that sample. Titers are expressed as log<sub>10</sub> values of TCID<sub>50</sub>/ml with the limit of detection at 0.699 log<sub>10</sub>TCID<sub>50</sub>/ml.



**Figure 18. Transmission supported through both T189A and G192R changes on the HA of P10 virus not the internal RCP10 proteins.**

Ferrets were inoculated intranasally (i.n.) with  $10^6$  TCID<sub>50</sub> of RCP10 (A189,G192) (A,B) RCP10 (T189,R192) (C,D) and 2WF10:6RCP10 (E,F) viruses. Twenty-four hours later, one naïve ferret (direct contact) was placed in the same cage as the infected ferret (A, C, E) and a respiratory droplet contact was placed in an adjacent cage separated by a wire mesh (B, D,F). Nasal washes were collected daily and titrated in MCDK cells. Black and grey bars represent individual ferrets, each color consisting of a group of 3 ferrets. Each bar represents a ferret sampled and the amount of viral shedding at that sample. Titters are expressed as log<sub>10</sub> values of TCID<sub>50</sub>/ml with the limit of detection at 0.699 log<sub>10</sub>TCID<sub>50</sub>/ml.

**Table 11. Summary of reassortant viruses tested for replication and transmission in ferrets**

Virus	Replication <sup>a</sup>	Transmission <sup>a</sup>	
		Direct	Aerosol
P10	4/4	4/4	4/4
2RCP10:6M98	4/4	4/4	3/4
RCP10(A189, G192)	4/4	4/4	0/4
RCP10(T189, R192)	4/4	4/4	0/4
<u>2WF10:6RCP10</u>	<u>4/4</u>	<u>3/4</u>	<u>0/4</u>

<sup>a</sup> Two separate studies of 2 infected, 2 direct and 2 aerosol contacts each

#### 5.4.5 Antigenic profile

Our results suggest that one of the important determinants for respiratory droplet transmission is located in close proximity to the RBS, overlapping a major antigenic site of the HA molecule (site B, Figure 15), therefore we wanted to determine the HI profiles for the reassortant viruses tested. Interestingly we found that the RCP10 virus displays a different antigenic profile from the parental WF10 virus, Table 12. The HI titers to the WF10 virus are greatly reduced if serum antibodies raised in response to the RCP10 are used instead of those against the parental WF10 virus, Table 12. The RCP10 serum also reacted inefficiently against other H9N2 viruses in HI assays. The opposite is also true: the HI titers to the RCP10 virus are greatly reduced if serum antibodies raised in response to the WF10 virus are used. More importantly, HI titers using anti-RCP10 antiserum were similar for the RCP10 and the RCP10(A189, G192) viruses, implicating amino acid 189 in antigenicity and in agreement with its position in the tip of the globular head of HA<sub>1</sub>, Figure 15.

This data carries huge implications for vaccine stocks for pandemic preparedness. It has been speculated that given natural conditions, immune pressure can select for variants with altered host specificity and an ability to escape host immunity, key factors in the evolution of avian H9N2 viruses (Webby and Webster, 2001); particularly this RCP10 virus containing a2-6 preference and an antigenically variant HA. This study highlights the potential discrepancy in antibody protection



from the avian field isolate (chosen to prepare the vaccine stock) versus the antigenic makeup of the virus that gains respiratory droplet (or human-to-human) transmissibility. This study also highlights the inherent limitations in the selection of vaccine seed stocks from current avian H9N2 strains. It will be important to determine if the changes seen in the HA of RCP10, namely amino acid 189 in the HA<sub>1</sub>, can confer transmissibility in additional H9 HAs and other avian subtypes and whether this should be considered a critical antigenic site for vaccine candidates. It is interesting to note that residue 189 has been implicated not only in H9 escape mutants but also in escape mutants of the highly pathogenic H5 and pandemic H2 viruses (Ilyushina et al., 2004; Kaverin et al., 2004; Kaverin et al., 2002; Tsuchiya et al., 2001). However, it must be noted that selection of alanine at position 189 in this study occurred in the absence of pre-existing immune pressure in the ferrets.

**Table 12. HI profiles - Implications for pandemic preparedness**

<b>Ferret Sera</b> <b>Virus</b>	<b>WF10</b>	<b>RCP10</b>	<b>RCP10(A189,G192)</b>	<b>RCP10(T189,R192)</b>
<b>WF10</b>	5120,5120	320, 640	640, 640	1280, 2560
<b>RCP10</b>	1280, 640	5120,5120	5120, 2560	1280, 640
<b>RCP10(A189,G192)</b>	1280, 640	5120,2560	5120, 5120	1280, 640
<b>RCP10(T189,R192)</b>	2560,2560	1280, 640	640, 1280	5120, 2560
<b>M98</b>	<10, <10	<10, <10	<10, <10	<10, <10
<b>Dk/Y280</b>	80, 80	80, 80	ND <sup>a</sup>	ND
<b>Ch/SF3</b>	160, 160	320, 640	ND	ND

<sup>a</sup> ND, Not done

### 5.5 Discussion

The threat of avian H9N2 strains, and for that matter any avian influenza subtype, becoming a pandemic virus is ever-present. However, the key mechanism, human-to-human transmission is an obstacle yet to be achieved and a process we cannot predict. Insight into the mechanism behind efficient human-to-human transmission can aid in surveillance, antiviral therapies, vaccine production and quick reaction/response to outbreaks. Previous studies have compared avian influenza viruses with early pandemic strains, particularly H1 and H3 strains, and studied the particular “adaptive mutations” that lead to respiratory droplet transmission using different mammalian and avian animal models (Garcia-Sastre and Whitley, 2006; Naeve et al., 1983; Taubenberger et al., 2007; Tumpey et al., 2007). These and other studies have confirmed the importance of  $\alpha 2,6$  receptor specificity for sustained transmission in humans (and ferrets) and defined position 226 in the RBS of HA as a key component in influenza host range (Matrosovich et al., 2000; Stevens et al., 2006). Our laboratory has recently shown that L226 in the RBS of the HA of H9 viruses also plays a crucial role in replication in ferrets; however, these viruses have yet to gain the ability to transmit by either respiratory droplets or aerosol (Wan et al., 2008).

Our study is the first to describe respiratory droplet transmission of an H9N2 influenza virus in ferrets and pinpoint the minimal changes necessary for respiratory droplet transmission in this model. It is important to note that this particular strain has

yet to establish itself in a mammalian host however after only ten passages of nasal washes we were able to establish infection and sustain respiratory droplet transmission that was reproducible in multiple studies. More importantly, we identified and located changes that dramatically alter the antigenicity of the virus, bringing to light the inherent limitations in the selection of vaccine seed stocks for avian H9N2 viruses and the possible inefficiency regarding the seed stock selection of other avian influenza strains. Our studies show that respiratory droplet transmission in mammals is not an exclusive property of the few virus subtypes that have caused human pandemics (namely H1, H2 and H3 influenza viruses). Other virus subtypes, like the H9N2 virus, can “learn” to transmit in a similar manner provided the ideal host and environmental conditions.

## Chapter 6: Conclusions

### 6.1 Conclusions from Dissertation Research

#### **6.1.1 Quail as intermediate hosts**

Our work with the A/mallard/Potsdam/178-4/83 (H2N2) virus suggests that its adaptation in quail creates a virus with expanded host range to other land-based birds. Adaptation of mall/178 did not increase the pathogenicity of the virus in quail however it gained the ability to efficiently replicate and transmit in chickens. The molecular changes that occurred during this quail adaptation were essential for its expanded host range to chickens, supporting the role of quail as a necessary intermediate host. Reverse genetics work highlighted a single amino acid change in the HA of qa-mall/178 as being a critical component of transmission in quail. However, all six amino acid changes are necessary for the transmission phenotype in chickens, indicating that quail were a necessary intermediate for the virus to gain replication and transmission phenotypes in chickens. Although a second adaptation of mall/178 lead to transmission in quail, the adaptive changes did not support transmission in chickens, indicating that adaptive mutations within a single virus can vary dramatically and that there is no categorical way in which mallard influenza viruses adapt in a particular host. This does not mean that the second adaptation in quail, qa-mall/178<sub>2</sub>, could not expand its host range to other

terrestrial species, however it was not investigated in this study. Regardless, the repeat adaptation does point to critical adaptive changes in the internal proteins during the initial adaptation that support transmission in chickens.

Further adaptation of qa-mall/178 in chickens created a virus with altered tropism and temperature phenotype, due to a 27 amino acid deletion in the stalk region of the NA. This deletion can support tropism and temperature phenotype changes even within the mallard backbone. It is important to note that the adaptive changes, which took place during the quail adaptation, were maintained through seven passages in chickens, confirming their importance in establishing this virus in the chicken and supporting the concept that the changes are biologically relevant in the adaptation of aquatic avian influenza in terrestrial birds. This data supports other observations of NA deletions during influenza adaptation in chickens and is the largest naturally occurring NA deletion of the N2 subtype to date.

There is little doubt that terrestrial bird species play a role in the expansion of influenza host range from the aquatic reservoir. In the past decade numerous outbreaks of avian influenza in Asia and Europe highlight the need to better understand the role that terrestrial birds play in the ecology of influenza evolution. Our findings support the role of quail as an intermediate host in the adaptation of a mallard influenza virus and highlight the need for a better understanding of the mechanism surrounding influenza adaptation and its host range expansion.

### **6.1.2 Avian H9N2 influenza A viruses; evaluation of pandemic potential**

The threat of avian H9N2 strains, and for that matter any avian influenza subtype, becoming a pandemic virus is ever-present. However the key mechanism, human-to-human transmission, is an obstacle yet to be achieved and a process we cannot predict. Insight into the mechanism behind efficient human-to-human transmission can aid in surveillance, antiviral therapies, vaccine production and quick reaction/response to outbreaks. Studies performed on avian influenza and early pandemic strains have confirmed the importance of  $\alpha$ 2,6 receptor specificity for sustained transmission in humans (and ferrets) and defined position 226 in the RBS of HA as a key component in influenza host range. We have recently shown that L226 in the RBS of the HA of H9 viruses also plays a crucial role in replication in ferrets; however, these viruses have yet to gain the ability to transmit by either respiratory droplets or aerosol.

Our study is the first to describe respiratory droplet transmission of an H9N2 avian-human reassortant influenza virus in ferrets and pinpoint the minimal changes necessary for respiratory droplet transmission in this model. It is important to note that this particular strain has yet to establish itself in a mammalian host however after only ten passages of nasal washes we were able to establish infection and sustain respiratory droplet transmission that was reproducible in multiple studies. More importantly, we identified and located changes that dramatically alter the antigenicity of the virus, bringing to light the inherent limitations in the selection of vaccine seed stocks for avian

H9N2 viruses and the possible inefficiency regarding the seed stock selection of other avian influenza strains. Work needs to be done within the H9 subtype, as well as other threatening subtypes i.e. H5, H7, to determine if similar changes observed in the RCP10 virus can support respiratory droplet transmission in other viruses and determine if changes, like those found in amino acid 189 should be incorporated into vaccine strains.

Our studies show that respiratory droplet transmission in mammals is not an exclusive property of the few virus subtypes that have caused human pandemics (namely H1, H2 and H3 influenza viruses). Other virus subtypes, like the H9N2 virus and possibly the H5 and H7 subtypes can transform in a similar manner provided the ideal host and environmental conditions.

## 6.2 Future Prospects

### **6.2.1 H2N2 project**

Work still needs to be done to clearly define the mechanism of the NA deletion. It is imperative that the molecular side of this project is performed to tie in the function of intermediate hosts in adaptive changes to viral genomes and the role each change plays. Insight into why regions of genes are more prone to adaptive mutations can aid in viral surveillance and a better understanding of viral-host interactions. Studies that need to be



performed include: 1) determining the mechanism that supports tissue tropism of the NA deletion in the lung and whether this change allows for increased host range to mammalian species, 2) identifying the minimal NA stalk deletion necessary for the changes in tropism and temperature phenotype, 3) characterization of the NA deletion in the mallard quail and chicken backbones *in vitro*, and 4) determine if the second adaptation in quail, qa-mall/178<sub>2</sub>, supports replication and/or transmission in other terrestrial bird or mammalian species.

#### **6.2.2 H9N2 project**

The newly isolated ferret-adapted P10 virus will have numerous applications. Critical studies to be performed include: 1) defining the host range of the virus (whether it can transmit in swine and terrestrial birds), 2) determining whether the T189A change on HA<sub>1</sub> can alter antigenic profiles of other avian influenza subtypes, 3) identifying the level of protection the current H9N2 vaccine stock provides for a RCP10 infection, 4) defining the potential role of a RCP10-like virus as a vaccine candidate, and 5) determining if the key combination of H183, A189, E190, L226 leads to aerosol transmissibility in other avian influenza subtypes.

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