VIRUS ORIGIN INFLUENCES TRANSMISSION EFFICIENCY AND FREQUENCY OF AMINO ACID EXCHANGES IN THE GLYCOPROTEINS OF LPAIV ISOLATES DURING SERIAL PASSAGE IN DUCKS

by

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(Under the Direction of Ralph Tripp)

ABSTRACT

Studies focusing on evolution of low pathogenic avian influenza virus (LPAIV) are critical in understanding the continual circulation of these viruses. In this study, changes in the genetic composition of the hemagglutinin and neuraminidase glycoproteins as a result of transmission of LPAIV to a new host under experimental conditions were investigated. LPAIV isolates of chicken and wild bird origin were used to establish a serial passage transmission model in Pekin ducks. LPAIV isolates of divergent origin displayed differences in transmission capabilities and shedding patterns. Passage of the viruses in ducks resulted in a higher frequency of amino acid mutations in the hemagglutinin of the chicken isolate in comparison to the wild bird isolate. Relative genetic stability of the NA protein of both LPAIV isolates was observed after passage in ducks. Identifying the genetic adaptations surrounding transmission of LPAIV in new hosts may help to understand the selection contributing to the evolution and circulation of these viruses.

INDEX WORDS: Low pathogenic avian influenza virus, transmission, hemagglutinin, neuraminidase

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DEDICATION

I would like to dedicate this thesis to my parents. Thank you for providing me with the tools to be successful, but giving me the freedom to do it on my own. I especially want to dedicate this to my mother who worked so hard and gave so much of herself to make sure her children had the opportunities to succeed in life. Of course, none of this would be possible without the patience, support, and encouragement from my amazing husband, Scott.

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CHAPTER 1

INTRODUCTION

Influenza A virus has been a part of our existence for centuries. It has been responsible for mild seasonal illnesses experienced by thousands of individuals each year as well as intermittent pandemics of devastating proportions. Highly pathogenic influenza A viruses (HPAIV) have caused four pandemics within recent history, including the catastrophic 1918 "Spanish flu", the pandemics of 1957 and 1968, and the most recent 2009 pandemic. Although the origins of these pandemic viruses have differed, the resulting effect in each case is the introduction of a novel virus to an immunologically naïve population. In poultry, influenza A virus has caused significant economic losses to producers around the world. It was not until 1997 that the first case of a human infection with influenza A virus through direct transmission from infected poultry was reported (1). This highly pathogenic H5N1 avian influenza A virus has continued to sweep through countries of Southeast Asia and eastward, devastating poultry flocks with intermittent spillover cases in humans. Fortunately, the lack of human-to-human transmission of influenza A virus has limited the distribution of highly pathogenic strains among the population.

The virus family *Orthomyxoviridae* contains the genera *Influenza A virus*, *Influenza B virus*, *Influenza C virus*, *Isovirus*, and *Thogotovirus*. This family of viruses is characterized by a single-stranded, segmented, RNA genome of negative polarity. While humans are affected by influenza A and B virus, and to a lesser degree influenza C virus, other host species are affected only by influenza A virus. The eight genomic segments of influenza A encode for up to 12 different proteins. The three genera of

influenza viruses are distinguished by their internal nucleoprotein and matrix 1 protein (2). Influenza A and B viruses are classified into subtypes based on the properties of their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To date, 17 HA subtypes (H1-H17) and 9 NA subtypes (N1-N9) have been identified (3).

Reassortment and high mutation rates resulting from error-prone replication have been responsible for the vast reservoir of influenza A virus strains. Such mechanisms have allowed influenza virus to produce strains containing genomic constellations advantageous to their continual circulation. Field surveillance has confirmed wild bird species, especially waterfowl, as the primary reservoir of low pathogenic influenza virus (LPAIV). Host specificity of influenza A viruses has been well-documented, but intermittent transmission between avian species as a result of mutation and/or reassortment has led to numerous outbreaks in populations naïve to the resulting novel viruses. The ability of influenza A viruses to mutate into highly pathogenic strains after introduction of wild bird isolates into naïve poultry species poses a significant threat to the human population. Understanding the mechanism of transmission between avian hosts is crucial to understanding the continual circulation of these viruses and potential control measures. The primary aim of this study was to investigate the transmission dynamics of three LPAIV isolates in ducks to potentially identify genomic changes associated with host adaptation.

During transmission and adaptation to new avian hosts, genomic changes in the two surface glycoproteins, HA and NA, are of particular interest. They contribute significantly to the characterization of individual strains. The mediation of viral attachment to host cellular receptors by the HA of influenza A viruses renders it the key determinant of host tropism. Influenced by host immune pressure, HA is the most antigenically diverse protein of the virus. Alterations in the genetic composition of hemagglutinin have been suggested to contribute to changes in pathogenicity, host adaption, and transmission of

influenza A viruses. The release of virions from cellular receptors is mediated by the NA of influenza A viruses to disseminate the virus within the host. A variation in the stalk length of NA has been observed between wild aquatic birds and domestic poultry. It is suspected that this variation plays a role in host adaptation by balancing changes in receptor binding affinity due to mutations within the HA (4). The exact mechanism of this modification is not entirely understood, but a recent study suggests it may be the result of selection of a minor virus subpopulation after passage of the virus in a new host (5). As part of the aim of this research, attempts were made to determine if influenza A viruses isolated from poultry harbor a minor virus subpopulation containing a variation in the NA stalk length, which could be selected for after passage of the virus in waterfowl. Investigating the NA stalk length after passage in waterfowl may help to understand the role of length variations in inter-specie transmission.

CHAPTER 2

LITERATURE REVIEW

History

Cases of influenza virus-like diseases have dated back to the Middle Ages (6). In 1878, an Italian veterinarian, Edoardo Perroncito, was the first to clinically describe what would later be termed highly pathogenic avian influenza (HPAI) as a contagious disease of poultry resulting in high mortality (7). At the time, Perroncito coined the disease "fowl plaque" believing it was related to fowl cholera. Two years later, the disease was determined distinct from fowl cholera based on its clinical and pathological properties (8). In 1901, the causative agent of this poultry disease was identified as a virus (9). That same year, an outbreak of the virus hit poultry farms in Italy and spread north where it remained endemic for several decades in Austria, Germany, Belgium, and France (10). The United States saw its first outbreak of the virus in a live bird market in New York City where the virus subsequently spread throughout the eastern shore and the Midwest (11). Outbreaks of HPAI in poultry during the mid 1930's were running concurrent with Newcastle Disease virus (NDV). It wasn't until 1942 that HPAIV was determined serologically distinct from NDV, albeit having similar clinical presentation (12). In 1955, the etiologic agent of this poultry disease was identified as a type A influenza virus (13). In 1981, at the first International Symposium on Avian Influenza, the term fowl plague was appropriately replaced with the term, highly pathogenic avian influenza (14).

The first identification of avian influenza virus (AIV) in wild birds was from a mass mortality of common terns in South Africa in 1957 (15). The event sparked an interest in the potential role of

influenza virus in animals as the origin of human outbreaks (16). A global serological surveillance study resulted in the identification of 21 species of wild birds harboring low pathogenic avian influenza virus (LPAIV) (17). Most species were within the orders *Anseriformes* and *Charadriiformes*, suggesting a role of these aquatic birds in AIV epidemiology. Currently, 105 species from 26 families of wild birds have been identified as AIV reservoirs, with waterfowl still constituting the majority (18, 19). Shortly after this surveillance, less virulent forms of AIV were also being isolated from domestic poultry, including H5 and H7 subtypes, refuting the previously popular dogma that all H5 and H7 subtypes were of high pathogenicity (20). Although the majority of H5 and H7 strains are of low pathogenicity, AIV isolates that have been identified as highly pathogenic have only been of H5 or H7 subtypes. The role of LPAIV in the evolution of HPAIV was exemplified in 1983 when a LPAIV H5N2 chicken isolate circulating in Pennsylvania mutated into a highly pathogenic strain, resulting in the death of 17 million chickens and turkeys (21). Although the predecessor LPAIV strain already contained a polybasic cleavage site (typical of HPAIV), it required only one amino acid change to alter the glycosylation of HA that led to the increased pathogenicity (22).

After determining that multiple subtypes of AIV could be found in a wide range of hosts, the classification system of influenza viruses that was originally based on the host species was revised. It established a nomenclature based on the antigenic properties of HA and NA regardless of the species of origin (23). The unified system of classification that is still used today is based on type of virus/host (if not human)/location of isolation/isolation number/year of isolation and HA-NA subtype labeled in parenthesis, for example (A/chicken/Pennsylvania/1370/1983(H5N2). Since its first clinical description, numerous global outbreaks of HPAI have resulted in multiple human infections, the loss of millions of birds, as well as billions of dollars in controlling the spread of the virus. The first human case resulting from direct transmission of HPAI H5N1 from infected poultry was in Hong Kong in 1997, resulting in 6 deaths of the 18 confirmed cases (1). The reemergence of this virus in 2003 rapidly spread across

several countries and continues to cause incidental spillovers into humans. There are currently 597 confirmed cases, of which 351 have resulted in death (24). HPAI H5N1 remains endemic in Bangladesh, Indonesia, Vietnam, China, and Egypt, but cases continue to be identified in additional countries (25). Incidental human cases of H7 and H9 AIV isolates have also been identified, suggesting a risk of these subtypes to cross species boundaries (26, 27).

Within the human host, influenza A viruses cause both seasonal, endemic infections as well as sporadic, pandemic-level outbreaks. Seasonal endemic outbreaks within the United States have resulted in an average of 200,000 hospitalizations per year and a total of roughly 36,000 deaths, most of which have been associated with elderly, very young, and immune-compromised individuals (28). At the pandemic level, influenza A viruses have caused millions of deaths. The most extensive pandemic was the "Spanish Flu" in 1918, an H1N1 virus likely of avian origin that caused over 20 million deaths worldwide. In 2005, the genome of this virus was completely sequenced using archived lung tissue specimens from victims of the pandemic (29, 30). Researchers are currently using this reconstructed genome to identify the source of the virus as well as features contributing to its extreme virulence (29, 31). After the pandemic, the H1N1 strain continued to circulate in humans until it was replaced in 1957 with the "Asian" H2N2 pandemic strain. It has been determined that while the majority of the H2N2 virus was of human origin, the HA, NA, and PB1 genes were derived from an avian source (32). In 1965, researchers Tumova and Pereira (33) discovered that the neuraminidase of the H2N2 "Asian" strain was identical to the neuraminidase isolated from a turkey, identifying for the first time genetic interaction between influenza viruses of human and animal origin. One year later, Laver and Kilbourne (34) were able to support the premise of genetic reassortment by successfully infecting cells with two different viruses, H1N1 and H2N2, to obtain a hybrid virus containing genes from both strains. Reassortment of influenza A viruses has since contributed to two additional pandemics. In 1968, the H3N2 pandemic virus originating in Hong Kong contained an HA and PB1 derived from an avian source (32, 35).

Reassortment of human, avian, and swine influenza A strains followed by subsequent circulation of the virus in pigs resulted in the most recent 2009 “swine” H1N1 pandemic (36). The human H1N1 virus, after it re-surfaced in 1977, as well as the H3N2 “Hong Kong“ virus continue to circulate within the human population (37). A continual surveillance of influenza virus by the Global Influenza Surveillance Network (GISN) through the World Health Organization (WHO) has been developed to predict the emergence of novel circulating strains used in annual vaccine development (38).

Viral structure

Influenza A virus is a pleomorphic, enveloped virus. Its lipid envelope is derived from the host plasma membrane upon budding of the virion from the lipid raft domains of the infected cell (39, 40). Viruses may bud from cells as either spherical (80-120nm) or filamentous (~100nm x 2-20μm) virions (41, 42). Filamentous morphology is typical of clinical isolates, whereas laboratory-passaged viruses have predominantly spherical morphology (43, 44). It has been reported that viruses of both shapes contain only one copy of each segment of the viral genome and are equally infectious (45, 46). Anchored within the lipid envelope of the virus are two glycosylated surface proteins giving rise to the characteristic “spikes” of the virus, the rod-shaped hemagglutinin (HA) and mushroom-shaped neuraminidase (NA) . The third integral membrane protein of influenza A virus is the matrix protein 2 (M2), forming a transmembrane proton-selective ion channel. The matrix protein 1 (M1) peripherally lines the inner portion of the envelope via electrostatic interactions (47). Filamentous formation is a genetic trait that has been mapped to the M1 protein (48-50). Calder et al. (51) have shown that the helical pitch of M1 is different between spherical and filamentous strains. Using reverse genetics, Bourmakina and Garcia-Sastre (52) were able to determine that single amino acid mutations in the M1 gene conferred filamentous budding and morphology in an otherwise spherical isolate, and that both the C-terminus and N-terminus of the M1 gene contributed to the altered morphology. The

polymerization of the M1 helix has also been proposed to be the driving force of virion budding (51). Cholesterol-induced conformational changes within the M2 helix as well as direct interactions of M2 with the M1 protein are suggested to contribute to filamentous formation of the budding virus (53-55). The matrix protein in other enveloped, negative sense, RNA viruses have demonstrated a similar role in viral assembly and budding (56-58). It is suggested that the two surface glycoproteins, HA and NA, contribute to assembly and budding of progeny virions through interactions of their highly conserved cytoplasmic tails with the inner M1 protein, but this concept has been debatable (59-65). Variations of filamentous morphology dependent on cellular polarization phenotype and the integrity of host actin filament has indicated that non-viral, host cellular factors may also influence viral morphology (46). Although the source of viral shape has been examined, the contributing role of filamentous versus spherical morphology in virus pathogenesis remains to be elucidated.

Viral Genome

Influenza A viruses belong to the virus family of *Orthomyxoviridae*, an assemblage of viruses characterized by their segmented, negative-sense, RNA genome. The genome of influenza A virus consists of eight, single-stranded, RNA segments of negative polarity. Early studies identifying recombination between virus strains suggested a segmentation of the viral genome (66). Sucrose gradient analysis, polyacrylamide gel electrophoresis, and temperature sensitive mutants were later used to confirm this assumption (67-69). These eight segments encode for up to 12 proteins, two of which (PB1-F2 and the recently identified PB1 N40) have not been identified in all strains. The remaining ten proteins, PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NEP are present among all strains of influenza A viruses.

Each of the eight viral RNA (vRNA) segments of influenza A virus are encapsidated as a ribonucleoprotein (RNP) complex containing a stoichiometric ratio of RNA binding nucleoprotein (NP) to

the three proteins of the polymerase complex, PB2, PB1, and PA (70-72). The RNP complex is the minimal unit responsible for replication of its associated vRNA and transcription of viral mRNA (73). The eight RNP particles containing their individual genomic segment, each of varying length and molecular weight, form a tapered assembly at the apical end of each budding progeny virion (51, 74, 75). Within the RNP complex, the vRNA folds back onto itself in a left-handed double helical hairpin conformation, leaving the terminal ends of the vRNA free to associate with the polymerase complex at the opposite end of the hairpin loop (72). The terminal ends of the genome contain partially complimentary, highly conserved 5' and 3' noncoding regions (NCR) of 13 and 12 nucleotides, respectively (76). These regions form a secondary panhandle structure that is thought to act as the promoter region responsible for the activation of replication and transcription of the viral genome (77). These conserved NCRs also serve as a signal for polyadenylation of mRNA and may play a role in packaging the RNPs into progeny virions (78-80).

Viral Proteins

The RNA-dependent RNA polymerase of influenza A virus is a heterotrimer complex composed of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and the polymerase acidic protein (PA). PB1 and PB2 are the two largest viral proteins, encoded by the two largest genomic segments, each constituting 2341 nucleotides. Segment 1 of influenza A virus encodes for the PB2 protein. The protein contains a binding site for PB1 within its amino (N)-terminal region, and at least two binding sites for the NP protein, one within its carboxyl (C)-terminal and one within its N-terminal region (81, 82). The N-terminal NP binding site overlaps the PB1 binding site, suggesting possible competition between the two proteins for PB2 binding (82). When using N-terminal truncated viruses to analyze this competitive relationship for PB2 another, less stable PB1 binding site was identified near the C-terminal region of PB2 (82) The PB2 protein has been identified as capable of recognizing and

binding the 5' 7-methyl-guanosine (m^7G) cap of the host mRNA used by the virus to initiate transcription (83, 84). PB2 may also play a role in host range determination. Single amino acid substitutions at residue 627 and the more recently observed substitution at residue 701 have been implicated in the adaptation of the AIV to mammalian hosts (85-87).

Segment 2 of influenza A virus encodes for the PB1 protein. This protein represents the backbone of the polymerase complex, with a C-terminal binding site for PB2 and a N-terminal binding site for PA (81). Its role in nucleotide polymerization and elongation makes it the true polymerase protein of the complex (88, 89). After binding of the host mRNA cap structure by PB2 and subsequent endonuclease cleavage by PA, the 5' fragment is used by PB1 as a primer to synthesize viral mRNA (90, 91). PB1 contains four conserved motifs that are present among other RNA-dependent RNA polymerases (92). Mutations within these conserved regions abolish the transcriptional activity of the virus, indicating the protein's significance (93). Regions within both the C-terminus and N-terminus of this protein have been described as binding sites for the panhandle structure of the vRNA template. Binding of PB1 to the panhandle of the cRNA template has been localized to regions of the N-terminus as well as to residues within the central region of the protein (94, 95).

A shift in the 5' end open reading frame of the second segment of the virus encodes for an additional, overlapping PB1 protein, PB1-F2 (96). The majority of influenza A virus strains express this small 90 amino acid protein. It has been identified as possessing pro-apoptotic activity, and that when overexpressed may alter virus pathogenicity (97). Multiple studies have shown that a single point mutation (N66S) within the PB1-F2 contributes to the virulence of the virus (a phenotype seen in both HPAI H5N1 and the 1918 pandemic strains) by interacting with the mitochondrial antiviral signaling (MAVS) protein to inhibit the induction of interferon. Most recently, a third polypeptide encoded by the PB1 mRNA has been identified. PB1-N40 is an N-terminally truncated form of the protein arising from

an alternate start codon (98). No function has yet been associated with this third protein. Although considered not essential for virus replication, absence of this protein has led to slower virus replication kinetics (98).

The PA protein may be the least understood subunit of the polymerase complex, although it is clear that its presence is required for the expression of vRNA in systems reconstituted in vivo (99). The third viral RNA segment encoding PA is only slightly smaller than the other polymerase genes, with 2233 nucleotides in length. Several functions for PA have been proposed, including contributions to polymerase complex formation, vRNA replication, and proteolytic activity encoded within its N-terminus (100-102). More recently, PA has been implicated in the cap-snatching endonuclease activity of the virus, a function previously thought to reside in the PB2 or PB1 protein (103-105). The endonuclease active site is contained within the N-terminal domain of the protein, a region also critical for protein stability, nuclear translocation, and vRNA promoter binding (103, 106). Binding of PA to the PB1 protein is localized within the C-terminus of the PA protein.

The critical role of the hemagglutinin (HA) protein in binding to host cell receptors, virus internalization, and fusion of the viral membrane with the host membrane renders it the key antigenic protein of influenza A viruses. Defined by their reactivity to antisera and sequence analysis, 17 HA subtypes have been described (H1-H17) (3, 107). The most recent HA subtype has been identified in yellow-shouldered bats found in Guatemala (3). The divergence of these sequences from other known HA sequences suggests a 17th HA subtype. From the viruses obtained from bats, the NA sequences also deviate from the nine known NA sequences, suggesting the possibility of a novel NA subtype as well.

The HA protein exists as a homotrimer on the surface of the virus. Each full length, single chain, precursor monomer (HA0) is held in the viral membrane by a hydrophobic transmembrane anchor near its C-terminus (108). The stop-transfer signal of the protein has been localized to the transmembrane

domain (TMD), indicating its role in positioning the protein within the lipid bilayer of the cell membrane (109) . The TMD not only contributes to stability and transport of the protein, but it has also been suggested to play a role in the association and assembly of the three HA0 subunits into their trimer form (110).

Cleavage of the HA0 precursor form of HA is essential in membrane fusion and viral infectivity. The cleavage peptide that is located within a prominent loop extending from the surface of the HA0 molecule is recognized and cleaved by host cellular proteases (111). Recognition of this sequence by different proteases contributes significantly to the virulence of the virus. The resulting protein is cleaved into its two active subunits, HA1 and HA2, which are bound to each other by only a disulfide bond. The HA1 subunit constitutes the membrane distal portion of the protein, consisting of a globular head that contains both receptor-binding and antigenic sites (112). Terminating sialic acid (N-acetyl neuraminic acid) residues of host cellular glycoproteins or glycolipids attach to the receptor binding domain that is formed by a shallow pocket on the distal end of the protein (113). Several amino acid residues in this domain, including Try 98, His 183, and Trp 153, are largely conserved among the majority of HA subtypes, especially among avian influenza viruses (114). Additional, amino acids positioned behind the pocket acting to stabilize the structure of the binding site are also conserved (114-116). Linkage of sialic acid to its adjacent saccharide by either an α2,3 bond or an α2,6 bond plays a significant role in tissue tropism and species preference of the virus (114, 117-119). It is proposed that substitution within amino acids 190, 225, or 226 of the receptor binding domain of HA correlates with altered binding specificity (31, 118, 120, 121). Human and avian influenza A viruses have also shown variability in the glycosylation of their respective hemagglutinin protein, possibly attributing to host selection (114, 122-124). The membrane proximal portion of HA1 and all of HA2 comprise the stem portion of the protein, forming a trimeric coiled coil of α-helices that extends 76 Å from the viral membrane (125). Polar and non-polar interactions between the three long α-helices of each stem provide the main forces

stabilizing the trimer (126). Cleavage of the precursor HA0 produces a highly conserved fusogenic domain (fusion peptide) at the N-terminus of the HA2 protein that is critical for membrane fusion and infectivity of the virus (127-130). Uncleaved HA is able to mediate virus attachment but cannot initiate the fusion step required for infectivity (131). Once phagocytized by the host endosome a pH-induced conformational change of the HA protein is responsible for the membrane fusion and subsequent release of the virus' genetic material into the host cell (132). This process is discussed later in the replication section. The hemagglutinin is the prime target for virus-neutralizing antibodies. Most neutralizing antibodies generated against HA target the exposed loops that surround the receptor-binding site, interfering with virus attachment (108, 113, 133). The high variability of the amino acid residues within these neutralizing epitopes hinders the development of universal vaccines based on HA, thus requiring HA subtype-specific antibodies (134-136).

In addition to the heterotrimer polymerase complex, the nucleoprotein (NP) encoded by the fifth RNA segment constitutes a structural component of the vRNP complex (137). It is a type-specific protein, responsible in part for antigenically classifying A, B, and C-type influenza viruses (138). The protein is predominantly α-helical and each monomer has a curved, crescent –like shape (139). NP is rich in arginine, glycine, and serine, thus carrying a net positive charge that contributes to its interaction with the negatively charged backbone of vRNA (140). Encapsidating vRNA and importation of the vRNP complex into the host nucleus for the purposes of RNA transcription and replication are the primary functions of the protein. The nuclear localization signal (NLS) within the nucleoprotein was first suggested to reside in the N-terminal region (141). Constructed mutants containing a deletion of this region did not completely abolish importation, suggesting that the protein contains an additional NLS (142). Regions within the C-terminal tail loop of the protein have been linked to the self-association of NP molecules to form oligomers, playing a role in maintaining the RNP structure (72, 139, 143).

The stoichiometric interaction of NP with vRNA is estimated at one NP molecule per twenty-four nucleotides of vRNA (75). The nucleoprotein binds to vRNA with high affinity, but with minimal sequence specificity, suggested by a lack of homology to previously characterized RNA-binding motifs (144, 145). Although the N-terminal region of the protein has shown to display RNA-binding activity, additional studies have identified other regions distributed throughout the protein to be responsible for high-affinity binding (146, 147). In addition to vRNA, NP directly binds to PB2, PB1, NS1, and M1 (148-150). The association of NP with M1, along with the viral NEP protein and the host's nuclear export receptor (CRM1), is thought to contribute to the nuclear exportation of vRNPs during the late phases of infection (151-153). It is also believed that interactions between NP and M1 are important for virion assembly (154, 155). In addition to binding vRNA and numerous viral proteins, the nucleoprotein accomplishes many biological functions through interactions with host cellular peptides. These include the nuclear import receptor, importin α, filamentous actin (considered to play a role in cytoplasmic accumulation), and the DEAD-box helicase BAT1/UAP56 (a known cellular splicing factor) (142, 145, 156). Another potential role of nucleoprotein is its influence on the polymerase's switch from mRNA transcription to genome replication, of which several hypotheses for this function exist (157-160). The nucleoprotein of influenza A viruses may be phosphorylated at up to one serine residue per molecule, predominantly at the highly conserved third N-terminal serine residue (161, 162). Phosphorylation patterns have shown to be strain-specific, and often change throughout replication (163). In studies using viruses containing an NP that had been treated with protein-kinase inhibitors, Neumann et al. (164) were able to suggest a potential role of phosphorylation in RNP trafficking.

The lack of substantial selective pressure on NP as an internal protein has contributed to its conserved nature, and thus it is a potential target in diagnostics and control. The conserved regions of the tail loop involved in oligomerization, as well as the presumed vRNA-binding groove located between the head and body domains of the protein may provide attractive drug targets (165). Many peptides

encoded by these sequences have been identified as epitopes of both subtype-directed and cross-reactive influenza A-specific cytotoxic T lymphocytes (CTL), although amino acid exchanges within these epitopes have recently been identified in escape mutants (166-169).

Segment 6 of influenza A virus encodes for the neuraminidase (NA) protein. Along with the hemagglutinin protein, the NA determines important antigenic properties of each virus. To date, nine subtypes of NA have been identified, N1-N9 (170). NA, which is present on the virus surface in smaller numbers than the hemagglutinin protein, is a class II transmembrane glycoprotein anchored into the viral membrane via its hydrophobic N-terminus (171, 172). The protein is a homotetramer, taking on a mushroom shape characterized by a large globular head supported by a narrow stalk. It has been determined through X-ray crystallography that each monomer of the protein is composed of six, four-stranded antiparallel β-sheets arranged in a propeller-like configuration (173). Loops formed between each β-sheet within the head of the protein contain enzymatically and antigenically important amino acid residues. The active site of each subunit is contained within a deep pocket on the surface of the protein, located directly above each central β sheet (174, 175). A large number of amino acids lining the active site are conserved with respect to a majority of characterized isolates (173, 176, 177). Eight of these invariant residues have been identified to directly bind the sialic acid substrate, and an additional ten play a role in stabilizing the active-site structure (178). The head of NA contains the full enzymatic properties of the protein, as evidenced by studies in which cleavage of the head produced antigenic properties indistinguishable from the properties of intact virus (179, 180). Enzymatic activity of influenza viruses was first recognized by Hirst et al. (181). It has since been determined that the sialidase property of the NA protein is responsible for this enzymatic activity (182). NA cleaves the α-ketosidic linkage between the terminal sialic acid and its adjacent residue, usually a galactose, on a carbohydrate chain (183). The liberation of sialic acid destroys virus receptors localized on host cell surfaces, preventing virus aggregates and allowing efficient release of newly formed virions (184). It has

been suggested that the sialidase activity of NA also acts to enable more efficient transmission of the virus to the respiratory epithelium by cleavage of sialic acid within mucosal secretions (185). The surface expression of neuraminidase subjects the protein to amino acid exchanges in response to selective immune pressure. Antibodies against this protein show little neutralizing effect (186).

Variability in both the number and sequence of amino acid residues have been identified in the NA stalk region, even within the same subtype (171, 187). It has been shown that alterations to the length of the NA stalk in the presence of particular HA constructs can regulate virus growth, suggesting a significant role in balancing the HA-receptor binding activity (122, 188-191). In addition to anchoring the protein, it has been proposed that the transmembrane domain of NA functions as both the translocation signal for lipid raft domain localization and the determinant for apical sorting of the protein in polarized host epithelial cells (192-194). The function of the six highly conserved amino acids of the cytoplasmic tail of NA has yet to be identified, but has been suggested to contribute to particle shape (60, 63, 195, 196).

Matrix protein 1 (M1) is encoded by segment 7 of the influenza A genome and consists of 252 amino acids (197). M1 plays a significant role in virus assembly and budding of progeny virions. Early studies demonstrated that M1, when expressed alone, was sufficient to form budding virus-like particles (VLP), although more recent work has demonstrated that trafficking of M1 to the plasma membrane requires an interaction with matrix protein 2 (M2) (198, 199). The role of M1 in assembly of virions is highlighted by its simultaneous association with the lipid envelope and the internal components of the virus. Interaction of M1 with the lipid membrane of the virus was first identified with the use of liposome interactions and hydrophobic photolabeling (200, 201). In contact with the lipid bilayer is a cluster of 20 hydrophobic residues located at residues 114-133 of M1, as well as a smaller group of 13 uncharged residues found closer to the N-terminal domain of the protein (200). The oligomerization of

M1 forms a shell that lines the inner surface of the viral envelope (202). The lining acts as a bridge between the vRNP and the integral membrane proteins through interaction with their cytoplasmic tails (198, 203). Binding of M1 to vRNP had been previously mapped to the C-terminal region of the protein (204). More recent evidence has linked the direct binding of vRNA to the middle domain of M1, a region containing the highly conserved ^{101}RKLKR^{105} motif (205-207). In addition to vRNA binding, M1's ^{101}RKLKR^{105} sequence is implicated in additional functions. It has been identified as the nuclear localization signal, a recruiter of the nuclear export protein of influenza A viruses (NEP, see below), as well as a contributor to viral morphology (48, 208, 209). Additional amino acid residues located within both terminal ends of M1 have also been implicated in viral morphology (49, 52). M1 modulates the directionality of vRNP transport between the nucleus and cytoplasm. Its late-stage nuclear association with NEP and newly formed vRNPs allows export of the genome into the cytoplasm for virus assembly, while its dissociation from mature vRNPs allows import of the genome into the nucleus upon infection (151, 152). Recently, an additional stretch of conserved arginine residues at positions 76 through 78 has demonstrated a role in intracellular trafficking of the protein, virus assembly, and virus budding (210). M1's association with vRNPs has been found to also inhibit vRNA transcription and/or replication (204, 211, 212). M1 is the most abundant and most conserved influenza A protein (213). For this reason, it has played a critical role in diagnostics by providing conserved regions used in RT-PCR to detect influenza A virus genomes from multiple species (214).

The matrix protein 2 (M2) is translated from a spliced mRNA of segment 7. At the 5' end of the M1 vRNA, a second overlapping reading frame encodes for 97 amino acids, establishing M2, a protein distinct from M1 (215, 216). Additional splicing events give rise to a third and fourth mRNA of nine and fifty-one amino acids, respectively (197). Although $mRNA_3$ is found in all viral strains and $mRNA_4$ exists in only a few, neither of these proteins' functions have been identified (217). Matrix protein 2 is a type III integral membrane protein abundantly expressed on the infected-cell surface, yet only a few of these

copiously expressed proteins are actually incorporated into each virion (218-220). It is suggested that M2 is transported to the apical membrane of cells via kinetics similar to the glycoproteins, although M2 is localized to non-raft domains of the host membrane (221). The protein is a homotetramer consisting of two dimers, each linked by a disulfide bond within their extracellular N-terminal domains (219, 222, 223). A left-handed, parallel bundle of four hydrophobic α-helices, one from each subunit, forms the characteristic transmembrane channel of the protein. It is this structure that is responsible for proton translocation, a function necessary for maturation of HA into its fusogenic form and dissociation of vRNP from the M1 protein (223-226). Identification of amino acid exchanges in the transmembrane domain of M2 in viruses resistant to the antiviral drug, amantadine, led researchers to believe that this domain constituted the pore of the channel (227). Several hydrophilic, pore-lining residues within the transmembrane domain are highly conserved and contribute to critical physiological functions of the channel. Within the tetramer, the imidazole side chains of each His-37 are clustered together to form the pH sensor, which is activated at acidic pH levels (228). Just one helical turn away from this residue is Trp-41 (229). The clustering of these residues' indole side chains form the conductance gate. A destabilizing conformational change resulting from protonation of His-37 residues directs the indole of each Trp-41 residue to rotate away from the pore, permitting H+ to pass. The C-terminal base and the N-terminal disulfide bonds of each M2 subunit stabilize the tetramer during proton conductance (230). In its native, neutral state, Trp-41 remains in a pore-blocking position (231). In this closed state, Trp-41 residues are stabilized through hydrogen bonds with Asp-44 of the adjacent M2 subunit (230).

M2 has been suggested to also maintain a threshold pH through the trans-Golgi network, in doing so preventing the premature activation of newly-synthesized HA glycoproteins during transport to the cell surface (232). In addition to stabilizing the tetramer, specific regions of the highly conserved cytoplasmic tail of the M2 protein have been shown to contribute to virus assembly. This has been suggested through an interaction of the protein with M1 that mediates the recruitment and packaging

of internal viral proteins and vRNA (53, 233). This interaction site with M1 has been mapped to residues 71-73 within the membrane proximal region of the M2 tail (233). Recent studies have suggested that the cytoplasmic tail of M2 contributes to filamentous virus morphology, although to a lesser degree than M1 (53, 55). Functional homologs of M2, with respect to ion channel activity, have been identified in both influenza B and C viruses (234, 235).

Like segment 7, the eighth segment of influenza A viruses contains a +1 open reading frame coding for more than one protein. The non-structural protein 1 (NS1) is translated from the full-length collinear mRNA of segment 8, while the nuclear export protein (NEP, originally termed NS2) is translated from the spliced mRNA (236). There is an overlap of 56 nucleotides at the 5' end of the mRNA that is shared by both proteins (237). NS1 consists of 230-237 amino acids and is divided into two distinct domains, an amino-terminal domain (residues 1-73), and a carboxy-terminal domain (residues 74-230) (238). The length of NS1 is strain-specific and variations arise from either a truncated or extended C-terminal tail (239). The dimerization of NS1 forms a cleft of α-helices along the N-terminal ends that is responsible for dsRNA binding (240, 241). Two highly conserved residues within this cleft, Arg-38 and Lys-41, are critical for RNA binding (240). The C-terminal "effector" domain mediates interactions between virus and host cell proteins (242). The NS1 protein is located predominantly in the nucleus, although it can also be found within the cytoplasm (243). One conserved nuclear localization signal (NLS) is found within the N-terminal domain of NS1. Depending on the strain, a second NLS may be found within the C-terminal domain of the protein (244).

NS1 is a multifunctional protein mediating a number of regulatory activities. The protein is a key virulence factor of influenza A viruses for its effect in suppressing the antiviral response elicited by the host cell. This suppression is mediated by counteracting the host interferon (IFN) production (245). It has been shown that NS1 intercedes IFN-β production through pre-transcriptional mechanisms and/or

post-transcriptional mechanisms that seem to be strain specific (246). Pre-transcriptionally, the protein can limit IFN production by preventing activation of the molecule's transcription factors, IRF-3, NκFB, and c-Jun (247, 248)). Using the PR8 strain, NS1 has also been shown to block IFN-β by forming a complex with the pathogen recognition receptor, RIG-1 (249). Post-transcriptionally, NS1 suppresses IFN-β by inhibiting host pre-mRNA processing as well as inhibiting transport of polyadenylated cellular transcripts across the nucleus (250). NS1 also directly blocks the functions of protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (OAS) by preventing a required conformational change of the PKR molecule and by out-competing OAS for interaction with dsRNA (251, 252). In addition to inhibiting IFN production, NS1 also regulates the antiviral response by suppressing the induction of RNA interference, limiting the expression of several genes involved in dendritic cell maturation and migration, as well as its own anti-apoptotic activity (253-255). NS1 promotes influenza propagation through interaction and activation of the phoshatidylinositol 3-kinase (PI3K) as well as through its own selective ability to increase translation. This is accomplished through interactions with the cellular translation initiation factor, eIF4GI, the poly-A-binding protein (PABPI), and possibly the tubulin-binding protein, hStaufen, to initiate viral mRNA translation without affecting host mRNA (256-258). NS1 has also been implicated in controlling viral mRNA splicing of both segment 7 and its own segment of the influenza A genome (217, 250).

As previously mentioned, NEP is translated from the second open reading frame of segment 8 (236). It is the smallest of the influenza A proteins with just 121 amino acids (237). Based on the identification of a nuclear export signal (NES) within the protein and its capability to bind M1, which in turn binds vRNP complexes, it was proposed that NEP acts as an adaptor between vRNP complexes and the nuclear export machinery (152, 259). O'Neill et al. (259) supported this postulation by determining that NEP has nucleoporin-binding activity, and that it could functionally replace the nuclear export effector domain of the HIV-1 Rev protein. After determining this structural function of the protein, its

name was appropriately changed from non-structural protein 2 (NS2) to nuclear export protein (NEP). It has been determined that the interaction of NEP with the nuclear export machinery is through direct binding with the host-cellular nuclear export receptor, CRM1 (260). The ternary export complex of a cell is composed of CRM1, RanGTP (as an energy source), and the export substrate (261). RanGTP-dependent binding to CRM1 is mediated by the leucine-rich NES of NEP located within the N-terminal domain of the protein (259, 260). This study supported the role of NEP in the export of vRNPs from the nucleus into the cytoplasm, where they assemble into progeny virions. Influenza B and C viruses also contain nuclear export proteins that function in a process analogous to the NEP of influenza A virus (262). A suggested second role of NEP is regulation of replication and transcription of the influenza A genome by altering the expression level of each RNA product, but further studies are needed to confirm this (263).

Replication

The replication cycle of influenza A viruses is a complex system dependent upon both viral and cellular machinery. The viral replication cycle is initiated by virus attachment to the host cell and progresses through endocytosis, fusion of viral and cellular membranes, uncoating of vRNP and its nuclear transport, transcription and replication of the genome, cytoplasmic translation, post-translational processing of viral proteins, transport and assembly of individual proteins at the plasma membrane, and eventually budding of the progeny virions. Virus attachment is mediated by the hemagglutinin of the virus. The globular head of the protein specifically binds to sialic acid moieties terminally bound to cell surface glycoproteins (264). The amino acid sequence of HA determines its specificity for sialic acids with either an α-2,3 linkage to the underlying sugar, found predominantly in avian species, or an α-2,6 linkage found predominantly in mammalian epithelial tissue (265). For the most part, these conformation differences contribute to host specificity of the virus (114). Receptor

distribution is not exclusive though since mammals and some avian species can express both conformations, although with different tissue distribution (266, 267). Specificity of HA is also not absolute and may not always restrict interspecies transmission. Matrosovich et al. (122) was able to identify human isolates from the HPAI H5N1 Hong Kong outbreak that bound only to Siaα2-3Gal-containing receptors. Some strains have also shown to be capable of binding both α-2,3 and α-2,6 linked sialic acids, as seen with certain HPAI H5N1 isolates (268). It has been reported that as few as one amino acid exchange in the HA sequence can alter receptor specificity (31). Pigs are a particular species that express high levels of both sialic acid linkage conformations in their respiratory epithelium (269). This species, suggested as a mixing vessel, has demonstrated the ability to be infected with both human and avian influenza strains resulting in reassortment of the virus, a phenomenon likely responsible for the 2009 H1N1 pandemic (270).

Upon attachment to the cell surface, the virus is internalized via receptor-mediated endocytosis of clathrin coated pits (271). Previous data has shown that filamentous virions were too large to utilize a clathrin entry pathway, suggesting an alternate internalization mechanism for virions of this morphology (272). The acidic environment of the endosome activates proton conductance of the M2 channel protein (273). Influx of H^+ ions into the endosome induces an acidic state (pH 5-6) that causes an irreversible conformational change of the HA2 protein (274). A conformational extension of the previously folded HA2 protein relocates its N-terminal fusion peptide over 100 Å from its original buried position and inserts it into the endosomal membrane. A subsequent bend in the HA2 protein repositions the C-terminal membrane anchor within proximity to the endosome membrane, facilitating fusion of the two membranes (129). Simultaneously, protonation of the virus interior induces a conformational change of the M1 protein that mediates detachment of the vRNP from the viral membrane (271, 275). Fusion of the two membranes forms a pore by which the dissociated vRNP is released into the cytoplasm (276). The events leading up to the release of vRNP from the endosome has been reported to occur within just

twenty-five minutes after virus internalization (275). The vRNP is then actively transported to the nucleus via the NLS located on the NP protein (259).

Replication and transcription of orthomyxovirus genomes take place in the nucleus of infected cells (277). Initially, the genome is transcribed into an mRNA template containing a 5'cap and a polyadenylated tail. As previously mentioned, the central region of the PB2 subunit of the influenza virus polymerase complex binds to the 5' 7-methyl-guanosine (m^7G) cap of the host's pre-mRNA polymerase II transcript to initiate transcription (83). This "snatched" cap is cleaved 10-13 nucleotides downstream by the viral PA subunit, and the short oligomers are used as primers for transcription and elongation by the PB1 subunit (83, 93, 103). As previously noted, the panhandle structure formed by the conserved sequences of both the 3'- and 5'-terminal ends of the viral RNA serves as a regulatory signal for transcription and replication (77, 79). The panhandle structure also plays a role in transcription termination and polyadenylation (80). Polyadenylation occurs through a stuttering mechanism of the polymerase complex, most likely due to a self-imposed steric hindrance at a poly(U) sequence near the 5' terminus of the vRNA transcript (77, 278, 279). Influenza viruses are thought to utilize host RNA polymerase II not only for its 5' cap primer, but also for its cellular splicing activities to process the additional mRNAs located in segments 7 and 8 (280). As viral mRNA accumulates, translation of host mRNA is inhibited to allow for selective translation of viral proteins (281). Translation of cellular mRNA is hindered through several mechanisms, including the inhibition of polyadenylation and nuclear export via the NS1 protein, as well as degradation of pre-existing cytoplasmic mRNA (250, 282). Down-regulation of the host PKR protein kinase by the virus ensures that the competency of protein synthesis within the cells remains intact during infection (283). Sequences within the 5' non-translated region of the viral mRNA have shown to be critical in regulating selective translation of viral proteins (284). These sequences are utilized by the NS1 protein to selectively increase the rate of viral

translation (281), and according to Aragon et al. (285) this is accomplished by recruitment of the host's translation factor, eIF4GI, to these 5' regions.

Synthesis of viral mRNA and its translated proteins are necessary to proceed to RNA replication (286). The polymerase proteins each contain their own NLS, but it is believed that PB1 and PA form heterodimeric complexes in the cytoplasm that are transported back to the nucleus independently of the PB2 protein (287-290). The polymerase switches to a primer-independent mode of RNA synthesis, generating full-length positive-sense transcripts (cRNA) complimentary to the vRNA. These cRNA transcripts serve as intermediate templates by which progeny vRNAs are generated (291, 292). Viral cRNA are capable of serving as templates to vRNA synthesis since they lack the incorporation of the cellular cap structure sequences and lack of termination at the polyadenylation site allows the polymerase to extend through the entire transcript (291). This antitermination is dependent on the absence of capped RNA-primed initiation (160). The mechanism responsible for switching of the polymerase from transcription to replication has been extensively studied, but a clear resolution has yet to be determined (157-159). Progeny vRNA destined for packaging into virions have shown to be encapsidated by the polymerase and NP molecules into vRNP complexes and subsequently associate with NEP and M1 to facilitate nuclear export (151, 259).

The integral membrane proteins (HA, NA, and M2) associate in the endoplasmic reticulum where subsequent post-translational modifications are made. Here, the HA and NA are glycosylated and assembled into their mature multimeric structures (126, 293). The proteins are subsequently transported to the Golgi apparatus for additional processing. Fatty acyl groups are covalently attached to residues of the M2 protein as well as to several C-terminal cysteine residues of the HA protein that contribute to lipid raft association (294-296). Signals within the transmembrane domains (TMD) of the membrane proteins direct transport of these proteins to the apical plasma membrane of polarized

epithelial cells via pathways of the *trans*-Golgi network (192, 297). TMD signals of HA and NA also facilitate their association with glycosphingolipid- and cholesterol-enriched detergent-resistant lipid rafts of the membrane (192, 298). M2 is excluded from these domains (299). Assembly and subsequent budding of progeny virions is initiated at lipid raft domains where both HA and NA occupy the membrane (299, 300). The process of viral assembly is not well understood, but it is thought to include a series of protein-protein interactions. One critical interaction is the binding of M1 to the cytoplasmic tails of both HA and NA, an interaction thought to facilitate vRNP's association with the assembly site (205). The oligomerization of M1 at the assembly site is thought to mediate host protein exclusion, recruitment of viral components, and provide a "scaffolding" that initiates membrane curvature during budding (51, 198, 203). Once all vRNA segments have assembled at the plasma membrane, their genomes must be strategically packaged into individual virions. Two models of genome packaging have been proposed. The random model suggests that viral RNA is distinguished from cellular RNA and incorporation of individual viral segments into the virion is only by chance (213). The more accepted model is the specific packaging model in which each vRNA can be differentiated by segment specific sequences and only one copy of each segment is incorporated into the progeny virion (301). Studies supporting the specific packaging model have shown that virus particle formation requires, at minimum, the segment specific sequences that are located within the terminal coding regions and extend into the un-translated regions of each vRNA (302, 303). It has been proposed that these packaging signals of each vRNA segment are composed of numerous sequences, rather than just one discrete sequence, and that a full complement of all segments is thought to initiate viral budding (304). Proteins responsible for the initiation and completion of budding have not been specifically determined, but several candidates exist. Individual expression of HA, NA, or M2 has led to the release of VLP's *in vitro*, although combined expression of all three proteins greatly enhanced VLP release (305). During infection though, HA required the association of M2 to mediate viral budding (233). While M2 is not transported directly to

the lipid raft domain, it is suggested that it associates with the periphery of the lipid raft assembly site through M1 recruitment (306). The amphipathic nature of the cytoplasmic helical tail of M2 is thought to cause membrane curvature at the neck of the budding virion, contributing to membrane scission (306). NA mediates the final release of the budded virion by cleaving sialic acid residues from the surrounding cell surface that HA would otherwise attach to, preventing the release of the progeny virion (183, 184). A recent study using cryo-electron tomography has shown NA proteins concentrated at one location on budded virions, further supporting its role in mediating the release of virions from the cell surface (51).

Pathogenicity

Increased viral replication, systemic dissemination, and an intense inflammatory reaction often leading to hypercytokinemia are thought to be contributors to the high pathogenicity of influenza A viruses (307). The molecular pathogenesis of influenza A virus is polygenic in that mutations arising in a number of genes contribute to modifications in synthesis, regulation, or structure of the virus. During replication, the lack of proof-reading capabilities of the RNA-dependent RNA polymerase naturally predisposes RNA viruses to mutations (308). The low fidelity rate of RNA polymerases has been measured at approximately 10^{-4} mutations per nucleotide per infectious cycle, a rate far exceeding DNA-based organisms (309). The quasispecies theory is believed to significantly contribute the evolutionary dynamics of RNA viruses. It suggests that the fittest virus is selected from a subpopulation of related, yet genetically distinct viruses, created as a consequence of error-prone replication (310). The mutability and selection of fitness has allowed these viruses to readily adapt to a diverse range of avian and mammalian hosts. Mutations arising during synthesis, often as a result of host immune response pressure or host adaptation, are responsible for antigenic drifts common to influenza viruses. A more dramatic alteration of influenza viruses utilizes the segmented nature of the genome. These antigenic

shifts may be seen through the exchange of gene segments between two or more distinct viruses infecting the same cell. Viral genome constellations acquired through reassortment can often drastically influence the pathogenicity of the newly constructed virus through altered antigenicity or host selection specificities (311, 312).

Perhaps the most investigated element of avian influenza A viruses mediating pathogenicity is the cleavage site within the HA molecule. As previously mentioned, the HA protein is responsible for virus attachment and entry through fusion of the viral and host membranes. Part of the molecule's post-translational processing is cleavage into the two subunits, HA1 and HA2, a prerequisite for infectivity (127, 128). A correlation between the cleavability of the HA molecule, the ability of the virus to disseminate through a wide range of host cells, and its pathogenicity was initially determined using tissue culture and an *in vivo* chicken model (313). It was later established that the primary structure of the cleavage site and its sensitivity to proteases defined the cleavability of the molecule, its cell tropism, and resulting pathogenicity (314, 315). LPAIV isolates contain a monobasic (single arginine) cleavage site, usually consisting of an R-E-T-R motif (316). HPAIV isolates, on the other hand, contain at least two basic amino acid insertions at the cleavage site. This characteristic R-X-K/R-R motif has only been identified in H5 and H7 subtypes (317, 318). The acquisition of a cleavage site capable of increased cleavability has been attributed to several mechanisms. The HPAIV H5N2 strain resulting in the 1995 outbreak in Mexico acquired a twelve nucleotide insertion at the cleavage site, encoding for four additional basic amino acids (319). It has been suggested that a default in the transcription polymerase resulted in a spontaneous duplication of a purine-rich sequence within the site, giving rise to the insertion of the basic amino acids (319, 320). Another mechanism identified has been non-homologous recombination of the HA coding sequence with cellular 28s ribosomal RNA (321). In this case, a foreign peptide composed of non-basic amino acids was inserted near the region encoding the cleavage site of HA. This insertion rendered the monobasic cleavage site susceptible to increased cleavage, leading to a

higher pathogenicity of this particular H7N3 isolate. An additional non-homologous recombination event involving the HA and M genes of the same virus resulted in a 21 nucleotide insertion at the cleavage site of a more recent H7N3 isolate, also generating a more pathogenic virus (322). It has been suggested that these foreign peptide insertions may act to create a bulge in the protein that projects the cleavage site into solution, thus increasing its accessibility to ubiquitous proteases (323). All reports involving non-homologous recombinations have involved only H7 subtypes and only after passage in chickens, creating some concern for LPAIV isolates of this subtype in poultry (322). It has recently been demonstrated that the pathogenicity associated with a polybasic cleavage site may also be host-specific in mammals (324).

Two groups of proteases are responsible for *in vivo* cleavage of the HA molecule. Extracelluar, trypsin-like serine proteases secreted by non-cilated cells of the respiratory epithelium and gastrointestinal tract are capable of recognizing and cleaving LPAIV isolates (316). Furin is a highly conserved, subtilisin-like protease present in many eukaryotic cell types. It is able to recognize and cleave the polybasic cleavage sequence of HPAIV isolates (111). The broad range of cells that possess these enzymes allows HPAI viruses to disseminate more readily, causing a systemic infection versus the local infection induced by LPAIV (325). Other proteases capable of cleaving both HPAIV and LPAIV include a protease secreted from Clara cells of the bronchial epithelium, plasmin, and several bacterial proteases (326, 327). Bacterial proteases appear to play a role in the development of influenza pneumonia after combined viral-bacterial infection (328).

Other studies have determined that insertion of a polybasic cleavage site into LPAIV may not always be sufficient to increase the pathogenicity of an isolate, suggesting that other elements within the HA molecule or other genes may play a role in pathogenicity (329, 330). Point mutations occurring in regions adjacent to the cleavage site of HA have shown to alter the virulence of the virus, indicating that

amino acids outside of the cleavage site may contribute to an increase of virulence (331). Oligosaccharide side chains within the vicinity of the cleavage site have demonstrated interference of protease accessibility to the site, often requiring the addition of more basic amino acids to overcome this interference (332, 333). A point mutation in a residue near the cleavage site of A/chicken/Pennsylvania/83 (H5N2) caused the loss of a glycan, resulting in an enhanced cleavability associated with an increase in virulence of the virus (22). Many glycosylation sites are conserved within the stalk region of HA, while the globular head contains more variability between isolates in its glycan composition (334). Carbohydrates are often used by the virus to shield or modify antigenic sites in order to evade the host immune response (335). In addition to modulating proteolytic cleavage, several studies have determined that the loss or addition of glycans near the receptor binding domain of HA influences binding affinity and specificity. This modification subsequently affects pathogenicity through altered replication and transmission capabilities (122, 336, 337). Introduction of single amino acid substitutions within the receptor-binding domain of the HA of HPAIV H5N1 has been shown by several to increase SAα2,6 binding, suggesting a potential increase in specificity for human receptors (120, 338, 339). In a recent case, Chen et al. (340) described mutations in an HPAIV H5N1 isolate that permitted aerosolized transmission of the virus among ferrets, implicating a heightened pathogenicity of the strain. The biosafety concerns of such transmission studies have recently become quite controversial in the media (341). Mutations resulting in increased receptor binding affinities are often balanced by a modification of the NA stalk region to allow for efficient release and dissemination of the virus (4, 122, 190). The NA protein itself has also been associated with virulence since glycosylation of its globular head has been implicated in increased neurovirulence in mice (342).

Early studies of influenza A viruses determined that changes in host range correlated with alterations in virulence (343). Several of these host range determinants have been associated with the virus's polymerase activity. The amino acid substitutions, E627K, and to a lesser degree, D701N, within

PB2 have been implicated in adaptation of avian influenza A viruses to mammalian hosts in the case of HPAIV H5N1, 1918 H1N1, and H7N7 strains (85-87). In addition, differences between species in the ability of PB2 to associate with the host mitochondrial antiviral signaling protein is suggested to correlate with differences in virulence of influenza A virus isolates among hosts (344). A single polymorphism (N66S) within the PB1-F2 protein of the HPAIV 1918 H1N1 and Hong Kong H5N1 strains has been associated with increased virulence by suppressing the activation of type I interferons during the innate immune response (345). Other amino acid substitutions located within the mitochondrial signaling domains of PB1-F2 have been identified in HPAIV H5N1 strains and are suggested to play a role in triggering apoptosis in specific immune cells of the host (346).

Additional proteins demonstrating involvement in the virulence of AIV isolates in mammalian hosts or differences in virulence among avian hosts include PA and NP, respectively (312, 347-349). Variations to any of the numerous functions regulated by the NS1 protein can also significantly influence virulence of the virus. Studies using reassortant viruses containing the NS1 gene from a 1918 H1N1 or from a HPAIV H5N1 isolate described a more efficient blocking of IFN-regulated gene expression (350, 351). A PDZ ligand domain has also been identified in the NS1 protein of avian isolates, but is masked in human isolates (352). Introduction of these PDZ ligand sequences into human influenza A viruses increases the virulence of the isolate, potentially as a result of binding human proteins and disrupting certain cellular pathways (353). As one can see, numerous elements of influenza A viruses that contribute to modifications in receptor specificity, transmission efficiency, host adaptation, replication efficiency, and immune response mediation can influence the pathogenicity of the virus. Although many factors may be subtype and/or species specific, the cleavage site of HA is the most universal and definitive contributor to virus pathogenicity.

Diagnostics

The clinical presentation after influenza A virus infection can vary depending on host species, age, virus strain, concurrent infections, or environmental factors (354). Most infections of naïve and susceptible persons with influenza A virus result in an acute respiratory illness including fever, inflammation of the upper respiratory tract, headache, vomiting, and fatigue (1). Infection with highly virulent strains results in a more systemic disease that is commonly exacerbated by concurrent bacterial pneumonia, often contributing to higher mortality (355). In poultry, depending on the pathogenicity of the virus, the disease can range from subclinical infections, respiratory distress and egg production losses, to severe systemic infections resulting in high mortality (354). Influenza A virus infection in wild birds has previously been asymptomatic, but current circulation of HPAIV H5N1 has caused incidental clinical disease in these birds. The first report of such cases was an outbreak in 2005 along Qinghai Lake in western China killing thousands of birds migratory bar-headed geese (356).

Influenza A virus causes non-pathognomonic symptoms in its host, requiring rapid and accurate diagnosis for successful control of the viral infection. Passive surveillance is key to identifying influenza A virus as the causative agent in production losses or mortality events within poultry flocks. Active surveillance (monitoring) is also critical in identifying AIV hotspots and global circulation patterns of these viruses. Many factors determine the applicability of diagnostic tests, including cost, sensitivity, specificity, simplicity, and speed. Test samples obtained from poultry and wild birds primarily include oropharyngeal and cloacal swabs. Oropharyngeal swabs are commonly taken from human clinical cases, but a systemic virus can often be detected from plasma, pleural fluid, rectal swabs, and cerebral spinal fluid (307).

Several methods for detecting viral antigens have been established. As early as 1936, influenza A virus could be propagated by inoculating and passaging the sample in chicken-embryonated eggs

(357). This virus isolation (VI) system is still the most sensitive method used to determine the presence of AIV through embryo mortality, and is the current standard in developing influenza vaccines (170). Although highly sensitive and able to detect virus early in infection, using embryos to isolate virus is reasonably costly and labor intensive, with results taking upwards of 1-2 weeks. The low specificity of VI requires confirmation tests to identify the presence of influenza A virus from other bacterial or virus contaminants, such as Newcastle disease virus (170). The hemagglutination assay (HA) or antigen detection tests are often used to confirm the isolated virus. The HA assay requires additional confirmation since hemagglutination of red blood cells is characteristic of other viruses and bacteria. Although most antigen detection kits are licensed for human use, these type-specific tests are quite often used in poultry diagnostics. The tests are highly specific, but their low sensitivity requires samples with high viral loads, usually from symptomatic or dead birds. The low cost, rapid results, and simplicity of these tests are ideal for field or clinical use.

The use of nucleic acid detection through molecular diagnostics has greatly evolved. Reverse transcriptase polymerase chain reaction (RT-PCR), based on detection of the highly conserved matrix gene, has allowed the ability of detecting influenza A virus from a pool of genotypically diverse strains (214). RT-PCR is sufficiently sensitive to isolate and amplify virus from a variety of specimen collections, including VI fluid, tissue homogenates, or environmental samples (358). It also has the capacity to accommodate high sampling numbers. The circulation of HPAIV H5 and H7 subtypes as well as H9N2 continues to be the focus of surveillance programs. A multiplex RT-PCR system has been established that simultaneously detects and differentiates between these three subtypes with high sensitivity (359, 360). The adoption of a real-time RT-PCR (RRT-PCR) assay for influenza detection has decreased the turnover time for processing to just hours and has reduced the possibility of contamination since no manipulation of the product is necessary after amplification (361). A RRT-PCR assay has also been developed that differentiates between H5 and H7 subtypes (362). Amplified PCR products are

subsequently analyzed using a multitude of sequencing methods to determine virus identification, characterization, and phylogenetic resolution. Analysis of the cleavage site following sequencing as well as in vivo pathotyping are used to determine the pathogenicity of an isolate. HPAIV and H5/H7 LPAIV isolates are identified by the OIE as reportable diseases. HPAIV is defined by OIE as a virus that contains a polybasic cleavage site characteristic of HPAIV, and when intravenously inoculated into eight-week old chickens, 75% mortality is seen within 10 days post infection (363).

The production of antibodies to several viral proteins in response to influenza A virus infection has been utilized by numerous diagnostic tests. Antibodies to M1 and NP are important components in diagnostics because of their type-specific, conserved sequences (170). The agar gel immunodiffusion (AGID) test can be used to detect IgM antibodies to either the NP or M1 proteins or the antigens themselves, and is widely used in poultry diagnostic testing (364, 365). The enzyme-linked immunosorbent assay (ELISA) is a more sensitive, yet less specific, test used to measure IgG antibodies made to the NP of influenza A viruses (366). Both tests are relatively cheap, allow high sampling numbers, and the use of inactivated virus as reference antigens in these tests reduces the biosecurity concern. The AGID is most commonly used among poultry flocks for its simplicity and sensitivity, but it requires 24-48 hours for results and is less sensitive than an ELISA (367). An indirect ELISA offers more rapid (within hours) and less subjective results. Unfortunately, indirect ELISAs are species specific and only commercially available for poultry (368). A competitive ELISA has been established that utilizes a blocking mechanism rather than a direct interaction with the test serum, allowing detection of antibodies from a variety of avian species (368, 369). Prior to AGID and ELISA, type-specific complement fixation and indirect immunofluorescent antibody tests were regularly used to detect AIV infection (366, 370, 371). Neutralizing antibodies made to the HA and NA proteins of influenza A viruses are detected by hemagglutination and neuraminidase inhibition (HI, NI) assays. They are identified by either the inhibition of red blood cell agglutination or the lack of a colorimetric reaction in response to the

inhibition of the NA enzymatic activity. These tests are often used to confirm prior tests or to further characterize the virus subtype. The tests can be performed within a couple hours, and again the ability to use inactivated reference antigen lessens the biosecurity concern. HI is highly sensitive as well as specific and detects antibodies for a longer period of time than AGID tests or ELISAs (367). As a disadvantage, the HI assay is moderately costly in that it requires a large panel of reference HA subtypes to provide an antigenic coverage broad enough to give meaningful results. Influenza-specific antibodies are currently utilized by many to establish novel diagnostic approaches. Recently, a study using antibodies conjugated to gold nanoparticles has been proposed as a rapid and reliable diagnostic assay for detecting influenza virus (372).

Reverse Genetics

During early attempts to generate influenza A virus in the laboratory, it was determined that RNP complexes were sufficient for the replication of vRNA (160). Luytjes et al. (78) were able to reconstitute RNP complexes *in vitro* using purified NP and polymerase proteins, labeling the system, RNP transfection. These *in vitro*-generated RNP complexes were later used in a eukaryotic cell transfection system to transcribe a vRNA template containing a cloned DNA gene. Reproducing these influenza viruses containing site-specific mutations led the way for recombinant influenza virus research (373). This system was limited though by its dependence on an influenza helper virus to supply an intracellular source of NP and polymerase proteins. Co-infection required a selection system to isolate the rescued virus from the helper virus. Selection methods used included restrictions in growth temperatures, host range, or drug selectivity (374). Neumann et al. (375) modified the reverse genetics system in 1994 with the use of cellular RNA polymerase I to drive intracellular synthesis of viral RNA and subsequent RNP complexes. They found that transfection of eukaryotic cells with plasmids containing cloned influenza cDNA inserted between a murine RNA polymerase I promoter and terminator produced RNP

complexes, and that when co-infected with helper virus these complexes could synthesis vRNA . Two years later, Pleschka et al. (376) introduced the use of a human pol I promoter and was the first to demonstrate genetic manipulation of influenza A viruses using this system. Reverse genetics hit a significant milestone when Neumann et al. (374) demonstrated the ability to generate influenza A viruses without the use of a helper virus, eliminating the need for cumbersome selection systems. They found that protein expression plasmids containing cDNA encoding for NP and polymerase proteins were capable of providing a source of these proteins for RNP synthesis. Eight additional plasmids containing the cDNA encoding for each viral protein provided the viral RNA. Although highly efficient in the 293T human embryonic kidney cell line, the use of twelve plasmids limited the number of other cell lines capable of such transfection efficiency (377). This same group alleviated that concern just one year later when they developed a bi-directional transcription construct (378). Using an RNA polymerase I transcription unit nested within a RNA polymerase II transcription unit, they were able to synthesize negative sense vRNA and positive sense viral mRNA from the same cDNA template. This system circumvented the need for the four additional protein expression plasmids. With higher transfection efficiencies and less technical difficulties, the use of reverse genetics for the generation of influenza A viruses has become an indispensable commodity in scientific research. The plasmid-based system has provided a versatile platform by which countless studies have been able to address long-standing biological concerns of influenza A virus, such as regulatory sequences, pathogenicity, host cell tropism, structure-function relationships, and vaccine technology (379, 380).

Vaccines/Control

With entrenchment of HPAIV H5N1 across Asia and surrounding countries, the threat of a global pandemic is presently upon us. Current strategies used to control influenza A virus include eradication through culling of poultry, prevention of inter- and intraspecies transmission through vaccination, and

management of existing human infections through antiviral drugs. Control of AIV in poultry flocks is ideal for mitigating the potential of human pandemics at its source. Eradication of influenza A virus through culling of infected flocks has dated back to early outbreaks and continues to be used as a successful measure to control AIV in the poultry industry (381). The economic losses resulting from such practices and reoccurring outbreaks have led many AIV-endemic countries to implement a vaccination program in conjunction with their eradication policies (382). Vaccination was deemed a last resort measure for decades since its practice resulted in restricted trade due to the difficulty of differentiating vaccinated from infected birds (383). The development of a DIVA (differentiating infected from vaccinated animals) strategy has alleviated this concern by the use of a vaccine containing a heterologous virus that differed serologically from the field strain by its NA subtype (383). Since its development in 2002, other viral proteins and even the use of viral vectors have been examined as alternate ways to differentiate the source of infection (384, 385). The most common AIV vaccines currently used in poultry include whole, inactivated viruses complemented with an adjuvant (usually water-in-oil), subunit vaccines, and recombinant vaccines expressing AIV proteins inserted into viral vectors (usually fowlpox virus) (386). The immune response to AIV vaccines in poultry appears to be much broader than in humans, often eliciting cross-protection against a diverse range of strains (365).

Vaccination has shown to be an effective measure to prevent influenza illness in humans. Dating back to 1945, the use of whole, inactivated viruses was initially used in vaccines to control influenza in humans (387). In the case of whole, inactivated vaccines, a LPAIV antigenically similar to the circulating strain is inoculated into embryonated chicken eggs for its propagation. The virus is chemically inactivated and administered intramuscularly or subcutaneously. Detergent fractionation of whole virus particles into split virions or to just its glycoprotein subunits are alternative formulations of vaccines that are more commonly used to avoid the adverse reactions and biosafety concerns associated with whole virus (388). Subunit vaccines work by expressing the HA and NA of the currently

circulating strain to generate neutralizing antibodies to these proteins. Although split and subunit vaccines are safer, they are less immunogenic than whole vaccines and require a higher amount of antigen to protect the individual. For decades, adjuvants have been paired with poultry vaccines to elicit a more robust immune response (389). Adverse reactions of established adjuvants have hindered their use in human vaccines, but recent studies are attempting to identify safe and effective compounds that will enhance vaccine efficacy by enhancing the immunogenicity of the antigen to require less antigen per dose (390, 391). Attenuation of influenza virus has been an attractive vaccination method for stimulating high mucosal and cellular immunity. Cell-mediated immunity aids in targeting more conserved regions of viral proteins that do not elicit antibody production. Most viruses are attenuated through cold-adaptation, but the use of reverse genetics to genetically modify virulence factors associated with specific viral genes (HA, PB2, NS1) has also showed promising approaches to virus attenuation (392, 393). Live, attenuated vaccines are administered more conveniently via an intranasal spray (FluMist) versus the subcutaneous and intramuscular administration of inactivated vaccines. These vaccines have not been recommended for use in poultry for the concern of generating HPAIV mutants (386). The generation of inactivated and live, attenuated vaccine strains containing well-defined genetic composition have been obtainable through the use of reverse genetics. An example of this technology is the generation of an influenza vaccine entirely from cloned cDNA that can be used in poultry as a DIVA approach by its N3 marker (394). Reverse genetics has also been instrumental in the development of seed strains for the inactivated and live, attenuated H5N1 vaccines (388).

In the wake of seasonal and pandemic outbreaks, a reoccurring problem in vaccine development is the six-month production period from the time that a new strain is identified by WHO epidemiological surveillance experts to the time that vaccines are available for deployment to the public (395). Novel development strategies are being employed in an attempt to create vaccines that are more readily amendable to bulk production. Such technologies include the use DNA vaccines and recombinant vector

vaccines. Cellular synthesis of viral proteins induced by DNA vaccines has shown to be safe, relatively easy, and economical, as well as being capable of elicting both humoral and cellular immunity (396, 397). Several studies have paired DNA vaccines with alternative delivery systems, such as nanoparticles and microneedles (398-400). Attenuated viruses or bacteria have also been utilized as a backbone to express and deliver viral proteins. These vectors have included yeast, bacterial plasmids, vaccinia virus, fowlpox virus, as well as other large DNA viruses (401-404). The foreign antigens of these vectors aid in eliciting the robust immunity seen with such vaccines. Concurrent expression of multiple viral antigens has been used in both DNA and vector vaccines to induce cross-protective immunity (405, 406). To date, most vaccines are still produced using an egg-based propagation system. Inability to scale up this system and the threat of an egg supply shortage in the event of a pandemic have prompted researches to explore an alternative non-egg approach to vaccine production (388). Cell culture-based influenza vaccines have been investigated as an alternate source of production. Madin-Darby canine kidney (MDCK) cells have been considered a prospective substrate for influenza virus vaccine propagation since they have shown to support efficient replication of numerous inactivated influenza virus subtypes (407, 408). Such substrates would also aid in propagation of influenza subtypes that produce low viral yields in embryonated eggs, such as H5 and H7 isolates (409). The rapidly evolving nature of influenza A viruses has greatly complicated the efficiency of vaccines and treatment drugs, requiring frequent reformulations of these control measures. Vaccine strategies have focused on the HA protein since it elicits the most protective immunity, yet unfortunately it is also the most variable of the viral proteins. For this reason, numerous studies have begun to define and test more conservative and protective antigens in an attempt to create a more “universal” vaccine. Among the proteins tested, the highly conserved ectodomain of the matrix 2 protein (M2e) may be the most promising (386, 410).

In the absence of prevention through vaccination, a number of registered antiviral drugs have been made available to humans as both prophylactic and therapeutic treatments of seasonal influenza

(411). Antiviral drugs targeting the neuraminidase protein of influenza A and B type viruses include oseltamivir (trademarked Tamiflu) and the inhaled zanamivir (trademarked Relenza). Other neuraminidase inhibitors within this class include laninamivir and peramivir. This group of antiviral drugs inhibits the release of newly formed virions by mimicking sialic acid and binding neuraminidase (412). The antiviral adamantane drugs, amantadine (trademarked Symmetrel) and rimantadine (trademarked Flumadine), target the M2 ion-channel protein found exclusively in influenza A viruses (413). It is speculated that the drugs act by one of two methods: physically obstructing the pore by binding near the proton channel or by locking the transmembrane domain in its closed state through an allosteric mechanism (230, 414, 415). Obstruction of the proton transport mechanism inhibits the virus from uncoating and releasing its genomic material into infected cells (413). Unfortunately, these few available drugs have been challenged with resistant strains. A recent surveillance after the 2009 flu season found that 100% of the seasonal H3N2 and pandemic H1N1 virus samples tested were resistant to adamantanes (416). Resistance to these drugs seen by seasonal H3N2 and Eurasian-lineage swine influenza A strains has been widespread for several years (417). The most prevalent mutation identified in adamantane-resistant strains is a S31N amino acid substitution found along the inside of the M2 pore (418). The Centers for Disease Control and Prevention has since recommended the use of oseltamivir and zanamivir over adamantanes for controlling influenza A infections (416). Oseltamivir- and zanamivir-resistant strains, particularly of the H1N1 subtype, have also been identified, but to a much lesser extent (419). An H274Y amino acid substitution in the NA protein is characteristic of most oseltamivir-resistant strains (420, 421). Detailed structural studies highlighting protein-drug interactions are underway in an effort to identify molecular mechanisms associated with drug resistance (422). Such studies will contribute to developing novel antiviral drugs in an attempt to keep up with the continually adapting influenza A virus.

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CHAPTER 3

SERIAL PASSAGE IN DUCKS OF A LPAIV ISOLATED FROM CHICKEN REVEALS A HIGH MUTATION RATE IN THE HEMAGGLUTININ[1]

[1]Ridenour, C.A., Williams, S.M., Jones, L., Tompkins, S.M., Tripp, R.A., Mundt, E. To be submitted to *Archives of Virology*.

Abstract

A comparative study of the ability of three low pathogenic avian influenza virus (LPAIV) isolates to transmit from duck to duck was performed. Pekin ducks were inoculated with two LPAIV isolates from chicken [A/Ck/PA/13609/93 (H5N2), H5N2-Ck; A/Ck/TX/167280-4/02 (H5N3), H5N3-Ck] and one LPAIV isolate from a wild bird [A/Mute Swan/ MI/451072/06 (H5N1), H5N1-WB]. During the establishment of the passage model only two viruses (H5N1-WB, H5N2-Ck) were able to transmit between ducks. Transmission of these isolates was dependent upon inoculation dosage and route of infection. Analysis of swab samples taken from ducks revealed that the H5N1 wild bird isolate was primarily shed via the cloacal route. The H5N2 chicken isolate was isolated from both cloacal and oro-pharyngeal swabs. Based on histopathological examination of multiple organs, no significant differences were observed between viruses or between passages. Analysis of the amino acid sequences of the viral glycoproteins (hemagglutinin, neuraminidase) showed that the hemagglutinin (HA) of the H5N2-Ck isolate was under a stronger evolutionary pressure than the HA of the H5N1-WB isolate, as indicated by the presence of a higher number of amino acid exchanges observed through passage. The neuraminidase of both viruses showed either no (H5N1-WB) or few amino acid exchanges.

Introduction

Avian influenza virus (AIV), belonging to the genus *influenza A virus* of the virus family *Orthomyxoviridae,* is maintained in wild aquatic and shore birds (Slemons et al., 1974; Stallknecht and Shane, 1988; Webster et al., 1992). In such birds, 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified and can be distinguished by appropriate tests (Hinshaw et al., 1982; Kawaoka et al., 1990; Rohm et al., 1996; Fouchier et al., 2005). Recently, a 17th HA subtype has been identified from sequences of influenza A viruses found in yellow-shouldered bats (Tong et al, 2012). The NA sequences from these bat samples were also different from other known NA sequences, leading to the possibility of an additional NA subtype added to the genus *influenza A virus*.

AIV occasionally spreads from its natural reservoir to poultry or mammals. In general, AIV isolated from wild birds are poorly adapted to domestic Galliformes. Although, there have been reports that AIV isolates can replicate and transmit between chickens (Pillai et al, 2010) as well as induce mortality in turkeys (Spackman et al, 2010). Other studies using the same HA subtypes (either H5 or H7) contradict these results, showing that these AIV isolates were not able to transmit or induce mortality (Pillai et al, 2010; Spackman et al, 2010). The underlying mechanisms of transmission are not well understood, but it is likely that adaptation processes during passage may change the genetic information enough to alter the phenotype of the virus. A deletion in the stalk region of the NA gene has been observed in AIV isolates from chickens and may be the result of adaption to the new host (Banks et al., 2001; Campitelli et al., 2004). The mechanism for such changes in genetic information may possibly be the result of mutation with subsequent selection. An alternate mechanism suggested by Dlugolenski et al (2011) identified a minor virus subpopulation already harboring a deletion in the NA stalk region of an H5N1 wild bird AIV isolate. This minor virus population became the prominent virus population after passage in chickens. The properties of recombinant AIV isolates containing either a short or a long NA

stalk region have been compared in the chicken and duck model (Hoffmann et al, 2012). The AIV harboring the short-stalk NA replicated to higher virus titers than the long-stalk NA in pharyngeal swabs taken from chickens. The opposite phenotype was observed in ducks. A similar phenotype was described for an H2N2 virus in chickens in which a deletion in the NA stalk supported better replication in chickens (Sorrell et al., 2010).

Another important determinant of AIV pathogenicity in poultry is the HA. The presence of multiple basic amino acids at the junction between HA1 and HA2 has seemed to be a prerequisite for the highly pathogenic and fatal pathotype of H5 and H7 AIV in gallinaceous birds (Bosch et al., 1979; Webster and Rott, 1987; Li et al, 1990). In contrast, one AIV isolate contained such multiple basic amino acids at the HA cleavage site without the high pathogenic phenotype in chickens (Lee et al., 2005). A more recent study has also shown that the presence of a polybasic HA cleavage site did not necessarily lead to a high pathogenic phenotype in non H5-AIV and non H7-AIV (Veits et al, 2012), indicating that further adaptive mutations may be necessary in certain genomic constellations. The role of gene constellation is emphasized by comparing two recent studies in which the introduction of a polybasic HA cleavage site in an H9 AIV led to a highly pathogenic phenotype (Gohrbandt et al, 2011), while the presence of this polybasic HA cleavage site in an H3N8 duck isolate was not sufficient for a highly pathogenic phenotype (Stech et al, 2009). Beyond the mutations previously described, consequences of other mutations in the NA and HA leading to more efficient replication in poultry are not well understood. Such uncertainties would include the observed mutations identified in several genes of an H5N2 AIV after multiple passages in quails resulting in better transmission in chickens (Sorrell and Perez, 2007). Identifying the mutation(s) responsible for this altered phenotype was not explored. An additional study revealed that when passaged in chickens, an H5N1 wild bird isolate showed not only a truncation in the NA stalk region, but also a higher degree of mutation in the HA as compared to an

H5N3 AIV chicken isolate (Dlugolenski et al, 2011). The study suggests that adaptive mutations had occurred during adaptation to the new host.

The aim of this study was to investigate possible changes in phenotype and genotype of a wild bird AIV isolate (H5N1-WB) and a chicken AIV isolate (H5N2-Ck) during consecutive passage in Pekin ducks. The results from this study indicate that during passage in ducks, both glycoproteins (HA, NA) of the H5N2-Ck isolate mutated to a higher rate than those of the H5N1-WB isolate. The results suggest that after passage in ducks the H5N2-Ck AIV isolate most likely reverted back to a wild bird AIV. These findings are important in understanding AIV transmission and adaptation linked to changes in the genetic composition of HA and NA.

Material and Methods

Viruses

Two LPAIV H5 chicken isolates [A/Ck/PA/13609/93 (H5N2), H5N2-Ck; A/Ck/TX/167280-4/02 (H5N3), H5N3-Ck] and one LPAIV wild bird isolate [A/Mute Swan/ MI/ 451072/06 (H5N1), H5N1-WB] were used for the experiments. All three isolates were kindly provided by Dr. David Suarez (Southeast Poultry Research Laboratory, Athens, GA, USA). The viruses were propagated once in nine-day-old embryonated chicken eggs obtained from a specific-pathogen-free (SPF) leghorn chicken flock (Sunrise Farms, Catskill, NY, USA). The allantoic fluid from the infected eggs were pooled, aliquoted, and stored at -80°C until use. The 50% egg infectious dose (EID_{50}) for each isolate was determined by inoculating 10-fold serial dilutions of allantoic fluid into embryonated SPF eggs. The titer was calculated following the method as described by Reed and Muench (Reed and Muench, 1938)

Establishment of a transmission model in ducks

All animal studies were conducted in BSL-3 animal facilities at the Animal Health Research Center at the University of Georgia. Animal care was provided as approved by the Institutional Animal Care and Use Committee of the University of Georgia. The ducks were provided feed and water *ad libitum.* The experiments were performed in animal rooms containing HEPA filtered exhaust air. Animals were held in a single cage on the floor allowing commingling. Five two-week-old Pekin ducks (METZER FARMS, Gonzales, CA, USA) were used for each of the experiments. In the first experiment each group of ducks was infected with a dose equivalent to 10^6 EID_{50}/100 µl via the choanal route for each virus. Twenty-four hours after infection five naïve ducks were added as contact birds. All ten birds were swabbed at day 2 (day 1 for the contact birds), 4 (day 3), 7 (day 6), 9 (day 8), and 11 (day 10) post infection (p.i.). Fourteen days after infection all birds were bled via the leg vein and euthanized by exposure to $C0_2$ followed by cervical dislocation. This method of euthanasia was used through all subsequent experiments. In the second experiment ducks were infected with the same viral titer using both the choanal and nasal route of infection. The remaining portions of the study mimicked the first experiment. In the third experiment an increased viral titer of the H5N2-Ck and H5N3-Ck isolates was used to infect ducks. The maximum titer present in the allantoic fluid of H5N2-Ck (10^9 EID_{50}/100 µl) and H5N3-Ck ($10^{7.25}$ EID_{50}/100 µl) was used to infect each duck. The viral titer of H5N1-WB was kept at 10^6 EID_{50}/100 µl per duck. The inoculation and sampling of the birds was performed as described in the second experiment.

Serial passage of two low pathogenic avian influenza viruses

Based on the results from the first three experiments, serial passage of two LPAIV (H5N1-WB, H5N2-Ck) was performed in two-week-old Pekin ducks (METZER FARMS, Gonzales, CA, USA). The infectious dose

used for H5N1-WB and H5N2-Ck was $10^{6.75}$ EID_{50}/100 µl per duck and 10^{9} EID_{50}/100 µl per duck, respectively. The route of inoculation used was a combination of choanal (50 µl) and nasal (25 µl per nostril). Twenty-four hours after initially infecting five ducks, five naïve ducks were added to serve as contacts. Four days post infection (three days p.i. for contact birds) cloacal and tracheal swabs were taken from all ducks. Virus isolated from the initially infected ducks represented the first passage, while virus isolated from contact birds represented the second passage of the appropriate virus. Once swabs were taken the five initially infected passage 1 ducks were removed and euthanized, three of which were later necropsied (see below). The five passage 2 ducks were then moved to an adjacent room where they served as the infection source to a previously placed new group of five naïve ducks to establish a third passage. The isolated viruses obtained from the cloacal and tracheal swabs taken from the new ducks three days post exposure were regarded as passage 3. On the same day of swabbing, passage 2 ducks were removed, euthanized, and necropsied as described for passage 1 ducks. Using the same pattern, passage 3 ducks were moved to an adjacent room to commingle and serve as the infection source for five naïve ducks (passage 4). The experiment proceeded in the described pattern through six passages. The ducks of passage 6 were swabbed at day three post exposure and necropsy was performed in parallel with passage 5 ducks. The passage schedule has been schematically depicted in Figure 1.

Necropsy and processing of obtained samples

Three of the five euthanized ducks from each passage were necropsied and samples from thirteen organs (thymus, trachea, heart, proventriculus, lung, spleen, liver, cecal tonsils, bursa of Fabricius, kidney, pancreas, duodenum, and cerebrum) were taken for microscopic examination. The tissue samples were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) following standard procedures. Cloacal and tracheal swab samples were

transferred in 2 ml of viral transport medium (37g/L brain heart infusion broth, 4,000 U/ml penicillin, 400 µg/ml gentamycin, 8 µg/ml amphotericin B, 4,000 µg/ml streptomycin, 1,000 µg/ml kanamycin sulfate). Swab samples were centrifuged at 16000 x g and filtered thru a 0.45 µm Millex-HV filter with an 8 mm diameter (Millipore, Bedford, MA, USA). One hundred microliters of the filtrate was used to inoculate nine-day-old embryonated chicken eggs obtained from a SPF flock (Sunrise Farms, Catskill, NY, USA). Eggs were incubated at 37°C and 55% humidity and candled daily. Embryonic death within twenty-four hours after inoculation was regarded as unspecific and disregarded from the study. All remaining eggs were incubated a total of four days post inoculation. Allantoic fluid was removed from all eggs and tested for the presence of the respective AIV using a hemagglutination (HA) assay as described by OIE (2006). Serum samples were investigated for the presence of hemagglutination inhibiting (HI) antibodies by applying the HI test (OIE, 2006) using the respective LPAIV used for infection.

Sequence analysis of the HA and NA genes

The HA and NA coding regions were amplified by reverse transcription-polymerase chain reaction (RT-PCR) from all allantoic fluid positive for virus isolation. The obtained cDNA fragments were directly sequenced and the *in silico* translated sequences were compared to sequences of the parental viruses already published in the NCBI genbank (HA of H5N1-WB: CY034679, NA of H5N1-WB: CY034680; HA of H5N2-Ck: CY034681, NA of H5N2-Ck: CY034682) to identify mutations. RT-PCR and sequence analysis was performed as previously described (Dlugolenski et al, 2011).

Analysis of the NA truncation

A previous publication has shown that a truncation in the stalk coding region of the NA gene of an H5N1-WB isolate was selected for during passage in chickens (Dlugolenski et al, 2011). To investigate whether this ratio between truncated and full length NA gene was also occurring during passage in ducks, the same primer sets and reaction conditions were used as previously described (Dlugolenski et al, 2011).

The H5N2-Ck isolate used in our study already encoded for a truncated NA protein. In order to analyze whether a minor population of H5N2-Ck virus was present encoding for a longer NA protein the NA nucleotide sequences from several wild bird isolates were aligned and primers were delineated which may represent the sequence of the deleted region. For the alignment, the parental NA sequence of H5N2-Ck was used along with 30 NA sequences from H5N2 wild bird isolates [A/duck/Taiwan/DV30-2/2005(H5N2), CY110935; A/American black duck/Illinois/08OS2688/2008(H5N2), CY079454; A/duck/Victoria/26/1981(H5N2), CY077687; A/duck/Oregon/459674-3/2006(H5N2), GU049988; A/duck/Pennsylvania/465759/2006(H5N2), GU049914; A/duck/NewYork/483239/2007(H5N2), GU049937; A/duck/Minnesota/462960-2/2006 (H5N2), GQ923175; A/duck/Pennsylvania/446080-6/2006(H5N2), GQ923423; A/duck/ New York/504372/2007(H5N2), GQ923535; A/duck/Korea/A93/2008(H5N2), GU086249; A/duck/NewYork/445743/2006(H5N2), GQ117139; A/duck/NewYork /465976/2006(H5N2), GQ117155; A/duck/New York/481172/2007(H5N2), GQ117203; A/duck/NewYork/489761/2007(H5N2), GQ117243; A/duck/Ohio/470655/2007(H5N2), GQ923543; A/duck/EasternChina/64/2004(H5N2), EU429791; A/duck/NewYork /492652/2007(H5N2), GQ257435; A/mallard duck/ALB/645/1980(H5N2), CY004969; A/duck/France/080032/2008(H5N2), CY046176; A/duck/NY/185502/02(H5N2), AY300942; A/duck/NY/44018-1/00(H5N2), AY300933; A/duck/NY/44018-2/00 (H5N2), AY300934; A/northern pintail/Akita/714/2006(H5N2), AB490828; A/duck/Shimane/19/2006(H5N2), AB472053; A/duck/Tsukuba/536/2006(H5N2), AB472052; A/spotbill duck/Xuyi/18/2005(H5N2), GQ184331; A/Muscovy duck/NewYork/62095-1/2006(H5N2), CY036036; A/duck/NewZealand/41/1984 (H5N2), CY014641; A/duck/Pennsylvania/10218/1984(H5N2), AB295604; A/black duck/NewYork/184/1988(H5N2), CY014874] using an online available program (http://www.ebi.ac.uk/Tools/msa/clustalw2). Based on the consensus of nucleotide sequences located in the probable deleted region, several forward primers were delineated. Also used was an oligonucleotide located in the 3'-noncoding region of the NA segment as an additional forward primer

(N2-FP1, GCAAAAGCAGGAGTGAAAATGAATCC) of the H5N2-Ck virus. A reverse primer was delineated using an oligonucleotide located downstream of the deleted region (N2-RP1, GTTTGTTGTCCAGCGTGGTC). A wild bird isolate [A/duck/NC/674964/07 (H5N2), kindly provided by Dr Stallknecht, University of Georgia] was used as a positive control for the RT-PCR. The conditions for the RT-PCR varied, including annealing temperature gradients ranging from 45°C to 65°C. Location and sequences of the primers used are depicted in Figure 7B.

Results

Establishment of LPAIV transmission in ducks

Three low pathogenic AIV isolates [A/Ck/PA/13609/93 (H5N2): H5N2-Ck, A/Ck/TX/167280-4/02 (H5N3): H5N3-Ck, A/Mute Swan/ MI/ 451072/06 (H5N1): H5N1-WB] were used to infect Pekin ducks. In an initial experiment, ducks were infected with a dose equivalent to 10^6 EID_{50}/100 µl inoculated via the choanal route. No virus was isolated from any swab samples taken from all three virus groups (data not shown). Only two ducks inoculated with the H5N3-Ck isolate and one duck inoculated with the H5N2-Ck isolate had developed HI titers. Ducks inoculated with the H5N1-WB isolate had shown no evidence of seroconversion (data not shown). Since the aim of the study was to investigate changes during direct duck-to-duck passage a second attempt to establish transmission was performed. The infectious dose of each virus remained constant but the route of inoculation was modified. Half of the virus was inoculated via the previously used choanal route, and the remaining virus was inoculated via the nasal route. Using this method, virus was isolated from tracheal and cloacal swabs taken from ducks both directly infected with the H5N1-WB isolate as well as from contact ducks of this group (Figure 2A). Nearly all ducks (9/10) of the H5N1-WB group seroconverted, as indicated by the presence of HI titers from serum taken 14 d.p.i. (Figure 2B). The seroconversion and isolation of virus from contact birds indicated transmission of the H5N1-WB virus between ducks. As observed in the previous experiment, no virus was isolated from

ducks infected with either chicken isolate (H5N2-Ck, H5N3-Ck), and only three of the five H5N3-Ck infected ducks developed HI antibody titers at 14 d.p.i.. In a third experiment attempting to establish duck-to-duck transmission of the AIV chicken isolates, the maximum virus titer present in the allantoic fluid of H5N2-Ck (10^9 EID_{50}/100 µl) and H5N3-Ck ($10^{7.25}$ EID_{50}/100 µl) was used to infect each duck. The virus titer used to infect the H5N1-WB group remained at 10^6 EID_{50}/100 µl per duck as used in the two previous experiments. The combined choanal and nasal route of inoculation was used. The data obtained from serological tests indicated that directly inoculated ducks from all three groups (H5N1-WB, H5N2-Ck, H5N3-Ck) were infected with their respective virus (Figure 3B). Virus was isolated from infected and contact birds of both the H5N1-WB and H5N2-Ck groups (Figure 3A). In contrast, no virus was isolated from ducks infected with H5N3-Ck (data not shown), as previously observed in experiments 1 and 2.

Serial passage of LPAIV in ducks

Based on the above described results, only two viruses (H5N1-WB, H5N2-Ck) were chosen for subsequent serial passage in ducks. At day 4 p.i. (day 3 for contacts) virus was isolated from both infected and contact ducks from all passages, indicating transmission of both LPAIV isolates through at least six passages (Figure 4). The number of ducks from each passage in which virus was isolated from was slightly higher in the H5N1-WB group than in the H5N2-Ck group. All five ducks from each of the first three passages of H5N1-WB had shed virus via the cloacal route, while during passages 4 through 6 only four ducks from each passage were positive for virus isolation. Only one duck during passage of this isolate (passage 4) shed virus via the oro-pharyngeal route, as indicated by the positive tracheal swab sample. The H5N2-Ck virus was not as effectively transmitted between ducks as the H5N1-WB, as indicated by the fewer number of positive swab samples from each passage. While only one duck shed virus via the respiratory tract when infected with H5N1-WB, ducks infected with virus of chicken origin

(H5N2-Ck) shed virus more readily via the respiratory tract. Clinical signs of disease were not observed at any time point during transmission. The necropsy of ducks infected with H5N1-WB or H5N2-Ck revealed no gross pathological lesions. Tissue samples from cerebrum, lung, spleen, kidney, liver, trachea, duodenum, pancreas, proventriculus, heart, thymus, bursa of Fabricius, and cecal tonsils were investigated for microscopic lesions (Table 1). The presence and frequency of microscopic lesions did not change during passage, regardless of virus used for infection. During infection and passage of both H5N1-WB and H5N2-Ck, similar microscopic changes were observed. Lesions were observed primarily in the lung, spleen, kidney, trachea, proventriculus, and bursa of Fabricius. Only one sample from each virus group showed changes in the duodenum during passage. One sample displayed minor lesions in the heart during the first passage of the H5N1-WB isolate, and three samples showed minor changes in the thymus during passage of H5N2-Ck. The changes in the lungs were characterized by moderate to severe lymphocytic infiltration within the lamina propria of the secondary bronchi. Affected spleens showed an increased number of macrophages throughout the parenchyma, and the kidneys displayed focal areas of moderate to severe lymphocytic infiltration within the interstitium. The microscopic lesions in the trachea were characterized by multiple areas of deciliation, minimal lymphocytic and heterophilic infiltration within the lamina propria, and excess mucin. The microscopic lesions in the proventriculus were characterized by mild lymphocytic infiltration beneath the ductular epithelium of the submucosal glands. Microscopic lesions in bursa of Fabricius tissue samples were characterized by multiple areas of mild heterophils in the stroma between the follicles and interfollicular epithelium, as well as mild depletion of lymphocytes within the medullary regions of the follicles. The individual samples displaying lesions in the duodenum during passage of each virus showed a mild increase of heterophils within lamina propria of the gut villi. The one affected heart sample from the H5N1-WB group displayed a focal area of mild lymphocytic infiltration on the endocardial surface. The affected thymi during passage of H5N2-Ck showed a mild depletion of lymphocytes within the cortical regions of

the thymic lobules. In summary, the microscopic lesions were suggestive of transient and local replication of each respective LPAIV.

Sequence analysis of the HA and NA encoding region

RNA was isolated from every swab sample positive for virus isolation and used for RT-PCR. The resulting PCR fragments were directly sequenced with analysis focusing on amino acid exchanges. The analysis revealed that twenty-one amino acids (aa) were exchanged in the HA protein during passage of H5N2-Ck (Table 2), while only six aa were exchanged in the HA protein during passage of H5N1-WB (Table 3). Sequence analysis of the NA protein revealed two aa exchanges during passage of the H5N2-Ck (Table 2) and no exchanges in the NA during passage of the H5N1-WB isolate. The observed amino acid exchanges were not present in all sequences. The frequency of individual exchanges present in the HA of H5N2-Ck varied between 4% and 100%, the latter being exchange T172A that was observed in every positive swab sample. Of the two amino acid exchanges observed in the NA of H5N2-Ck, one was present in 1 out of the 19 analyzed samples (5%) while the second observed exchange was identified in 6 of the 19 samples (32%). The frequency of aa exchanges in the HA was lower during passage of H5N1-WB in ducks (4%-36%, see table 3). The location of each exchange within its respective viral protein was determined based on the model provided by Ha et al. (2002) (Figure 5). During passage of H5N2-Ck, most exchanges in the hemagglutinin (15/21) were localized within the globular head domain of HA1. Two aa exchanges were located within the HA2 portion of the protein. Amino acid 172 (T172A) and aa 518 (V518I) showed the highest frequency of aa exchanges with 100% and 96%, respectively. Interestingly, both exchanges (T172A, V518I) were already present as minor peaks in the sequence chromatogram of the parental H5N2-Ck virus, indicating a selection of viruses containing these aa exchanges (Figure 6A). No minor peaks were observed for any other aa exchanges during passage of H5N2-Ck. During passage of H5N1-WB, only half (3/6) of the aa exchanges were located within the globular head domain, while the

remaining exchanges were located in the HA2 portion of the hemagglutinin protein. The nucleotide exchange leading to the aa exchange I73V was already present as a minor peak in the sequence chromatogram of the H5N1-WB parental virus (Figure 6B). This again suggests that selection was the driving force for the observed aa exchange. These amino acid exchanges from both isolates were observed as early as three days post infection (four days for passage 1). This indicated that the observed mutations were based on fit-for-transmission evolution rather than based on escape from antibody responses. Certain aa exchanges within the HA during passage of H5N2-Ck led to the addition or loss of potential glycosylation sites, as analyzed by prediction programs for N- and O-linked glycosylation sites (http://www.expasy.org). The aa exchange T172A resulted in the loss of a potential N-linked glycosylation site while T226I resulted in the loss of a potential O-linked glycosylation site. The aa exchange P251S resulted in the addition of a possible O-linked glycosylation site.

Analysis of NA region harboring truncation

In a previous study in which the same H5N1-WB AIV isolate was passaged in chickens, a truncated form of the NA gene of the virus was selected for (Dlugolenski et al, 2011). In the present study, further experiments were performed in order to investigate whether the ratio between the full length and truncated forms of the NA gene of this isolate changed during passage in ducks. RNA isolated from allantoic fluid of the parental H5N1-WB virus as well as from a H5N1-WB virus obtained from passage 6 were investigated by RT-PCR in simultaneous experiments using primer sets established by Dlugolenski et al. (2011). The primers used included a set of oligonucleotides flanking the deleted region of the NA (N1FP/N1RP), an oligonucleotide located within the deleted region (N1-INT) to identify the presence of the full-length NA, and an oligonucleotide designed to amplify only the truncated form of NA (N1-Del). The analysis of the RT-PCR products revealed that there were no differences in the signal strength between the RT-PCR products after six passages in ducks (Figure 7A). These results, along with the

sequence analysis of the NA encoding region, indicate that the ratio between the full length and truncated versions of H5N1-WB did not change during passage in ducks. The full length NA population remained the dominant population through passage of the virus.

Similar investigations were performed in the NA encoding region of the H5N2-Ck virus using a combination of oligonucleotides attempting to target the full length NA following the strategy of Dlugolenski et al. (2011). Since the NA gene segment of H5N2-Ck encodes for a truncated NA gene, oligonucleotides (INS-FP1, INS-FP1-1/ INS-FP1-2/ INS-FP1-3/ INS-FP1-4/ INS-FP1-5/, INS-FP2, INS-FP2-1/ INS-FP2-2/ INS-FP2-3/ INS-FP2-4/ INS-FP2-5, INS-FP3) which were located in the extended N2 region of other H5N2 viruses encoding for a longer NA protein were used to amplify a full length NA virus population (Figure 7B). The primers were delineated based on a nucleotide sequence alignment of N2 sequences obtained from wild bird H5N2 isolates encoding for the long version of NA. Additionally used were oligonucleotides flanking the proposed truncated region of the NA of H5N2-Ck (N2-FP1, N2-RP1). RNA of parental H5N2-Ck was used as a positive control for the truncated version of NA and a wild bird isolate, A/duck/NC/674964/07 (H5N2), was used as a positive control for the full length NA. The RT-PCR conditions were varied, including the use of annealing temperature gradients ranging from 45°C to 65°C. When using the oligonucleotide pair N2-FP1/N2-RP2, RT-PCR fragments of appropriate size were amplified from the parental H5N2-Ck isolate as well as the wild bird isolate. When using these primers with isolated RNA from passaged viruses, amplified RT-PCR fragments were similar in size to only the parental H5N2-Ck. When using the oligonucleotides located within the 72 nt truncated region along with N2-RP1, appropriate-sized RT-PCR fragments were amplified only from the RNA of the wild bird positive control. As expected, no cDNA fragment was amplified using the internal primers with RNA of the parental H5N2-Ck isolate. When using these internal primers with RNA from passaged viruses either no cDNA fragment was amplified or cDNA fragments that were amplified did not reveal internal NA sequences, as analyzed by comparing sequences to that of parental H5N2-Ck RNA. The results suggest

that the parental H5N2-Ck isolate did not harbor a virus subpopulation containing a full length version of the NA gene. It should be noted that it is also possible that the targeted internal sequence may have been different from the oligonucleotides used for amplification, thus amplification was not possible.

Discussion

Sustainment of life cycles of viruses is based on their ability to infect a new host. Some viruses infect the host and develop latency (e.g. *Herpesviridae*), while other viruses develop a persistent infection in the host by escaping the immune response through high rates of mutations in epitopes responsible for virus neutralization (e.g. hepatitis C virus, HIV). These groups of viruses do not require immediate transmission; rather their properties allow them to be transmitted solely by chance to preserve their genetic line. A third group of viruses are cleared by the immune response of the infected host in a comparatively short period of time (e.g. *Birnaviridae, Picornaviridae, Orthomyxoviridae*). These viruses have developed different mechanisms to maintain their ability to infect a new host. Infectious bursal disease virus, bovine enterovirus and bovine parvovirus have a high resistance to environmental conditions, (Monteith et al, 1986; Gay and Mundt, 2011) allowing them to contaminate the environment in order to be ingested by a new host. In contrast, AIV can be easily disinfected (Gay and Mundt, 2011) and require transmission in a relatively short period of time. It is generally accepted that the natural hosts of AIV are wild birds, specifically those living in close proximity to water (Slemons and Esterday, 1977; Stallknecht and Shane, 1988). The ability to replicate and subsequently shed from its host is a prerequisite for the distribution of AIV.

From this study, it became evident that Pekin ducks were not as susceptible to AIV isolates which have had a history of passage in chickens (H5N2-CK, H5N3-Ck) as they were to a virus which has been isolated from a wild bird (H5N1-WB). The route of inoculation was important for infection and subsequent duck-to-duck passage using the wild bird isolate, H5N1-WB. Virus titer was also critical for

transmission of one of the chicken isolates (H5N2-Ck). Thus, route of inoculation and virus titer are important determinants of infection and subsequent transmission between ducks. A similar experiment using the same virus isolates was performed by Pillai et al. (2010) in turkeys, chickens and Pekin ducks. In ducks, they observed transmission with the H5N1-WB and H5N2-Ck isolates. No transmission was established with H5N3-Ck, as observed in the present study. Interestingly, they used only the intra-choanal route of inoculation for all experiments, which in our case was not successful. In the present study, the addition of the intranasal route was required for successful infection and transmission. The observed differences may be explained by the difference in volume of virus inoculum used. Pillai et al (2010) used 200 µl to infect ducks, while only 100 µl was used in the present study.

During duck-to-duck passage it was observed that the H5N2-Ck isolate was shed via both the respiratory and digestive tract of ducks, while the H5N1-WB isolate was shed almost exclusively via the digestive tract. These data indicate that both viruses were efficiently shed via the digestive tract of ducks while only the chicken isolate was readily shed via the respiratory tract. During passage in chickens, the same H5N1-WB isolate was shed exclusively via the oro-pharyngeal route (Dlugolenski et al., 2011) which is clearly opposite of the observed shedding patterns of H5N1-WB in ducks. A similar phenotype was observed during the initial characterization of these isolates in ducks. H5N1-WB was predominantly shed via the cloacal route while both AIV isolates from chickens were shed via the oro-pharyngeal route (Mundt et al, 2009). The exact reason for these differences is unknown, but the results indicate that AIV isolates are primarily shed via the respiratory route in chickens regardless of their origin. In ducks, AIV isolates from chickens are shed via both routes, but when infected with a wild bird isolate virus is predominantly shed via the digestive tract. Recently an investigation has been described in which ducks were infected with AIV generated with reverse genetics containing neuraminidases of different stalk lengths (Hoffmann et al., 2012). No difference was observed in the respiratory shedding pattern between the two viruses, indicating that the truncation of the NA was likely not the sole

determinant of this phenotype. Pillai et al. (2010) also observed no differences in viral shedding patterns in ducks between an H5N1 wild bird isolate and an H5N2 chicken isolate. Spackman et al. (2007) reported that an H5N1-WB isolate (the same virus used in this study) was present in oro-pharyngeal as well as cloacal swabs of ducks, but in the latter to a 100-fold higher number of viral RNA copies. Differences in shedding patterns have also been observed between two AIV wild bird isolates (H5N2, H7N2) when inoculated into ducks (Achenbach and Bowen, 2011). Both isolates were efficiently shed via the cloacal route, but only the H7N2 isolate was able to shed to high viral titers in oro-pharyngeal swabs. Spackman et al (2010) described investigations on three different bird species (ducks, turkeys and chickens) using a variety of AIV isolates obtained from domestic fowl (chicken, turkeys) and wild birds (mallard, pintail, ruddy turnstone). In general, isolates from chickens, regardless of host, shed to higher viral titers via the respiratory route. It was noted that wild bird isolates were also able to shed via the respiratory route, especially when inoculated into domestic fowl hosts. In this study (Spackman et al., 2010), as well as in the above described studies (Spackman et al, 2007; Pillai et al. 2010), the viral RNA was measured by real time RT-PCR as equivalent to infectious virus. No virus isolation was performed which makes a direct comparison of the data not possible.

The present study has shown that both viruses were capable of transmission in Pekin ducks based on a model which mirrors natural transmission as close as possible. Upon necropsy, microscopic lesions were observed predominantly in the trachea, lung, spleen, proventriculus, and bursa of Fabricius from ducks of both groups. Interestingly, a similar frequency and appearance of microscopic lesions was observed between passages, indicating that the phenotype of each virus did not change during passage. The similarity in appearance and frequency of observed lesions between the two viruses suggests that the tissue tropism in ducks was similar between the AIV isolate from chicken and from wild bird. The analysis of the sequence data of the HA and NA clearly showed that the highest selective pressure was on the HA of the H5N2-Ck isolate when passaged in ducks. Twenty-one different amino acid exchanges

were observed in the HA during passage of H5N2-Ck. Most of them were observed in the globular head domain of the HA1, a region responsible for attachment and binding to sialic acid receptors. This suggests that during passage in ducks a kind of back evolution of the AIV chicken isolate may have occurred. This is rather hypothetical since the characteristics of the virus isolate which was originally introduced into the chicken population is unknown. The observed genetic stability of the HA aa sequence of H5N1-WB in our study was similar to the genetic stability of an HA of a highly pathogenic H5N1 human isolate which was passaged five times in ducks (Shinya et al, 2010). In the case of the human isolate, only one mutation was observed in the HA and no mutations were observed in the NA after passaging in ducks.

In the present study, a genetic stability of the NA amino acid sequence of H5N2-Ck was observed through intense investigations by RT-PCR of the parental virus and viruses from passage 6. From this, it is suggested that an H5N2-Ck subpopulation containing a full length NA was not present in the parent isolate to become selected for, implying its lack of necessity in transmission of a chicken isolate in ducks. The possibility that the length of the NA protein is not critical for replication and transmission in ducks has also been suggested through investigations described by other research groups (Shinya et al, 2010; Hoffmann et al, 2012). As for the parental H5N1-WB isolate and its respective virus from passage 6, the ratio between the truncated NA sequence (minor population) and the full length NA sequence (major population) did not change. Based on this finding it can be assumed that, although still present, the truncated NA of H5N1-WB had no advantage for transmission in ducks. In contrast, a shorter NA nucleotide sequence encoding for a truncated protein became the major virus population during passage of the same H5N1-WB virus in chickens (Dlugolenski et al, 2011).

Our study suggests that LPAIV isolates can be efficiently transmitted in ducks irrespective of their origin. The results show that domestic ducks can support replication and transmission of influenza

A virus from both waterfowl and poultry species without prior adaptation, although with variable efficacies. Differences between the two viruses were observed in their shedding patterns as well as mutation rates of the HA gene. While the chicken isolate was shed via both routes in ducks (oro-pharyngeal and cloacal), passaged virus of the wild bird isolate was shed primarily via the cloacal route. The mutation rate of the HA of the H5N2-Ck was much higher than that of the H5N1-WB during passage in ducks, indicating a selection pressure required of the chicken isolate to adapt to replication in waterfowl. The low mutation rate of the NA gene in both viruses suggests a lesser contribution of this protein to replication adaptation in waterfowl.

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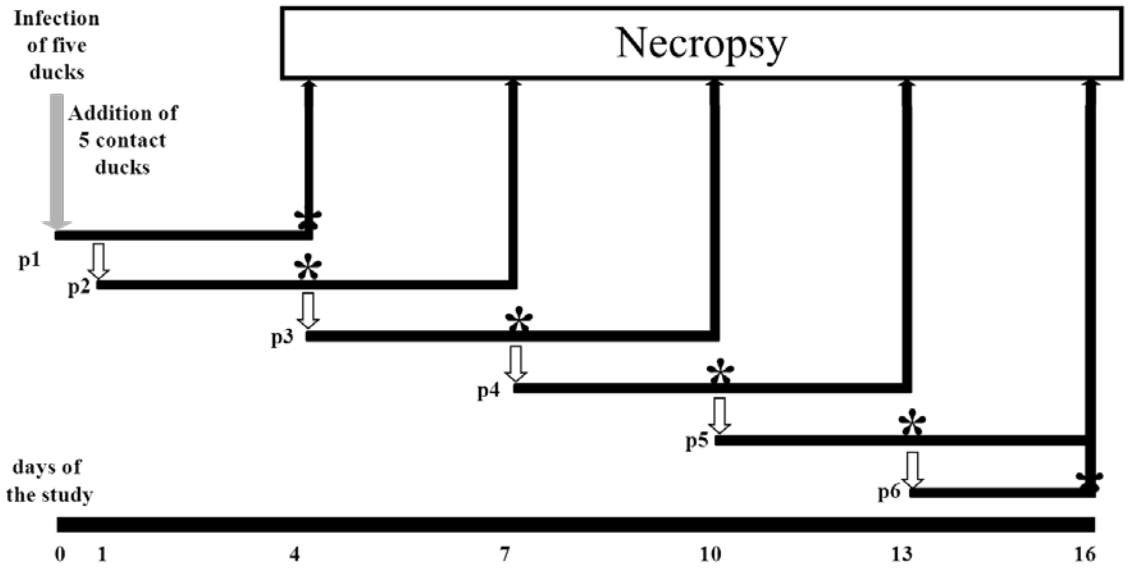

Figure 1. Schedule of serial passage of LPAIV isolates in Pekin ducks. Five ducks were infected on the first day of the study (day 0), indicated by a grey arrow. The additions of five naïve contact ducks are marked by open arrows. The removal of ducks for necropsy is shown with black arrows. The days in which swab samples were taken are marked by an asterisk above the respective group. The appropriate passage (p) number is indicated for each group of ducks.

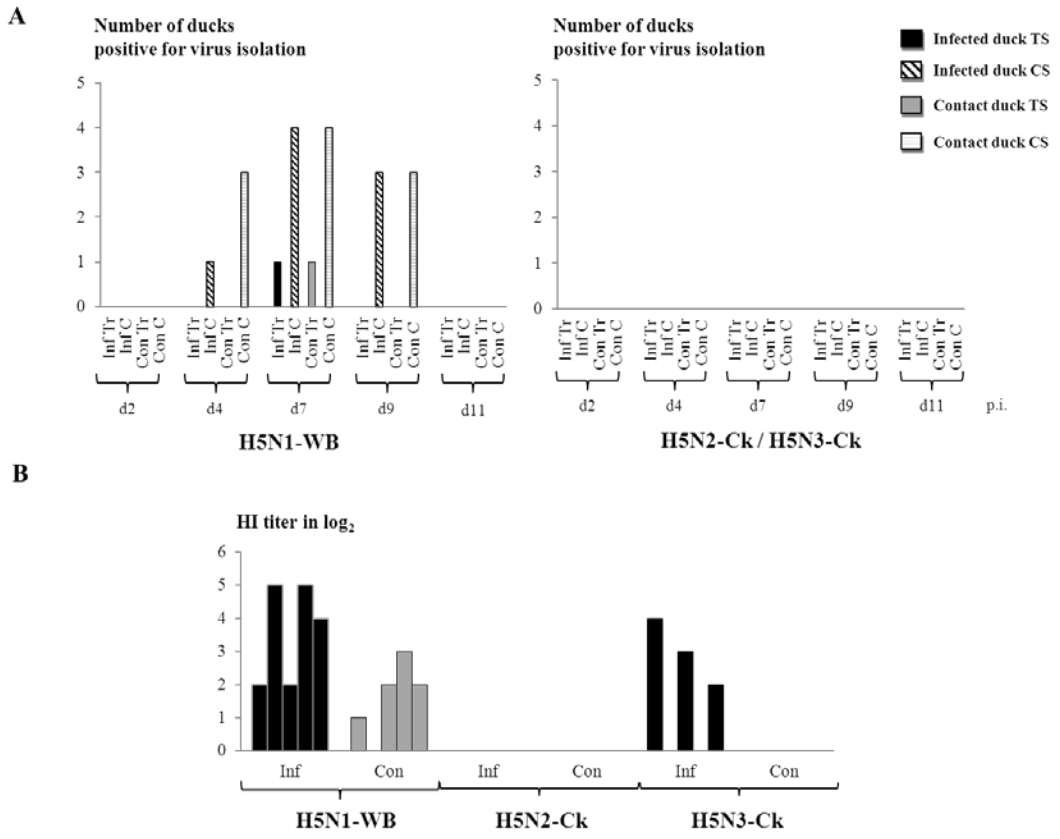

Figure 2. Replication and seroconversion of low pathogenic avian influenza viruses in Pekin ducks after a combined intra-choanal/intra-nasal route of infection. (A) Five, two-week-old Pekin ducks were infected (Inf) with one of three H5 isolates of low pathogenic AIV [A/Mute swan/ MI/ 451072/06 (H5N1), H5N1-WB; A/Ck/PA/13609/93 (H5N2), H5N1-Ck; A/Ck/TX/167280-4/02 (H5N3), H5N3-Ck] using an infectious dose equivalent to 10^6 EID_{50}/100 µl per duck via a combined intra-choanal/intra-nasal route. Twenty-four hours later, five naïve ducks were added to each group as contacts (Con). Tracheal swabs (TS) and cloacal swabs (CS) were taken at the indicated days after infection (d.p.i.) and inoculated into nine-day-old embryonated SPF chicken eggs. Bars indicate the number of swab samples positive for virus isolation. In this case, ducks from only the H5N1-WB group were positive for virus isolation. (B) Fourteen days after virus inoculation (thirteen days for contact birds) blood samples were taken from infected (black bars) and contact (grey bars) ducks and the serum investigated for the presence of respective LPAIV antibodies in an appropriate hemagglutination inhibition test (HI test). Bar height indicates the log_2 of the reciprocal of the last dilution where hemagglutination inhibition was observed for each duck.

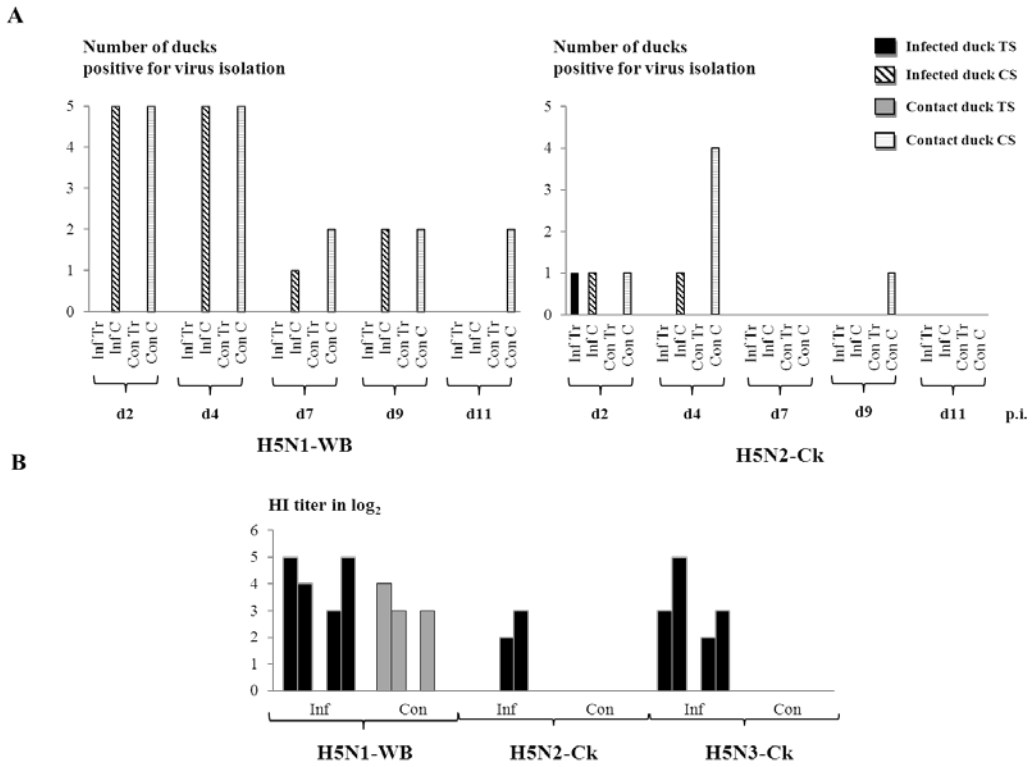

Figure 3. Replication and seroconversion of low pathogenic avian influenza viruses in Pekin ducks after a combined intra-choanal/intra-nasal route of infection and a higher virus dosage. (A) Five, two-week-old Pekin ducks were infected (Inf) with one of three H5 isolates of low pathogenic AIV [A/Mute swan/ MI/ 451072/06 (H5N1) H5N1-WB, A/Ck/PA/13609/93 (H5N2) H5N1-Ck, A/Ck/TX/167280-4/02 (H5N3) H5N3-Ck] using an infectious dose equivalent to 10^6 EID_{50}/100 µl, 10^9 EID_{50}/100 µl, and $10^{7.25}$ EID_{50}/100 µl per duck, respectively. Ducks were infected via a combined intra-choanal/intra-nasal route. Twenty-four hours later, five naïve ducks were added to each group as contacts (Con). Tracheal swabs (TS) and cloacal swabs (CS) were taken at the indicated days after infection (d.p.i.) and inoculated into nine-day-old embryonated SPF chicken eggs. Bars indicate the number of swab samples positive for virus isolation. In this case, ducks from both the H5N1-WB and H5N2-Ck group were positive for virus isolation. All ducks of the H5N3-Ck group were negative for virus isolation (data not shown). (B) Fourteen days after virus inoculation (thirteen days for contact birds) blood samples were taken from infected (black bars) and contact (grey bars) ducks and the serum investigated for the presence of respective LPAIV antibodies in an appropriate hemagglutination inhibition test (HI test). Bar height indicates the log_2 of the reciprocal of the last dilution where hemagglutination inhibition was observed for each duck.

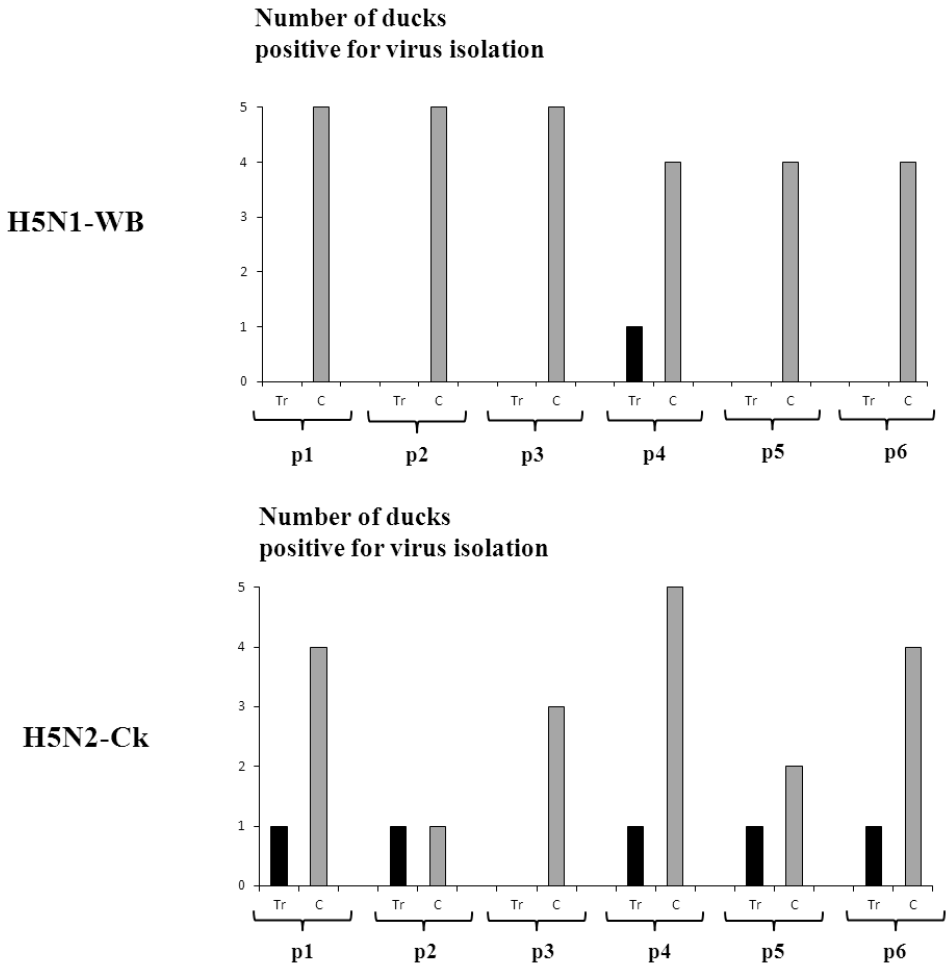

Figure 4. Replication of two low pathogenic avian influenza viruses through six serial passages in Pekin ducks. Five, two-week-old Pekin ducks were infected (Inf) with 10^6 EID_{50}/100 µl of A/Mute swan/ MI/451072/06 (H5N1) [H5N1-WB] or 10^9 EID_{50}/100 µl of A/Ck/PA/13609/93 (H5N2) [H5N2-Ck] via a combined intra-choanal/intra-nasal route. The initially infected group represents passage 1 (p1) of each respective virus. Twenty-four hours later, five naïve ducks were added to each group as contacts (p2). Each subsequent passage was performed as described in Materials and Methods. Tracheal swabs (Tr, black bars) and cloacal swabs (C, grey bars) were taken from ducks four days post inoculation (p1) and three days post contact exposure (p2-p6). Isolation of AIV from swab samples was performed in nine-day-old embryonated SPF chicken eggs. Bars indicate the number of swab samples positive for virus isolation.

Table 1. **Microscopic changes observed during passage of H5N1-WB and H5N2-Ck.**

	Passage number	C [a]	Lu	Sp	K	L	Tr	D	P	PV	H	T	B	CT
H5N1-WB	p1	0/3 [b]	1/3	0/3	0/3	0/3	1/3	0/3	0/3	1/3	1/3	0/3	0/3	0/2
	p2	0/3	1/3	1/3	1/3	0/3	3/3	0/3	0/3	2/3	0/3	0/3	2/3	0/2
	p3	0/3	1/3	1/3	1/3	0/3	2/3	0/3	0/2	1/3	0/3	0/3	3/3	0/2
	p4	0/3	1/3	1/3	1/3	0/3	2/3	0/3	0/3	0/3	0/1	0/3	2/3	0/2
	p5	0/3	0/2	3/3	0/3	0/3	2/3	1/3	0/3	2/3	0/2	0/3	2/3	1/3
	p6	0/3	0/3	1/3	2/3	0/3	2/2	0/3	0/3	1/2	0/2	0/3	2/3	0/2
	total	0/18	4/17	7/18	5/18	0/18	12/17	1/18	0/17	7/17	1/14	0/18	11/18	1/13
	percentage	0% [c]	24%	39%	28%	0%	71%	6%	0%	41%	7%	0%	61%	8%

	Passage number	C [a]	Lu	Sp	K	L	Tr	D	P	PV	H	T	B	CT
H5N2-Ck	p1	0/3 [b]	2/3	1/3	0/3	0/3	3/3	1/3	0/3	2/3	0/3	0/3	3/3	0/0
	p2	0/3	1/3	1/3	1/3	0/2	3/3	0/2	0/3	0/3	0/3	1/3	3/3	0/0
	p3	0/3	0/3	3/3	1/3	0/3	2/3	0/3	0/3	2/3	0/3	0/3	0/3	0/0
	p4	0/3	0/3	1/3	0/3	0/3	2/3	0/3	0/3	1/3	0/3	0/3	1/3	0/0
	p5	0/3	1/3	0/3	0/2	0/3	1/3	0/3	0/3	1/3	0/3	0/3	2/3	0/0
	p6	0/3	0/3	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/2	2/3	3/3	0/1
	total	0/3	4/18	6/18	2/17	0/17	13/18	1/18	0/18	6/18	0/17	3/18	12/18	0/1
	percentage	0%[c]	22%	33%	12%	0%	72%	6%	0%	33%	0%	17%	67%	0%

[a] Tissue samples were taken at day 6 p.i. of each passage (day 4 for passage 1).
cerebrum: C, lung: Lu, spleen: Sp, kidney: K, liver: L, trachea: Tr, duodenum: D, pancreas: P, proventriculus: PV, heart: H, thymus: T, bursa of Fabricius: B, cecal tonsils: CT.

[b] Number of ducks from each passage displaying microscopic lesions in the indicated tissue sample (see text). Lesions indicative of viral infection are highlighted in grey.

[c] Percentage of total ducks necropsied displaying microscopic lesions in the indicated tissue sample.

Table 2. **Amino acid exchanges in the HA and NA protein of H5N2-Ck during passage in Pekin ducks.**

Passage number	HA protein							
	M4I [a]	V73I	N112R	Q126H	E128K	I132V	D142E	T172A
p1	2/5 [b]	0/5	2/5	2/5	2/5	0/5	2/5	5/5
p2	1/2	0/2	1/2	1/2	1/2	0/2	1/2	2/2
p3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3
p4	0/6	0/6	0/6	0/6	0/6	1/6	0/6	6/6
p5	1/3	1/3	1/3	1/3	1/3	0/3	1/3	3/3
p6	1/5	0/5	1/5	1/5	1/5	0/5	1/5	5/5
total	5/24	1/24	5/24	5/24	5/24	1/24	5/24	24/24
percentage	21% [c]	4%	21%	21%	21%	4%	21%	100%

Passage number	HA protein							
	Q177K	L191I	T226I	K228E	I242M	R250K	P251S	I281M
p1	2/5	1/5	2/5	1/5	2/5	1/5	1/5	0/5
p2	1/2	1/2	1/2	½	1/2	1/2	1/2	1/2
p3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
p4	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
p5	1/3	1/3	1/3	1/3	1/3	1/3	1/3	0/3
p6	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
total	5/24	4/24	5/24	4/24	5/24	4/24	4/24	2/24
percentage	21%	17%	21%	17%	21%	17%	17%	8%

Passage number	HA protein					NA protein	
	K282R	V298L	E317G	L452F	V518I	K148R	Y260F
p1	0/5	0/5	0/5	0/5	4/5	1/4	0/4
p2	1/2	1/2	0/2	½	2/2	1/2	0/2
p3	0/3	0/3	0/3	0/3	3/3	2/3	0/3
p4	0/6	0/6	1/6	0/6	6/6	2/5	1/5
p5	0/3	0/3	0/3	0/3	3/3	0/2	0/2
p6	1/5	1/5	0/5	0/5	5/5	0/3	0/3
total	2/24	2/24	1/24	1/24	23/24	6/19	1/19
percentage	8%	8%	4%	4%	96%	32%	5%

[a] numbering of the amino acid position in accordance with the sequence published in NCBI genbank, HA (CY034681), NA (CY034682); amino acids are indicated by single letter code.

[b] number of samples from each passage expressing the indicated amino acid exchange at that position

[c] percentage of the total number of samples expressing the indicated amino acid exchange

Table 3. Amino acid exchanges in the HA protein of H5N1-WB during passage in Pekin ducks.

Passage number	I73V [a]	A200E	G276R	E420K	K424E	K425R
p1	2/5 [b]	2/5	1/5	0/5	0/5	0/5
p2	2/5	4/5	0/5	1/5	2/5	1/5
p3	1/5	3/5	0/5	0/5	1/5	1/5
p4	1/5	0/5	0/5	0/5	0/5	3/5
p5	1/4	0/4	0/4	0/4	0/4	0/4
p6	3/4	0/4	0/4	0/4	0/4	0/4
total	10/28	9/28	1/28	1/28	3/28	5/28
percentage	36% [c]	32%	4%	4%	11%	18%

[a] The numbering of the amino acid position in accordance with the sequence published in NCBI genbank, HA (CY034679). Amino acids are indicated by single letter code.

[b] number of samples from each passage expressing the indicated amino acid exchange at that position

[c] percentage of the total number of samples expressing the indicated amino acid exchange

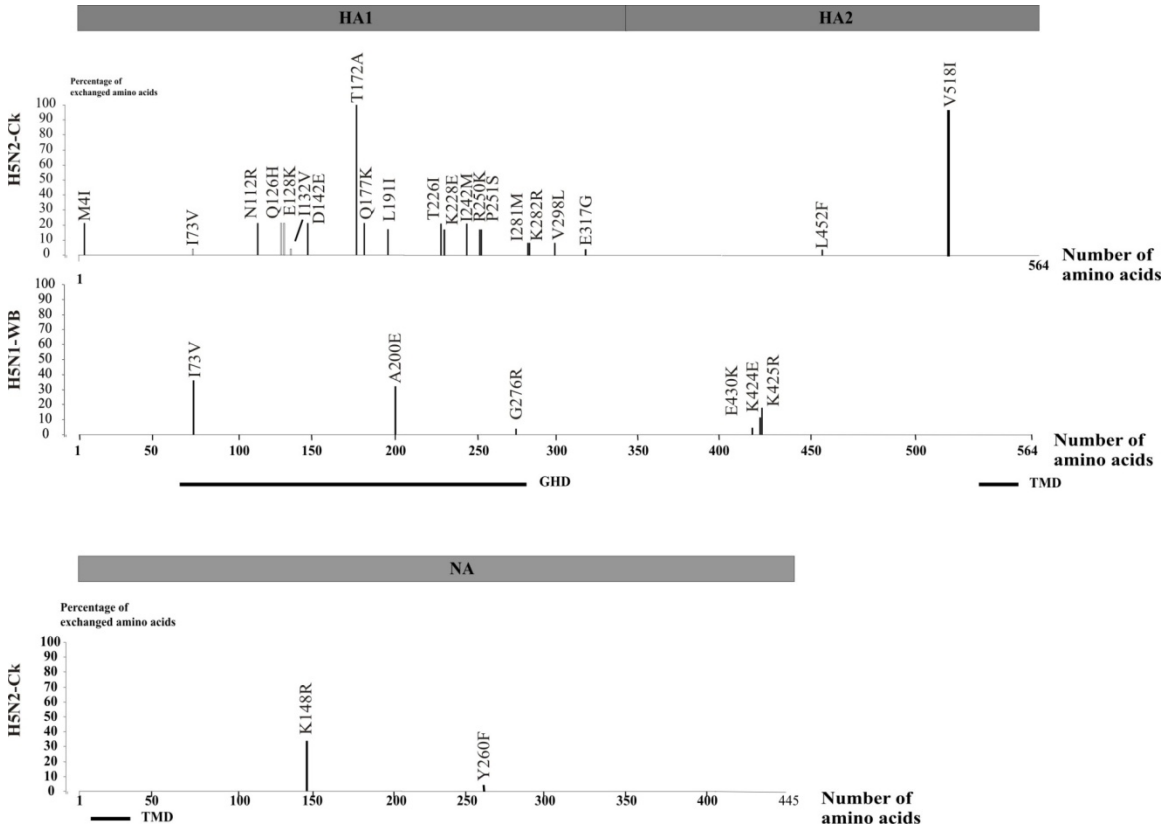

Figure 5. Location of amino acid exchanges in the glycoproteins of two low pathogenic avian influenza viruses. Pekin ducks were infected with A/Mute swan/ MI/ 451072/06 (H5N1) [H5N1-WB] or A/Ck/PA/13609/93 (H5N2) [H5N2-Ck] and a duck-to-duck transmission study was performed over six serial passages. From each swab sample positive for virus isolation the hemagglutinin (HA) and neuraminidase (NA) nucleotide sequences were determined and translated *in silico* into the appropriate amino acid sequences. Exchanges in each amino acid sequence with respect to its parental virus are indicated using single letter code. The frequency of each exchange, identified as a percent of the total number of positive swab samples, is indicated by the height of its respective bar. The location of the globular head domain (GHD) and transmembrane domain (TMD) are highlighted below the respective protein. The numbering for each amino acid sequence is in accordance with the parental sequence published in NCBI genbank [HA of H5N1-WB (accession number CY034679), HA of H5N2-Ck (CY034681), NA of H5N2-Ck (CY034682)]. No amino acid exchanges were identified in the NA of H5N1-WB during passage in ducks.

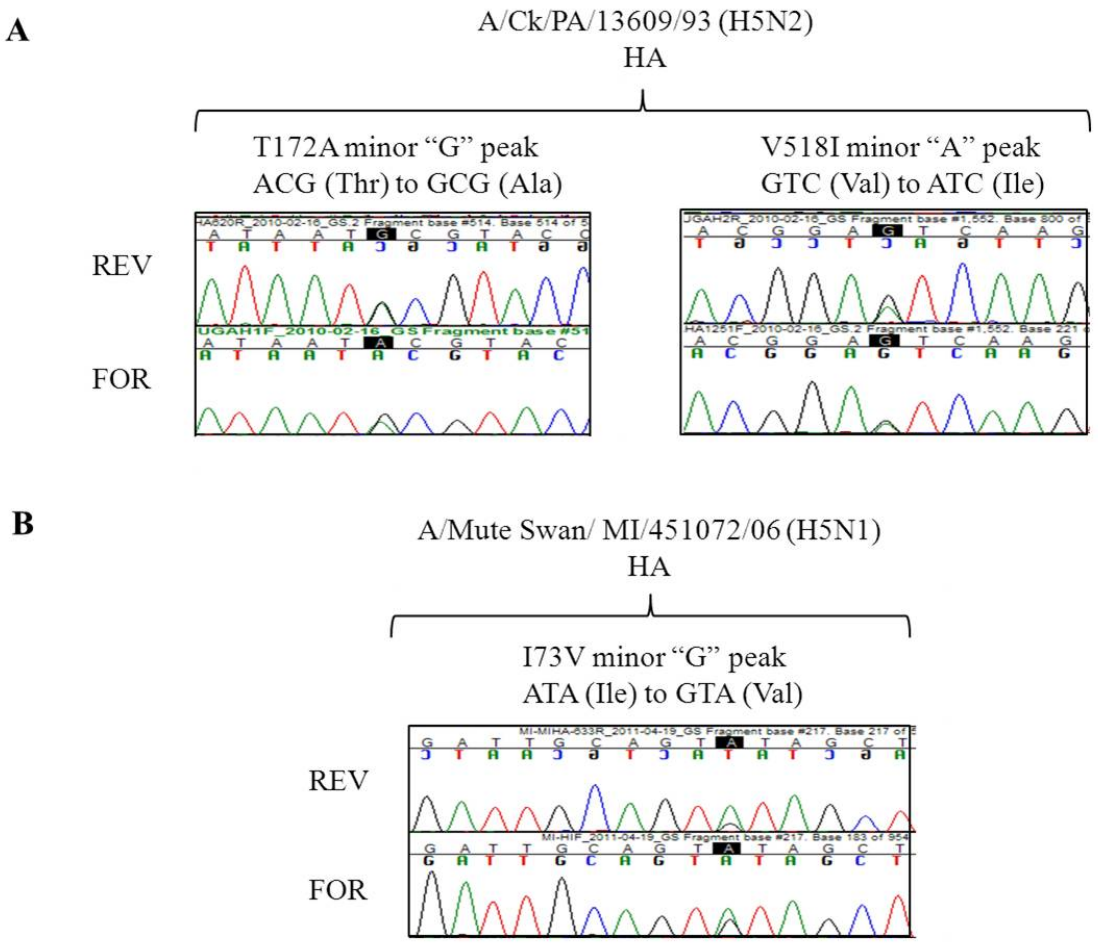

Figure 6. Analysis of chromatograms of the HA gene sequences. Allantoic fluid from the parental virus of (A) A/Ck/PA/13609/93 (H5N2) and (B) A/Mute Swan/ MI/ 451072/06 (H5N1) were used for sequence analysis. The amino acid exchanges observed during passage were analyzed for the presence of minor peaks present in the chromatograms of the parental forward (FOR) and reverse (REV) nucleotide sequences. The observed minor sequencing peak representing the corresponding change in the codon is shown at the top of each chromatogram. The first nucleotide of the affected codon is indicated by a black box.

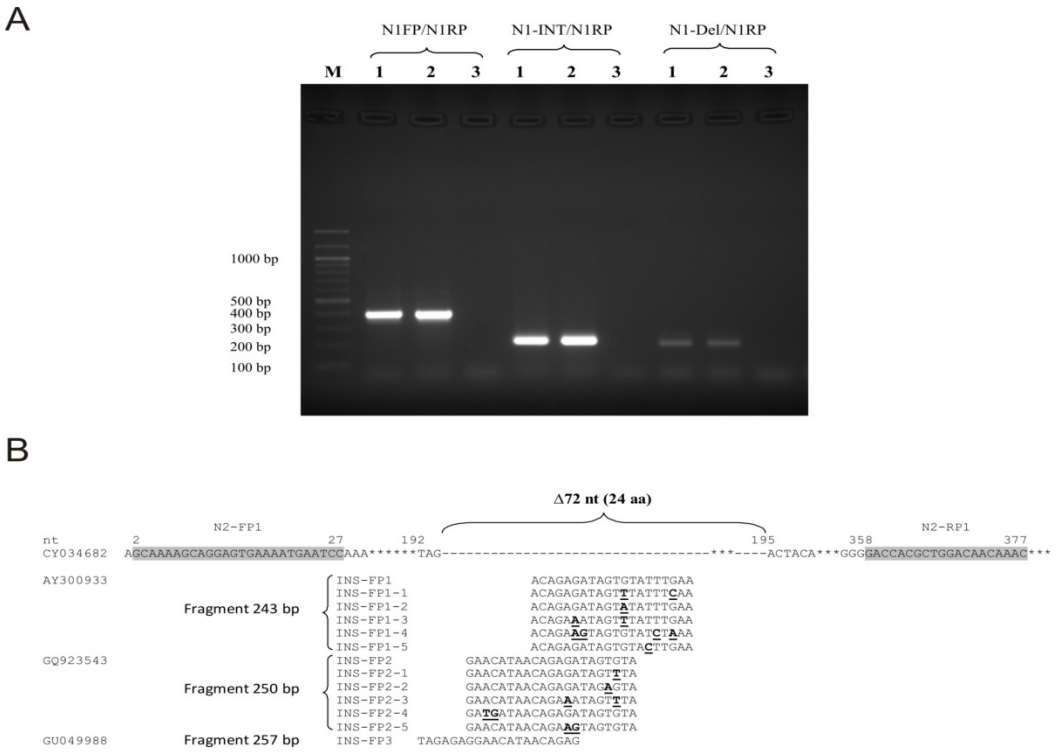

Figure 7. Investigations of the region harboring truncation in the NA protein. (A) RT-PCR was performed using RNA isolated from allantoic fluid of (1) the parental H5N1-WB virus, (2) a virus obtained from an H5N1-WB passage 6 cloacal swab, and (3) a negative control obtained during RNA preparation containing the same volume of phosphate buffered saline. The RT-PCR was performed with three sets of primer pairs, one set targeting sequences flanking the deleted region of NA (N1FP/N1RP), one set targeting the extended region of the full length NA (N1-INT/N1RP), and one set targeting the truncated form of NA (N1-Del/N1-RP1). The sequences for these primers have been previously published (Dlugolenski et al, 2011). The reaction products and a 100 bp DNA ladder (M) were separated on a 1.5% agarose gel. (B) The location of the oligonucleotides used in RT-PCR for the analysis of the truncated region of NA of H5N2-Ck is shown. Oligonucleotides N2-FP1 and N2-RP1 bind in known regions flanking the deleted portion of the H5N2-Ck NA genome segment based on its published sequence (Genbank accession number CY034682). The nucleotide sequences for the oligonucleotides INS-FP1, INS-FP2, and INS-FP3 were based on full length H5N2 NA sequences published in NCBI genbank, AY300933, GQ923543, and GU049988, respectively. The remaining oligonucleotides (1-5) were delineated based on a nucleotide alignment using 30 sequences of the full length N2 gene of H5N2 wild bird isolates (see Material and Methods). The sequences of the oligonucleotides are located within the 72 nucleotide (nt) region not present in the parental H5N2-Ck virus. This truncated region, highlighted by dashes, likely resulted when the original H5N2 virus was introduced and passaged in chickens. The sizes of the expected RT-PCR fragments are indicated for each set of primers. The asterisks represent the nucleotides which are present in the sequence but are not shown.

CHAPTER 4

CONCLUSIONS

Increased urbanization into wildlife habitats along with the continual circulation of influenza A viruses in wild reservoirs poses a significant risk of transmission to domestic poultry. Even though host specificity is well-documented, it is widely accepted that influenza A virus from wild birds are able to transmit to poultry. Understanding the abilities of different low pathogenic avian influenza viruses (LPAIV) to transmit between avian hosts is essential, especially when considering their ability to mutate into highly pathogenic strains after introduction in poultry. It is well-established that spillover cases of direct poultry-to-human transmission of highly pathogenic avian influenza viruses can have detrimental effects on the human population. Understanding the viral evolution occurring between different species, especially between wild and domestic avian hosts, may provide insight into how these viruses maintain their constant circulation. Part of understanding this evolution is identifying changes in their viral genome as a result of host adaptation leading to efficient transmission between these species.

In order to identify genetic changes associated with host adaptation, serial passage of LPAIV isolates of different origins were performed in Pekin ducks. A transmission model attempting to mimic the natural transmission of influenza A viruses was used to passage two different LPAIV chicken isolates (H5N2-Ck, H5N3-Ck) and one LPAIV wild bird isolate (H5N1-WB). Differences in transmission capabilities between these viruses were dependent on inoculation dosage and route of infection, with only the H5N1-WB and H5N2-Ck establishing consistent transmission in ducks. Isolation of virus from swab samples also indicated differences in shedding patterns of LPAIV of different origins. These results

support previous findings that LPAIV of chicken origin is shed via the respiratory tract more readily than LPAIV of wild bird origin (1). Interestingly, similar microscopic lesions were observed during infection and passage of both H5N1-WB and H5N2-Ck, suggesting a similar tissue tropism of both virus isolates in ducks. From these results, it has been determined that LPAIV isolates of different origin transmit between ducks with variable efficacies and with variable shedding patterns.

Virus origin not only influenced transmission capabilities in ducks, but also influenced the frequency of amino acid exchanges observed in each isolate during passage. The study published by Dlugolenski et al. (2011) showed a clear example of how a virus can modify its genome to best suit its survival and transmission in a new host (2). In support of host adaptation through genome modification, the current study identifies numerous amino acid exchanges within the hemagglutinin of a LPAIV chicken isolate when passaged in ducks. A higher frequency of exchanges in the HA of H5N2-Ck compared to H5N1-WB suggests a stronger selection process of the poultry isolate during passage in waterfowl. It was observed that two of the exchanges present in the HA during passage of the chicken isolate (T172A and V518I) were already present as minor virus subpopulations in the parent virus, suggesting that these viruses were more selectively fit for survival in the waterfowl host. Additionally, the same occurrence was evident in the HA of the wild bird isolate during passage in ducks, indicating that the I73V minor virus subpopulation identified in the parent virus was more fit for survival in ducks. The majority of amino acid exchanges present in the HA during passage of the chicken isolate were located within the globular head of the protein. Located within this region are receptor-binding domains as well as antigenic recognition sites. Amino acid exchanges within these domains would likely influence the binding specificity and recognition of the virus in the new host. Three of the exchanges localized to the globular head of HA had also potentially altered the glycosylation patterns of the protein, a phenomenon that can affect host selection and/or pathogenicity of the virus (3, 4). No

modifications to potential glycosylation sites were observed in the HA during passage of the wild bird isolate.

Amino acid exchanges within the NA were much rarer, with the wild bird isolate showing no exchanges during passage and the chicken isolate expressing only two exchanges. The genetic stability of the NA protein after passage of the chicken isolate suggests that this isolate did not harbor a full length version of the protein to be selected for during passage in waterfowl. The chicken isolate, containing a truncated version of the NA, was successfully passaged through ducks, suggesting that a full length version of the protein was not necessary for efficient transmission of this virus in ducks. Interestingly though, the majority of sequences from wild bird isolates contain a long version of the NA sequence. From a previous study (2), it was confirmed that the wild bird isolate used in this study contained a minor virus subpopulation harboring a truncated NA gene. In the present study, passage of this virus in ducks showed that, while the short version of NA still existed, the full length version was the dominant population during all six passages. Thus, perhaps while a full length NA may be most fit for survival and transmission of influenza A viruses in waterfowl, it may not be essential. Further studies would certainly be needed to confirm this. Other studies continuing to identify the genetic adaptations surrounding transmission and adaptation of LPAIV in new hosts may help to understand the selection contributing to the emergence of novel viruses in avian hosts.

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