PASSAGE OF LPAIV H5 ISOLATES IN CHICKENS RESULTS IN GENOTYPIC CHANGES IN THE GLYCOPROTEIN GENES AND DEVELOPMENT OF A SPECIES INDEPENDENT COMPETITIVE ELISA SYSTEM.

By

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(Under the Direction of Egbert Mundt)

ABSTRACT

The determination of the hemagglutinin (HA) subtype is restricted to inhibition and virus neutralization assays. Both assays are labor intensive and not suitable for automation. The presence of HA5 and HA7 antibodies are of major interest since these subtypes of AIV can develop a highly pathogenic phenotype. To develop a test which can be used in automatic settings the HA5 protein of a wild bird isolate [A/duck/NC/674964/07 (H5N2)] was cloned and subsequently expressed in a baculovirus system. In addition, a monoclonal antibody specific for the HA5 antigen (H5-mAb) was generated. Both reagents were used to establish a competitive ELISA (cELISA) system. The cELISA performed with influenza antibody free sera or sera of animals infected with AIV HA subtypes other than H5 showed no significant inhibition of H5-mAb binding, indicating a high specificity of the test. In contrast, sera of animals (chickens, turkeys, mallards, redheads, wood ducks, and cats) experimentally infected with H5 subtype AIV or vaccinated with inactivated vaccines were able to significantly inhibit the binding of the H5-mAb. The cELISA showed a significant inhibition (> 25%) of mAb binding in hemagglutination inhibition (HI) positive serum samples in chicken, duck, and turkey sera with a

reproducibility of >95%. This test provides a platform for further development of other subtype specific HA ELISAs.

Additionally, to better understand the mechanisms of LPAIV transmission and adaptation serial passages of LPAIV isolates were performed in chickens. Sequences of the HA and NA genes were determined for the stock viruses and at each subsequent passage. Three groups of 3-week-old SPF chickens were infected with three different LPAIV, one wild bird isolate (H5N1), and two chicken isolates (H5N2, H5N3). At 1 day post inoculation (pi), 5 contact birds were added to each group. In addition, subsequent passages in chickens for each virus were performed. Tracheal and cloacal swabs were taken at 2, 4, 7, and 9 d pi., and used for virus isolation (VI) in embryonated SPF eggs. LPAIV positive allantoic fluid was detected by hemagglutination assay. Existence of HI antibodies was tested on serum samples taken before and 21d after infection. The NA and HA genes of the appropriate virus of VI positive swab and allantoic fluid samples were sequenced and amino acid (aa) sequences were deduced.

INDEX WORDS: Low Pathogenic Avian Influenza, Transmission, ELISA, H5N1, Hemagglutinin, and Neuraminidase

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DEDICATION

I would like to dedicate this thesis to my family and friends who have supported me through this process and provided exponential guidance and help especially my mother, sister, and brother, Debbie, Sasha, and David. Without your continued support and guidance I would not be where I am today. Your strength and confidence have provided me with the drive and determination to complete whatever I set my mind too. I would also like to dedicate this thesis to my closest friends Josh and Kyle your continued support and distraction helped me keep a level head and even have a bit of fun.

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CHAPTER 1

INTRODUCTION

Influenza A virus has caused three major human pandemics in the last century and one that emerged in 2009 resulting from a triple reassortment of viral genomic segments in pigs. Influenza A virus is a member of the family *Orthomyxoviridae*. Influenza A virus is a pleomorphic enveloped particle that contains a lipid membrane derived from the host cell. Influenza A virus has a segmented negative sense RNA genome consisting of 8 segments that encode for 11 proteins. The viral proteins PB1, PB2, PA, NP, and the viral RNA comprise the viral ribonucleotide protein (vRNP) complex. In addition to these viral proteins the virus encodes the hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), a small protein PB1-F2, and the nuclear export protein (NEP). The only non-structural protein is the NS1 protein. The HA and NA proteins are surface glycoproteins responsible for binding and release of the viruses respectively. The M2 protein forms a hydrogen ion pore in the envelope of the virus. The M1 protein forms a layer below the envelope that interacts with the vRNP complex for organization of the viral RNA segments; while, it also functions for the export of the RNP from the nucleus. NS1, NEP, and PB1-F2 are regulatory components of the virus mediating export of proteins, vRNP's, and modulation of the host immune response to viral infection.

The HA protein mediates binding of the virus to targets cells through interactions between the HA receptor binding domain and sialic acids on the host cell. HA also mediates membrane fusion of the virus allowing release of the vRNP complexes from the cellular

endosome into the cytoplasm and is considered to be the key determinant for host tropism of influenza A virus. HA binding to host cells is mediated by the orientation of the sialic acids presented on the outer cell membrane of the host. For instance, avian species typically express an α 2,3 orientation of terminal sialic acid residues where mammal cells typically express an α 2,6 orientation on the cells in the upper respiratory tract. HA is also a very important determinant of pathogenicity associated with low pathogenic (LPAI) or high pathogenic avian influenza (HPAI) in chickens. LPAI have a characteristic HA cleavage site in their amino acid (aa) sequence between the HA subunits that is monobasic in nature and exhibits only a limited ability to infect deeper layers of cells in the respiratory and intestinal tract due to the lack of trypsin-like proteases in these cells. This monobasic cleavage site is mainly cleaved by furin-like proteases which are present at the surface of epithelial cells. HPAI encode for a polybasic cleavage site between the HA subunits that can be cleaved by host furin-like proteases which are constitutively expressed in most cells, thus allowing for systemic infections of the avian host to occur. HA is a very important antigenic viral protein and induces neutralizing antibodies against the virus.

The NA protein mediates viral release and clearance after viral budding occurs at the outer cellular membrane in cleavage of terminal sialic acids. The NA protein also supports viral spread by allowing the virus to penetrate the mucosa of the respiratory tract. NA has been shown to be involved in viral pathogenicity while the exact function has not yet been shown. Neutralizing antibodies against the NA protein are described but have limited neutralizing activity; especially, when compared to neutralizing antibodies induced by the HA protein.

Because influenza A virus is a segmented RNA virus, it can rapidly undergo genetic shift. The HA and NA proteins are the most variable proteins that undergo genetic mutations which

can result in antigenic drift. So far, 16 different HA subtypes and nine different NA subtypes have been described.

Rapid evolution of influenza A viruses can present problems for the development of diagnostic assays for influenza viruses. The development of viral antigen or viral genome based detection systems has been rapidly evolving and new technologies are currently emerging including microarray, RT-PCR based assays, and isothermal amplification based techniques. But, the development of antibody detection assays has remained constant. The gold standard for serology of avian influenza being the agar gel immunodiffusion test (AGID), hemagglutination inhibition (HI) test , and neuraminidase inhibition (NI) test.

The AGID test is an application of the Ouchterlony immune-double diffusion assay to influenza A virus. AGID allows for the visualization of the reactivity of influenza A specific antibodies with antigen after gel diffusion. The AGID test can either be used to detect antigen or antibodies using appropriate reference antibodies or antigen. The test is relatively inexpensive until it comes to quality assurance and the hiring of quality researchers that can accurately interpret the results. AGID test results can be obtained within 24-48 hours making it a relatively quick test.

The HI test is readily used to characterize the HA subtype. The test can be used to either determine the HA antigenic subtype in viral samples or to subtype antibodies in serum samples. The HI assay may require the use of biological containment facilities when the antigen used is infectious virus but inactivated viruses can be used in this test. The advantage is that this test can be rapidly performed but isn’t automatable in the field.

The NI assay is used to determine the NA subtype of influenza viruses. The test relies on the presence of antibodies that inhibits the enzymatic activity of the neuraminidase protein. The NI test is sensitive, specific, and extremely useful in neuraminidase subtyping of newly isolated viruses. The NI test is relatively expensive and requires slightly more time to complete when compared to the HI test.

The first goal of our research was to develop an H5 specific species-independent competitive ELISA (cELISA) system for the detection of H5 specific antibodies in serum samples from different species. cELISA systems can be highly specific and sensitive. The cELISA would allow for an automation that can be readily used in field situations. cELISA systems are relatively cheap and do not necessarily require the use of infectious virus for testing.

Additionally, not much is known about host adaptation of LPAI when transmission occurs between a wild bird and domestic poultry or from poultry to mammals. One well known adaptation that appears to be conserved during passaging of wild bird isolates into chickens is the addition of a single N-glycosylation site at the globular head of the protein. It is believed that this additional glycosylation site increases the binding affinity of the HA to the targets cells of the respiratory tract of chickens. Additionally, it has been well described that a deletion of a stretch of amino occurs in the stalk region of the NA protein. It is believed that this aa deletion occurs as a regulatory response to the increased binding efficiency of the HA protein. Truncation of the NA potentially leads to less efficient clearance of the virus creating a balance between viral binding and release. The second goal of this study is to obtain a better understanding of the mechanisms involved in LPAI transmission and adaptation when passaged through chickens. During our research, we focused on the surface glycoproteins HA and NA which might undergo the highest level of selective pressure when passaged from one host to another.

CHAPTER 2

LITERATURE REVIEW

History

Avian influenza virus was first described in 1878 by Peroncito which occurred in response to an outbreak of highly pathogenic avian influenza virus (HPAI) in northern Italy and other European countries [1]. The disease was first characterized as a contagious poultry disease that resulted in high mortality and was initially termed the fowl plague. Additionally, the Italian outbreak in the northern part of the country was termed “malattia gravissima” by the residents which is translated “most severe disease”. HPAI was initially termed fowl plague because researchers at the time believed that HPAI was actually the acute septicemic form of fowl cholera. In 1880, Rivolta and Delparto reported by Stubs at Iowa State University clearly defined fowl plague as a novel disease [2].

Influenza viruses have caused four pandemics in humans over the past 100 years: 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1). The standard subtyping and nomenclature of influenza viruses is based on the HA and NA proteins as observed from 16 known HA subtypes and 9 NA subtypes. For example, the standard nomenclature A/duck/NC/674964/07 (H5N2) first states the influenza virus subtype (A, B, or C) the host in which the virus was isolated from (duck), the location in which the virus was isolated (North Carolina), the reference number (674964), the year in which the virus was isolated (2007), and finally the HA and NA subtype which is determined by the HI and NI assays (H5N2).

Virus structure and Genome

Influenza A virus is a pleomorphic virus with an envelope derived from the cellular membrane. The diameter of the virus ranges from 80-120 nm and has been found as small, spherical, or long filamentous particles. It was previously described that clinical isolates of influenza A virus showed the presence of filamentous virions in virus preparations [3]. The virus tends to revert back to the spherical form after continuous passaging in cell culture or embryonic eggs [4-5]. The filamentous morphology of influenza A virus is genetically determined and is primarily linked to the M gene segment encoding the M1 and M2 proteins. M1 and M2 appear to be the significant contributors for morphological differentiation of influenza A viruses. M2 and M1 specific antibodies have been shown to completely inhibit formation of filamentous viral particles. Using reverse genetics, Bourmakina and Garcia-Sastre were able to show that M2 was not a significant contributor to viral morphology [6]. They described that the M1 protein was the key player and more importantly the amino and carboxy terminal regions were the sequence determinants for influenza virus morphology [5]. It has been shown that other mutations or changes in other proteins including the HA and NA can lead to irregular virus shapes [6]. Also, some non-viral associated factors can play contributing roles in determination of the virus morphology. It has been shown that the actin skeleton of some host cell types may play a significant role in the formation of filamentous influenza A virus [7].

Influenza A virus is a member of the family *Orthomyxoviridae* which is composed of enveloped viruses which contain a segmented genome consisting of RNA with negative polarity. Influenza A virus contains 8 negative sense RNA segments which encode for 11 proteins including PA, PB1, PB2, HA, NA, NS1, NEP/NS2, M1, M2, NP and sometimes PB1-F2. PB1, PA, PB2 and NP all encode proteins that compose the ribonucleotide protein (RNP) complex.

The vRNP along with the viral RNA (vRNA) are involved in transcription of mRNA and replication of vRNA. HA and NA are surface glycoproteins and the two key antigenic proteins of the virus which characterize the antigenic subtype. M1 is the matrix protein that lines the inner side of the viral envelope, and the M2 protein forms a proton ion channel in the viral envelope. NS1 acts as immune modulator and has multiple other functions involved in host immunity and viral replication. NS2 is also referred to as nuclear export protein (NEP). The small protein PB1-F2 is not encoded by all Influenza A virus isolates but has been found in such strains as the 1918 H1N1 strain and has been shown to have pro-apoptotic activity.

The Influenza A virus RNA polymerase complex is a heterotrimer composed of the PB1, PB2, and PA proteins and part of the vRNP complex. Each vRNP complex is an independent molecular machine. vRNP complexes appear to be flexible supercoiled structures [8]. The key determinant of the vRNP complex superhelical structure is the binding of the polymerase complex to the vRNA promoter which is formed by complementary 5' and 3' terminal sequences [9]. The influenza A virus RNA polymerase is responsible for transcription of positive stranded viral mRNA containing a host cell derived cap structure at the 5' end and a poly adenosine tail at the 3' end. The polymerase complex is also responsible for replication of full length positive stranded viral cRNA and synthesis of negative stranded viral RNA [10].

The PB2 protein is a component of the RNA dependent RNA polymerase complex and is required for cap snatching. PB2 is encoded by RNA segment 1. It recognizes and binds to cellular type I mRNA cap structures. PB2 has been shown to be associated with host range restriction of influenza virus [11] There are two separate regions in the PB2 protein that are responsible for NP binding along with PB1 binding [12]. PB2 may also exhibit cap-dependent endonuclease activity [13].

PB1 is the backbone of the RNA polymerase complex and is one of the two proteins encoded by RNA segment 2. PB1 exhibits nucleotide polymerization activity [14]. The PB1 protein contains amino acid sequence motifs that are homologous to other RNA dependent RNA polymerases [15]. The N-terminus of PB1 binds and interacts with the C-terminus of PA whereas the C-terminus of PB1 interacts with the N-terminus of PB2 [16]. Residues 712-732 are responsible for efficient binding to PB2. PB1 also contains the nuclear localization signal for the polymerase complex along with multiple nucleotide binding domains [17-18].

PB1-F2 is the second protein encoded by RNA segment 2. PB1-F2 is encoded by a shift in the open reading frame in the PB1 gene. Recent research has found that an overwhelming majority of sequenced PB1 genes have a conserved PB1-F2 open reading frame. Most often the PB1-F2 protein is approximately 90 amino acids in length but has been observed as a shorter protein in multiple isolates [19]. PB1-F2 is the only influenza A virus protein that is transcribed from a +1 open reading frame opposed to splicing events observed with the viral proteins NEP and M2. The PB1-F2 protein was originally discovered from the strain A/Puerto Rico/8/34 (PR8) [20]. Recent research has shown that PB1-F2 has pro-apoptotic activity along with regulation of host immune responses. It is suspected that PB1-F2 functions as an endogenously expressed apoptosis stimulator or an exogenous protein that is released similar to the pro-apoptotic protein found in HIV [21-22]. Additionally, some research has shown that PB1-F2 sensitizes infected cells to apoptotic stimuli such as TNF-alpha through the interaction with the mitochondrial permeability transition pore complex [23].

The PA protein is the third subunit of the influenza RNA polymerase complex and is encoded by RNA segment 3. Much of the function of the PA protein is unclear, but PA has been shown to have cap binding activity, endonuclease activity, and function in vRNA binding and

replication [24]. PA consists of two distinct domains. The C-terminal domain contains the PB1 binding site which interacts with the N-terminus of PB2. PA interacts with PB1 through multiple hydrogen bonds and hydrophobic contacts [25]. The C-terminal domain of PA has 13 α helices and 9 β strands. PA has a total of 423 aa residues [25]. The N-terminal domain contains aa residues important for protein stability, promoter binding, cap-binding, and endonuclease activity [24].

Hemagglutinin (HA) is the key surface glycoprotein which is responsible for viral binding to host receptors, internalization, and membrane fusion during the endosomal pathway after infection. HA is encoded by RNA segment 4. HA is the most abundant protein on the viral envelope and contains the major neutralizing epitopes for antibodies are formed by HA [26]. The major difference between avian and human HA structures is the receptor binding site for sialic acids [27]. The HA molecule exists as a homotrimer in its native form, and each monomer consists of two polypeptides known as HA1 and HA2. The homotrimeric form of HA has a molecular weight of approximately 220kDa containing multiple glycosylation sites [26]. The full length uncleaved polypeptide precursor HA is referred to as HA0. The HA1 subunit folds to form the receptor binding site and esterase domain. The HA2 subunit folds into a membrane proximal domain with a central alpha helical domain [27]. It has been shown with comparisons between H5 of LPAI and H1 from mammalian influenza A viruses that the two HA molecules have structural differences. As stated earlier HA is a glycosylated protein with multiple N-glycosylation sites observed on the surface of the protein. It has been suggested that the structural differences observed between avian influenza virus and mammalian influenza virus HA proteins is partly responsible for glycosylation site differences. It is suggested that glycosylation differences might also result in changes in host tropism [27]. HA2 constitutes the

core fusion machinery in the region terminal part of the protein and is dominated by the long central helix that forms a trimeric coiled-coil and the shorter α helix that packs against the central helical bundle [28-29]. The HA1 constitutes the membrane distal end which consists of the globular head of the protein which can be broken down into two subdomains including the receptor binding region (R region) along with sites for neutralizing epitopes and the esterase region (E region) which is a region that shows high homology to the esterase domain of Influenza C virus hemaggutinin-esterase-fusion protein [30]. The receptor binding site is localized in a shallow pocket in the R region. The nature of the receptor binding site sialic acid linkage to vicinal galactose is the primary determinant for host tropism [26]. The receptor binding domain of all HA subtypes have three distinct structural elements including an α helix and two loops. There are also three conserved residues in the receptor binding domain including Tyr98, Trp153, and His183 [31]. HA1 also acts as a supposed latch that locks HA2 in its prefusion conformation and prevents premature triggering of the fusion machinery [28].

It has been suggested that the transmembrane (TM) domain of HA2 has multiple functions apart from a simple membrane anchor. The TM domain is believed to promote progression to the full fusion pore by breaking the hemifusion diaphragm by a mechanism that is still poorly understood [6]. Additionally, the TM domain contributes to folding and delivery of the HA to detergent resistant lipid rafts in the cell membrane [32].

Along with the polymerase complex, the vRNP complex is also composed of the nucleoprotein or NP protein. NP is encoded by RNA segment 5. NP plays an important role in interaction with the polymerase complex and the vRNA. NP is required for nuclear import of the vRNA into the nucleus and is necessary for replication and transcription of influenza virus genomic information. NP is involved in the maintenance of host-specific gene pools and this has

been proven by using reassortment experiments that reduced the viability of the virus when the NP genes were reassorted between multiple influenza A viruses [33]. NP has been shown to play an important role in determination of host specificity [34].

After x-ray crystallographic study of the NP protein its been shown that the C- terminal tail of the NP mediates interaction between neighboring NP molecules while the N-terminal tail and central regions bind primarily to vRNA [35]. The NP gene is relatively well conserved and can only be divided into two host-specific classes as suggested by research done on 16 NP gene sequences that supported the division of just two distinct classes [36]. The NP gene encodes an internal protein which contributes to its conserved nature which makes the NP protein and gene a good target for diagnostics of all subtypes of influenza. Being an internal protein, the protein lacks substantial selective pressure by host immune responses which reflects the host-specific adaptations observed in the NP [37]. Gorman et al suggests that the basis for difference between avian and human nucleoproteins may be due to difference in tissue tropism observed in the gut and respiratory tract of the host [37]. It has been reported that the NP gene is so well conserved. At the amino acid level, the percent identity between all NP genes appears to be approximately 90% [38].

NP is approximately 498 amino acids in length and is rich in arginine, glycine, and serine. NP has a net positive charge with a neutral pH [39]. Most Influenza A virus strains have a phosphorylated NP at a serine residue [40]. The phosphorylated serine is not always conserved at the same amino acid position.

Along with its RNA binding activity, the NP protein also forms an oligomeric complex with PB1, PB2, and the M1 protein. It has been previously shown that NP interacts with multiple

host cell polypeptide families including but not limited to importin alpha, filamentous actin, nuclear export receptor, and a DEAD box helicase [39, 41].

Influenza A viral segment 6 encodes for the neuraminidase (NA) protein which is one of the two key antigenic proteins of influenza A viruses. Influenza NA is a surface glycoprotein that is responsible for receptor cleavage and viral clearance through the cleavage of sialic acids localized on the cell surface. The neuraminidase activity of NA cleaves the α-ketosidic linkages between terminal Neu5Ac and neighboring saccharides [42]. It has also been suggested that NA plays a role in enabling the virus to penetrate mucosal secretions that are rich in sialic acids especially those seen in the respiratory tract so that the virus can reach the respiratory epithelium.

NA is considered a mushroom shaped protein. NA exists as a homotetramer in its quaternary form and is anchored to the viral envelope via a single hydrophobic sequence consisting of 29 amino acids that are localized at the N-terminus of the protein. The total molecular weight of the tetrameric form of NA is approximately 200 kDa that are all similarly glycosylated [42-43]. The monomeric form of neuraminidase displays a symmetrical folding pattern of six 4-stranded anti-parallel β-sheets which form a propeller like shape [44]. The central and first strand of each sheet is parallel to the propeller axis and the outermost strand is nearly perpendicular to it. The outermost strand of one sheet is the connecting strand to the central strand of the next sheet. This connecting loop contains the majority of the antigenic sites of the protein and enzymatically active residues [42]. The mushroom head of the tetrameric protein is organized with circular four-fold symmetry and the active site of each subunit is located in a deep pocket localized directly over the 6 central β strands of each sheet. The amino acids localized in the active site of neuraminidase are all invariant with respect to a majority of most characterized viruses [42]. The active site of neuraminidase contains three conserved

arginine residues Arg118, Arg292, and Arg371 that are responsible for carboxylate binding to the sialic acid [45].

Because neuraminidase is a surface glycoprotein which is naturally exposed to the immune response, high selective pressure from the host results in amino acid changes of the protein. Even though neuraminidase is considered one of the important antigenic viral proteins, antibodies directed against neuraminidase have very limited neutralizing ability potentially due to the size properties of the neuraminidase active site.

Matrix protein 1 (M1) is encoded by RNA segment 7. M1 is the most abundant protein localized within the virion, and it has been shown to play a crucial role in viral assembly and viral budding [46]. The M1 protein is localized at the inner surface of the viral envelope in close proximity to the vRNP complexes [47]. The M1 protein has dual activities exhibiting binding activity to the cellular membrane becoming later the viral envelope and vRNP [48]. The membrane binding activity allows for accumulation of M1 at the cell membrane where HA and NA have localized. M1 has a function for nuclear export of the vRNPs via the M1's vRNP binding activity [49]. The C-terminal domain of M1 plays an additional role in vRNP nuclear export through interaction with the NEP protein [49]. M1 interacts with the viral envelope similar to integral membrane proteins supported by the fact that a protease-resistant peptide of approximately 50 kDa was isolated after treatment with trypsin. The postulated site for M1 insertion into the viral envelope is located in the middle region of protein containing a cluster of 20 hydrophobic amino acids or one fourth of the way down the amino terminal where there is also a stretch of approximately 13 uncharged amino acids that could be the possible insertion site [50]. Additionally, most of the M1 protein is attached to the viral envelope via electrostatic

interactions [51]. The M1 protein has also been shown to have interaction with the cytoplasmic tails of the viral glycoproteins [52].

The second protein encoded by viral segment 7 is the matrix protein 2 (M2). M2 is translated from a spliced mRNA [53]. M2 is abundantly expressed but only small amounts of the protein are incorporated into the virion [54]. The M2 protein of influenza A virus forms a tetrameric proton channel that is vital for the influx of protons into virus particle which causes a change in the structure of the M1 protein resulting in an release of the vRNP. Also, the cytoplasmic tail of the M2 protein has been shown to be an influential player during viral assembly [55-56]. Later on during the replication cycle of the virus, M2 mediates the pH of the trans-golgi network and prevents premature conformational changes of the HA [57]. The M2 protein is a 97 residue single pass membrane protein. The carboxy terminus is located outside of virion whereas the N-terminus is located within the virion. M2 exists as a homotetramer in its native state [58]. The four transmembrane helices form an ion channel in the viral envelope. His37 acts as the pH sensor and Trp41 acts at the ion channel gate [59-60]. The closed confirmation of the M2 channel is a homotetramer in which all the N-termini are obstructed. Additionally, each subunit has a channel forming transmembrane helix, a short flexible loop, and C-terminal amphipathic helix. In the homotetrameric structure, each transmembrane helix forms into a four helix bundle with a left handed twist creating a well defined pore. In the closed state, the M2 homotetramer has a C-terminal end that extends into a loop that connects the amphipathic helix with the transmembrane domain. The amphipathic helices are packed in a right handed manner [60]. The gating mechanism involves the tight packaging of the transmembrane helices which brings the indole ring of Trp41 into contact with each other using Van der Waals forces to form the ion channel gate. The gate is stabilized by hydrogen bonds

created with Asp44. When the pH of the endosomes is lowered, the imidizole rings of His37 become protonated causing destabilization of the helix-helix packing by electrostatic repulsion. This breaks interactions between Trp41 and Asp44 causing the gate to flip open [60].

Non structural protein 1 (NS1) is encoded by RNA segment 8 along with nuclear export protein (NEP, or in the past NS2). NS1 has a molecular weight of approximately 26 kDa, and NS1 is divided into two distinct domains the N-terminal domain or RNA binding domain and the C-terminal effector domain [61]. The RNA binding domain is formed by aa residues 1-72 and the effector domain is formed by aa residues 84-220 [62]. The RNA binding domain contains three large α helices connected via short loops [63]. The effector domain has seven β-strands and three α-helices [62]. The effector domain and RNA binding both have dimeric interactions but in the full NS1 structure the resulting formation of NS1 molecules is a chain with alternating RBD and effector dimers ([62].

NS1 is a multifunctional protein with many regulatory activities. Some recently described functions are, dsRNA binding, sequestration of dsRNA or interaction with RNA helicase RIG-1 which prevents activation of some important transcription factors, suppression of the induction of RNA interference, direct binding to PKR and inhibtis PKR activity, inhibition of host mRNA processing, and inhibition of the nuclear export of polyadenylated cellular transcripts [64]. NS1 is one of the key virulence factors of influenza A virus through conferring resistance to antiviral effects through modulation of the host innate response acting as a an IFN antagonist [65]. NS1 appears to act as IFN antagonist in two distinct strategies. First, NS1 specifically blocks IFN induction and activation of PKR and 2’-5’ oligoadenylate synthase through the N-terminal RNA-binding domain. Secondly, NS1 may act to block pre-mRNA processing mediated by two domains in the C-terminal effector domain. These regions are speculated to be required for

binding to host CPSF which results in cellular mRNA processing [64]. CPSF stands for cleavage and polyadenylation specificity factor and is involved in the cleavage of 3' signaling regions from newly transcribed pre-mRNA. NS1 may interact with components of the nuclear pore therefore blocking mRNA export [66]. It has been shown that NS1 also interacts with tumor necrosis factor alpha (TNFα) [67]. NS1 also has a direct effect on influenza replication through interaction with phosphatidylinositol 3-kinase [68]. NS1 also forms a trimeric complex with eukaryotic translation initiation factor eIF4GI and the cellular poly-A-binding protein I which enhances translation of viral mRNAs [69]

The Nuclear export protein (NEP) formerly known as NS2 is responsible for export of vRNPs from the nucleus into the cytoplasm. Similarly to viral segment 7, NEP is translated from a spliced viral mRNA of segment 8. NEP is a 14.5 kDa protein that shows similarities to HIV-1 Rev protein which acts in transport of vRNPs by mediating the association of Crm1 with vRNPs [70]. NEP is a modular protein with an N-terminal domain which mediates RanGTP-dependent Crm1 binding and a C-terminal domain which is responsible for M1 binding [52]. The nuclear export of vRNPs is mediated by the cellular protein Crm1/exportin1 which is a member of the importin B family of nuclear transport receptors[71]. Crm1 mediates nuclear export of vRNPs through identifying proteins which have a nuclear export signal consisting of a leucine rich region in the protein. NEP contains a leucine-rich NES which is recognized by Crm1. NEP is also speculated to associate with M1 which binds vRNPs in high affinity.

Replication

The replication cycle of influenza viruses is very complex and some of the more specific mechanisms have yet to be described. Initiation of viral infection requires binding of the HA protein to the host cell receptor sialic acid. Sialic acids are terminal sugars located on N or O linked glycoproteins made of neuraminic acid. Host cell tropism for influenza A virus is determined by the specificity of the HA for Neu5ac α (2, 3) - Gal or Neu5ac α (2, 6) - Gal linkages between sialic acids. It is important to state that this is not the only determinant for host specificity but is believed to be one of the major contributors, because different animals have different tissue patterns expressing different sialic acid linkages. For instance, the α 2, 3 linkages are typically expressed in avian species but can be observed in other mammals such as swine. The α 2, 6 sialic acids are typically found on the surface of human cells but can be found in a wide variety of other animals including swine. Because swine express both linkages in high amounts in the respiratory epithelium, swine can become an excellent host for multiple infections where reassortments of viral RNA segments can occur. Additionally, even some avian species show differential expression of both sialic acids with different tissue expression. Another key point is that not all HA molecules bind preferentially to specific sialic acids but can bind to both α 2, 3 and α 2, 6 sialic acids.

After viral attachment to the target cell, the virus is internalized via receptor mediated endocytosis. The endosome then becomes rapidly acidified and this induces the opening of the M2 ion channel that allows the influx of protons into the virion. This results in the rapid acidification of the contents of the virion. Acidification of the endosome initiates the conformational change of the HA protein. The HA2 fusion peptide becomes exposed to the endosomal membrane and fusion between the viral envelope and endosomal membrane occurs.

In parallel, protons now present in the endosome flow into the viral particle. This initiates a conformational change of the M1 protein allowing the release of vRNPs into the cytoplasm of the infected cell.

Interestingly once the pH of the endosome reaches between pH 5 to 6, the HA protein undergoes a pH-induced and irreversible conformational change that exposes the HA2 N-terminal fusion peptide. The conformation of the HA1 receptor binding domain does not change [72]. At neutral pH, the fusion domain of the HA2 protein is buried in a pocket of ionizable residues that is adjacent to the HA0 cleavage site. When the virion is acidified, the fusion domain is released from the buried position and causes insertion of HA2 into the endosomal membrane [73]. Cleavage of HA is an absolute requirement for infectivity and the nature of the HA cleavage site is important as a virulence factor in influenza A virus infection [74]. Acidification of the core of the virion via the M2 channels facilitates the release of vRNP from M1 into the cell cytoplasm [74]. The vRNP complex is then actively transported into the nucleus via the nuclear localization signal of the nucleoprotein [75].

The negative sense vRNA is initially transcribed into positive sense viral mRNA by the viral polymerase complex. To this end, the virus utilizes the host machinery to begin mRNA synthesis. For translation of influenza viral mRNA, the mRNA requires a 5’ capped primer. The 5’ capped primer is stolen from host mRNA and attached to viral mRNA in a process known as cap snatching. The viral mRNA is then actively transported to the cytoplasm where viral protein translation occurs using host cell machinery. The positive sense RNA additionally serves as a template to transcribe negative sense viral RNA.

There are two viral proteins M1 and NEP involved in nuclear export of viral proteins [76]. M1 also plays a crucial role in viral assembly and budding. Viral assembly is primarily mediated by the three integral membrane proteins HA, NA, and M2. HA and NA are post translationally modified in the endoplasmic reticulum where they are folded and glycosylated before they are transported to the plasma membrane of the cell. M2 contains fatty acids and is anchored in the viral envelope via a C-terminal stretch of hydrophobic amino acids. M1 plays a critical role bridging the association of the vRNP complex with the HA and NA proteins in the plasma membrane.

Assembly and budding of influenza involves a few distinct steps. First, all viral components must be directed and brought to the assembly site. Second, the viral components must all interact in an orderly fashion to assemble into infectious virions. Next, bud formation must occur; finally, budding must occur causing separation of the virus particle from the host cell [74]. The viral components involved in viral budding and assembly are the vRNP complex, vRNA, NP, NEP, M1, HA, NA, and M2. For assembly to begin, all viral components must be directed specifically to the apical domain of the plasma membrane in polarized epithelial cells. Since the vRNPs are synthesized in the nucleus, they must be actively transported into the cytoplasm. This is believed to occur via the cellular Crm1 mediated nuclear export pathway with interaction with three viral proteins including M1, NEP, and NP [71].

It has been shown that M1 provides a critical function in nuclear export of the vRNP complex because vRNPs remain in the nucleus in the absence of M1 [77]. NEP is essential for nuclear export but does not directly interact with the vRNP but interacts with the N-terminal domain of M1 [52]. NEP mediates RanGTP-dependent binding to the cellar export protein Crm-1 via the leucine rich nuclear export motif located at the N-terminal domain of NEP. M1 does in

fact interact directly with vRNP via its C-terminal domain [78]. Additionally, NP is believed to interact directly with Crm-1 [71]. Experimental observations have shown that there are two types of vRNPs in the nucleus. One of which is involved in active transcription and the other which remains transcriptionally inactive [74]. It is not well known how the vRNPs remain transcriptionally active in the nucleus, but it is suggested that a few M1 molecules bind at the critical site on the vRNA or the polymerase complex which renders the vRNPs transcriptionally active [74].

All three of the viral envelope proteins HA, NA, and M2 preferentially accumulate at the site of virus assembly. The site of virus assembly is the apical plasma membrane in polarized epithelial cells [79]. The HA, NA, M2 proteins contain specific regions that are potential determinants for sorting and targeting of these proteins to the apical plasma membrane. Apical sorting of the M2 protein is not well known, but much is known for the HA and NA protein. The HA and NA proteins both contain two specific determinants for apical sorting including one in the ectodomain, and the other is located in the transmembrane domain of the protein [74]. HA and NA have been shown to interact with non-ionic detergent resistant lipid microdomains or lipid rafts. Determinants that mediate interaction with lipid rafts are located in the transmembrane domains of the HA and NA proteins [80]. Neither HA or NA are required for virus budding since viral budding can still occur in absence of these two proteins [81].

As stated previously, M1 is crucial in the processes of virion assembly and budding, and for budding to occur. Both M1 and the vRNP must be transported to the budding site but it remains unclear on how this occurs. Virus budding does not occur in the absence of the M1 protein , and it has been shown that budding does in fact still occur when cells are transfected with the M1 protein alone [82]. Additionally, it has been shown that M1 and NP are localized at

the apical plasma membrane even though it doesn't use the exocytosis pathway for transport [83]. It is believed that the M1-vRNP complex is directed to the apical plasma membrane through a piggy back type interaction that is initiated during exocytoic transport of HA and NA [84]. It has been shown that cytoskeletal components interact with M1-vRNP and may facilitate transport of the complex to the viral assembly site [85]. Previously, the interaction between M1 and the vRNP was emphasized but it's important to note that the M1 protein interacts with itself to form dimers and multimers and this interaction is thought to be critical in concentration of viral components at the budding site, host protein exclusion, asymmetry formation in the lipid membrane, and initiation of membrane bending and recruitment of host components to the budding site for release [86-87].

It is believed that viral glycoproteins are the key determinants for localization of the assembly and budding site of the virus. This is believed to be the case because viral glycoproteins tend to accumulate at the site of viral budding even when expressed alone in transfected cells. Budding requires selection of assembly site, initiation of the budding process, bud growth, and bud completion and release of the virus [74]. Each of these steps requires both host and viral interactions. Bud formation and bud release are the last steps in viral replication and production of new infectious particles. Initiation of bud formation requires curvature of the plasma membrane and involves a transition form more planar membrane structure to a curved structure [88]. Also, assembly of lipid bilayers into specific lipid microdomains such as lipid rafts at the site of budding is likely to contribute to virus budding [74]. M1 proteins are also key components in bud formation and pinching off along with other key host components [89-90].

Each of the eight viral gene segments has 12-13 nucleotides at the 5' and 3' ends of each segment that play a critical role in viral packaging and viral assembly. vRNA packaging is an

extremely inefficient process possibly resulting in improper packaging which results in additional viral segments being packaged or some segments left out. After viral packaging and assembly, budding of the viral particle must occur. Budding and separation of the virus particle from the host membrane is reliant on the enzymatic activity of the NA protein. This protein is involved in cleavage of terminal sialic acids that prevents aggregation of the virus at the cell surface. There have been two proposed mechanisms for RNA packaging random and specific. Random packaging predicts the presence of common structural elements in all vRNPs causing them to be incorporated randomly [91]. Specific packaging model suggests that structural features in each vRNA segment enable them to be preferentially incorporated into virions [74].

Another host component involved in virus budding was briefly described earlier is the role of lipid rafts. Lipid rafts have multiple functions in virus budding. Lipid rafts are lipid microdomains enriched in sphingolipids and cholesterol and are relatively resistant to non-ionic detergent at low temperature [92]. Lipid rafts play crucial roles in virus entry and uncoating, viral protein transport and targeting, selection of the viral assembly site, interaction among viral components, and bud initiation and completion [93].

Other host proteins have been shown to be involved in virus budding of influenza A virus, including microfilaments, G proteins, and some protein kinases. In particular, proteins in the family involved in vacuolar protein sorting which interact with the L domains of the Gag and matrix proteins. These proteins include, but are not limited to Tsg101, ESCRT, and proteins containing WW domains [94-95].

Finally, the last steps in viral replication are bud completion and viral release. Buds are released by a mechanism of fusion of the opposing membrane and fission of the bud from the

cell membrane. Viral clearance and aggregation is mediated by the NA protein which is involved in cleavage of terminal sialic acids located on the cellular membrane.

Molecular Pathogenesis

The molecular pathogenesis of influenza A viruses is multifactorial and many genes have been shown to be contributors to differences in pathogenicity, but the most influential and recognized contributor to pathogenicity in avian influenza virus is the cleavage site of the HA protein. Each determinant of pathogenicity most likely plays a critical role in one of three phases of the influenza A virus replication cycle including RNA synthesis, regulation, or structure. Changes in the pathogenicity of avian influenza viruses is often contributed to segmented nature of the RNA genome and the lack of proofreading activity associated with the RNA polymerase complex. In general, RNA viruses are the most mutable of all known biological entities [96]. The two major subtypes of avian influenza viruses associated with changes in pathogenicity have been observed with those encoding either for H5 or H7 subtype.

As described earlier, HA is responsible for receptor binding, membrane fusion, and is the key antigenic determinant of influenza A virus in respect to induction of neutralizing antibodies. The HA cleavage site contains specific amino acid sequences which are necessary for cleavage of the HA0 protein into the HA subunit proteins HA1 and HA2 where the later reveals the HA fusion domain. Typical HA cleavage sites especially those observed in LPAI isolates have a typical proteolytic cleavage site with the amino acids motif XR/GLFG. The fusion peptide is the amino acid sequence which allows fusion with the host membrane [97]. The protelytic cleavage site of the HA protein is the key virulence factor of avian influenza virus [98]. LPAI have a

characteristic HA cleavage site as that described above which is monobasic and can only be cleaved by trypsin-like proteases and some mammalian type II serine proteases which limits the ability of LPAI to cause a systemic infection [99-100], but a HPAI has a cleavage site which consists of a number of basic amino acids (polybasic) that can be cleaved by widely distributed furin-like enzymes which increases its ability to replicate efficiently in multiple types of tissues and locations throughout the body [101].

Changes in the HA cleavage site can readily occur through multiple methods including simple site mutations, accumulated nucleotide insertions, tandem duplications of stretches of purines, and RNA/RNA recombination events. Single site mutations are the result of naturally occurring single nucleotide changes that occur as a result of the error prone RNA polymerase of influenza. This type change has been well characterized in the development of a HPAI isolate H5N2 that resulted in an outbreak in Pennsylvania in 1983. The initial amino acid sequence of the HA cleavage site was PQRETR/G and the mutated sequence was PQRKKR/G [102]. Accumulated nucleotide insertions must result in an amino acid codon addition and must result in a functional codon. So, three consecutive amino acids must be introduced for a polybasic cleavage site. According to researchers, this mechanism for changes in the HA cleavage site to occur is likely but evidence is poor. Speculation has been made that for this to occur quasi-species must previously exist that include two amino acid insertions that cause a frameshift to occur and is just waiting for the third insertion to occur to become functional. Proof that tandem duplications of stretches of purines resulted in HPAI was provided by the 1995 H5N2 outbreak in central Mexico that showed insertion of duplications of nucleotide sequence of AAAGAAA which resulting in the introduction of RKRKRK in the HA cleavage site resulting in a HPAI [103-104]. Finally, the last proposed mechanism for changes that occur in the HA cleavage site

is RNA/RNA recombination events that potentially occur between 28s rRNA, the M protein, or quasi species that exist in the viral population [109].

As stated previously, avian influenza virus pathogenicity is multifactorial and many other genes contribute to pathogenicity of the virus. For instance, the NA gene has been shown to be involved in increased pathogenicity associated with host adaptation especially from waterfowl to land based poultry. When passaged from waterfowl to chickens a deletion of multiple amino acids is observed in the stalk region of the protein. It is believed that the truncation of the stalk region is most likely a regulatory effect to counter balance the increased binding affinity of the HA protein that resulted from the addition of a glycosylation site at the globular head of the protein [105-106]. Additionally, the NA gene has been contributed to pathogenicity with the addition of a glycosylation site to the globular head of NA, which has been shown to increase pathogenicity in chickens. It is believed that the addition of this glycosylation site increases sialidase activity which results in the virus being more efficiently able to spread through the host [107-108]

The NS gene has also been associated with attenuating pathogenicity. Recent studies have shown that amino acids changes in the NEP protein resulted in increased or attenuated pathogenicity. It has been previously shown that the NS1 and NS2 proteins can effect many intracellular functions of the virus including RNA splicing, RNA binding, M1 protein binding , nuclear export, inhibition of cellular RNA capping, polyadenylation, and inhibition of the cellular interferon response[109]. Any changes in the effects of these two proteins can dramatically enhance or attenuate the pathogenicity of the virus. An amino acid exchange observed in the NS1 protein was shown to have a dramatic affect on increasing the virulence of an H5N1 isolate [110-111]. An additional study showed that two amino acid exchanges in the

NS2 protein produced increased virulence in chicken embryos [110]. The function of the NS1 protein to functionally inhibit the interferon (IFN) α/β cascade is definitely linked to Influenza A pathogenesis. Studies have been conducted on hundreds of viral isolates and thousands of NS genes has clearly demonstrated the wide variability in the composition of the NS gene [102]. It has also been observed that a PDZ ligand motif located at the C-terminus of the NS1 protein is present in highly virulent strains of Influenza including the 1918 H1N1 but not avirulent or low virulent strains [102]. The PDZ ligand motif could play a critical role in the trafficking of membrane proteins and may play a role in many other intracellular activities [102, 112].

Finally, changes in the polymerase complex of influenza have been clearly associated with measured variations in pathogenicity [113]. For instance, a single amino acid exchange (E627K) in the PB2 protein has been shown to increase virulence and adaptation to mammals [114]. Additionally, it has been shown that PB1 contributes to pathogenicity because it has been shown to attenuate disease in mice inoculated with PB-1 reassortments [113]. Additionally, the PB1-F2 gene is not observed in all strains of influenza A virus but is under massive positive selection and may play a critical role in increased pathogenicity of the virus due to its pro-apoptosis activity [102] The M gene has also been attributed to pathogenicity due to host adaptation and specificity especially the M2 protein which has been shown to be a contributor to host adaptation [115]. So, there are many molecular contributors to pathogenicity, but a majority of the changes discussed are subtype specific and often even strain specific determinants to pathogenicity. The only clearly defined contributor to pathogenicity is the basic composition of the proteolytic cleavage site of the HA protein of HPAI.

Diagnostics

Clinical presentation of avian influenza infection is heavily dependent on host species and the strain of the influenza virus. Influenza virus infection is not pathognomonic. So, laboratory diagnosis of influenza is a requirement for detection of the viruses. Most diagnostic tests for influenza virus are type or subtype specific. Diagnostic technologies can be based on a combination of factors including fitness of purpose, technical ease, speed, diagnostic sensitivity, specificity, and cost [116]. There are a wide array of diagnostic tests including virus isolation, antigen detection, RT-PCR, agar gel immunodiffusion, enzyme linked immunosorbent assays, hemagglutination inhibition test, and neuraminidase inhibition test. Current advances in influenza diagnostics and instrumentation, along with computer analysis provides laboratories the ability to now rapidly subtype, and rapidly sequence the complete genome of influenza viruses.

Clinical presentation of influenza viruses is restricted primarily to laboratory diagnosis because influenza A virus doesn't cause pathognomonic lesions grossly or microscopically [116]. Asymptomatic infections in wild birds are common and these wild birds continuously shed the virus which can than potentially infecting other species. Pathological lesions of influenza are completely dependent on subtype, pathotype, species, and the presence of secondary infections. LPAI causes an upper respiratory tract infection with inflammation of the sinuses, conjunctivae, and the trachea. HPAI causes more dramatic lesions due to a systemic infection.

Virus isolation and identification is the time honored method for diagnosis of influenza A virus. Samples are inoculated into embryonated eggs, and egg inoculation is the gold standard for

diagnosis of avian influenza virus and internationally recognized as the standard method by the O.I.E.

Potential samples are inoculated into the chorioallantoic sac, the yolk sac, and potentially the chorioallantoic membrane [117]. The embryonated chicken egg is widely believed to be the most sensitive method for viral isolation of avian influenza virus. Virus isolation from embryonated chicken eggs can be utilized to isolate viruses from a myriad of samples including tissue homogenates, cloacal swabs, and environmental samples. Virus isolation is apparently extremely sensitive, but is not highly specific, and the risk of contamination in embryonated eggs is an ever present issue. Embryonal mortality is not the only method for determination of the presences of influenza A virus. The allantoic fluid of eggs is tested using the hemagglutinating assay which test for hemagglutinating activity of the sample on chicken erythrocytes. Although this test is a reliable method for diagnosis on the presence of influenza virus this test is also readily used on other viruses and bacteria that exhibit hemagglutinating activity including Newcastle disease virus. This presents a problem for misdiagnosis and virus isolation. After virus isolation is done, additional testing must be performed to confirm the identity of the virus which includes the determination of the virus subtype.

Antigen capture and lateral flow ELISA assays are an extremely rapid onsite method for detection of influenza A virus. These tests were initially developed for use in humans and rapidly adopted for use in multiple hosts [118]. Lateral flow ELISAs detect influenza viral antigen. Typically they are specific for the nucleoprotein using either polyclonal or monoclonal antibodies specific for the nucleoprotein. Antigen capture tools have been developed that recognize the hemagglutinin protein which allow for rapid detection of distinct subtypes especially H5 [119]. Lateral flow ELISA based techniques are extremely portable, simple, and

relatively quick [116]. Antigen capture ELISA's are intended for use as on-site confirmatory tests of avian influenza infection outbreaks [120]. Lateral flow detection assays are not sufficient tools for surveillance programs and there are many limitations to these techniques including assay disruption from peroxidases found in some samples and non-reactivity due to secondary infections [116]. There are alternative tests being investigated for use to replace antigen capture assays including biosensor technologies, colorimetric sensors, surface plasmon resonance, and quartz-crystal microbalance technologies [121].

Molecular diagnostics for avian influenza virus detection is constantly evolving. Molecular diagnostics primarily include nucleic acid detection assays including PCR specifically RT-PCR in the case of influenza A virus. Real time RT-PCR along with full genome sequencing techniques and subtyping using PCR amplification and sequencing of the hemagglutinin and neuraminidase genes for both surveillance and diagnostics have also been utilized in the past years [122-123] . RT-PCR based assays provide rapid detection and are extremely sensitive and have a detection limit in the range from 5-100 viral RNA copies, and multiplex RT-PCR based techniques provide the ability to analyze multiple targets from one sample for subtyping [124]. Opposed to RT-PCR there are other molecular techniques that can be used for diagnostics of influenza virus including some isothermal based techniques for detection and subtyping including loop mediated and nucleic acid based amplification [125-126]. Isothermal amplification approaches differ in methodology compared to PCR because they require a single temperature whereas PCR requires varied temperatures for differing times.

After RT-PCR amplification of specific targets and full genome amplification, standard cycle sequencing or Sanger sequencing, and just recently pyrosequencing have been used for epidemiological studies and viral identification and characterization [127]. These techniques

allows for high phylogentic resolution. A new technique metagenomics, using pyrosequencing, allows for the analysis of biological and environmental samples to fully discover viral populations that may be present. This technique can be currently utilized to discover the presence of new strains or even novel viruses.

Finally, microarrays have traditionally been used to perform basic research, but recently microarrays have been utilized for rapid sample subtyping for detection and diagnostics. [128]. Microarrays allow for the detection of thousands of different types of sequences and are used to detect single nucleotide polymorphisms. Microarray technology is becoming increasingly less expensive and may become one of the key techniques used for subtyping and detection of influenza A virus.

As described previously, there have been a multitude of antigen capture and identification assays for Influenza virus, but there are limited tests for antibody detection. The gold standard for antibody detection of influenza viruses is agar gel immunodiffusion test, hemagglutination inhibition test, and neuraminidase inhibition test. All serological tests are frequently used for global surveillance and epidemiological studies. Recent studies have focused on using the ELISA system to develop competitive ELISA systems that are not species specific and are highly sensitive and specific [129].

The agar gel immunodiffusion (AGID) test is an application of the Ouchterlony immune-double diffusion to avian influenza virus. AGID allows for the visualization of reactivity of avian influenza specific antibodies with antigen after gel diffusion. AGID can either be used to detect antigen or antibodies using reference antibodies or antigen. The AGID test is relatively in expensive until it comes to quality assurance and highering of quality researchers that can

accurately interpret results. AGID results can be obtained within 24-48 hours. The hemagglutination inhibition test is readily used to subtype the HA protein in viral samples by identifying HA specific antibodies in variable samples including serum. The hemagglutinating inhibition assay is relatively expensive due to high labor costs and requires the use of biocontainment facilities when the antigen used is infectious virus. But inactivated viruses can also be used in this test. Although this test is extremely rapid, it isn't automatable in the field.

The neuraminidase inhibition assay is used to determine the NA subtype. The neuraminidase inhibition test relies on the presence of neutralizing antibodies that inhibits the enzymatic activity of the neuraminidase. The neuraminidase inhibition test is sensitive and specific and extremely useful in neuraminidase subtyping of newly isolated viruses. The neuraminidase inhibition test is relatively expensive and requires slightly more time to complete when compared to the hemagglutination inhibition test.

Control/Vaccination

The ideal way to control and prevent the emergence of new influenza virus outbreaks and pandemics in humans and poultry is to inhibit or at least reduce the likelihood of interspecies transmission. The most common way in which this was done is the culling of infected poultry which has been consistently successful in previous outbreaks including the outbreak in Hong Kong in 1997 and the Netherlands in 2003. Culling infected poultry causes the reduction of the viral load and reduces the likelihood of transmission to humans. Unfortunately, culling and quarantine methods are unlikely to be successful with the emergence of new highly pathogenic strains of influenza [130] The new and current strategy to in dealing with influenza virus

outbreaks in poultry is culling along with vaccination. Current research has added more emphasis on the development of alternative vaccine strategies and rapid vaccine production. The viruses that may pose the greatest problems with vaccine production are the HPAI H5 and H7 strains due to the low viral yields generated by these viruses in eggs and the requirement for high-level biocontainment facilities for vaccine propagation [131].

Because of the high antigenic diversity among strains of influenza virus, there are many problems with vaccine design and propagation. Currently there are multiple types of vaccines being developed for humans, but the two major types currently in circulation are inactivated virus vaccines and live attenuated vaccines. A majority of the current licensed influenza vaccines are in the form of inactivated antigen preparations [132]. All vaccines containing inactivated influenza virus vaccines work primarily through generation of antibodies directed against the hemagglutinin protein. Inactivated vaccine efficacy is measured by the reduction of confirmed influenza illnesses of 70% in the age group between 14 and 60 years of age [133]. Efficacy of influenza inactivated vaccines decreases drastically with the young and the elderly [134].

There are three formulations of inactivated virus vaccines for influenza. The first formulation is the whole virion which have been utilized since the 1940's [132]. The second formulation is composed of a split virion which is formulated by disrupting whole viral particles with detergents. The last and final formulation is a subunit composition which is developed by enriching viral surface glycoproteins following disruption of viral particles [132]. The reactogenicity of whole virus vaccines in children spawned the development of split vaccine formulations back in the 1960's [135]. Split and subunit vaccines have been proven to be relatively safe and delivered for decades, but the relatively poor immunogenicity associated with split and subunit vaccines requires two doses of theses vaccines to generate a sufficient immunity

in naïve individuals [136-137]. Another drawback to inactivated viruses vaccines is the production system for these vaccines. Inactivated virus vaccines are propagated in embryonated chicken eggs which present an obstacle along with the length of time between selection of vaccine strains and the availability of the first dose of formulated vaccines [138-139]. Inactivated vaccines cannot be stock piled because of the dependence on antigenic matching of the vaccine strains with circulating strains, but there have been inactivated vaccines stock piled for virus strains that pose a considerable zoonotic risk [140].

The other current licensed vaccines are live attenuated vaccine formulations that are administered intranasally of a replication competent virus. Current live attenuated vaccines are generated by introduction of the HA and NA genes of circulating strains into a viral backbone of a live attenuated cold adapted strain of influenza [141]. There is a variety of approaches to develop attenuated virus, but new targets such as the M2, PB2, and NS1 genes are primary targets [142]. There are some potential advantages of live attenuated viruses versus inactivated virus vaccines. First live attenuated vaccines mediate both neutralizing antibody production and a cell mediated response. Cell mediated immunity is vitally important because aids in targeting of more conserved regions of the virus [143]. Much like inactivated vaccines live attenuated vaccines most often require multiple doses. Additionally, some drawbacks do exist with live attenuated vaccines. Some HA and NA formulations do not yield live virus formulations that lack the ability to replicate in the upper respiratory tract. Also, vaccination with a live virus always presents some risks if the virus were to undergo selection/mutation [144].

Methods for the development of new influenza vaccines are currently being investigated, especially the use of reverse genetic systems to develop new vaccines [145]. Reverse genetics allows for the generation of very specific live vaccine formulations. Reverse genetics does not

allow for quicker preparation of vaccine stocks, but does allow for adequate preparation of seed strains [132].

DNA vaccines are also a new technology being utilized for formulation of new vaccines. DNA vaccines are currently being investigated in use for influenza viruses [132]. DNA vaccines expressing multiple HA and NA combinations along with other accessory genes have been shown to provide protection in mice and other animal models [146]. DNA vaccines are relative cheap, safe, and rapid for use in humans. The one drawback to influenza DNA vaccines is the poor immunogenicity that they confer in humans [147]. Additionally, recombinant fowl pox vaccines have been tested containing the H5 and NP genes of influenza virus and have shown protective efficacy in poultry [148]

Because current vaccines have poor immunogenicity and require multiple doses to be truly efficacious, the use of adjuvants is extremely important to the vaccine formulation of current vaccines [132]. Adjuvants allow poor immunogenicity vaccines to be more effective and mediate higher antibody titers and elicit a possible cell mediated response. Adjuvants are not a new technology. Materials such as alum salts have been added to vaccines for decades [149]. The addition of alum to current vaccine formulations has only provided a slight improvement on the mediated immune response [150]. Oil in water adjuvants including MF59 and AS03 have provided much more pronounced effects and require six times less antigen [151]. Oil in water adjuvants have been shown to induce higher antibody titers and have shown to induce cross reactive responses when administered with some split and subunit vaccines [152].

Even though vaccination is the ideal method to reduce the intraspecies and interspecies spread of influenza viruses, vaccine preparation can take up to 6 months once the right vaccine

strain has been chosen. So, an interim method for control must be administered such as antiviral drugs[130]. There are currently a few different types of antivirals on the market for treatment of influenza infections. There are two distinct types of prophylactic drugs including M2 ion channel blockers and neuraminidase inhibitors. The M2 ion channel blocker include amantadine and rimantidine which blocks the replication of influenza A viruses at a certain stage [153]. The two neuraminidase inhibitors are zanamivir and oseltamivir which reduce viral release from infected cells which results in aggregation of virions at the cell surface and prevents viral penetration of the mucosa, and prevents viral spread [154]. There are some major drawbacks to the use of M2 blockers including the rapid emergence of resistance to M2 blocker and the lack of effectiveness of M2 blockers to Influenza B viruses [155]. Influenza strains that acquire resistance to M2 blockers are characterized by mutations in the transmembrane domain of the M2 protein [156]. Neuraminidase inhibitors are much more stable but are relatively more costly. Neuraminidase inhibitors are also effective against influenza A and B viruses and have fewer side effects in comparison to M2 blocker [157]. Antivirals are the most important short-term resource for control of influenza virus infection. Antivirals often provide many benefits in response to viral outbreaks including a broad antiviral spectrum and potency, prophylactic and therapeutic effectiveness, favorable pharmokinetics, availability, tolerability, and safety [130] [158].

References

1. Perroncito, E., *Epizoozia tifoide nei gallinacei.* Annali Della Reale Accademia d' Agricoltura di Torino, 1878. **21**: p. 87-12637

2. Stubbs, E.L., ed. *Fowl pest*. 3 ed. Diseases of Poultry, ed. B.a. Devries. 1943, Iowa State College Press: Ames. 493-502.

3. Chu, C.M., I.M. Dawson, and W.J. Elford, *Filamentous forms associated with newly isolated influenza virus.* Lancet, 1949. **1**(6554): p. 602.

4. Choppin, P.W. and I. Tamm, *Studies of two kinds of virus particles which comprise influenza A2 virus strains. II. Reactivity with virus inhibitors in normal sera.* J Exp Med, 1960. **112**: p. 921-44.

5. Bourmakina, S.V. and A. Garcia-Sastre, *Reverse genetics studies on the filamentous morphology of influenza A virus.* J Gen Virol, 2003. **84**(Pt 3): p. 517-27.

6. Jin, H., et al., *Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape.* EMBO J, 1997. **16**(6): p. 1236-47.

7. Roberts, P.C. and R.W. Compans, *Host cell dependence of viral morphology.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5746-51.

8. Pons, M.W., et al., *Isolation and characterization of the ribonucleoprotein of influenza virus.* Virology, 1969. **39**(2): p. 250-9.

9. Tiley, L.S., et al., *Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5' ends of the viral RNAs.* J Virol, 1994. **68**(8): p. 5108-16.

10. Honda, A., K. Mizumoto, and A. Ishihama, *RNA polymerase of influenza virus. Dinucleotide-primed initiation of transcription at specific positions on viral RNA.* J Biol Chem, 1986. **261**(13): p. 5987-91.

11. Subbarao, E.K., W. London, and B.R. Murphy, *A single amino acid in the PB2 gene of influenza A virus is a determinant of host range.* J Virol, 1993. **67**(4): p. 1761-4.38

12. Poole, E., et al., *Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites.* Virology, 2004. **321**(1): p. 120-33.

13. Galarza, J.M., et al., *Influenza A virus RNA-dependent RNA polymerase: analysis of RNA synthesis in vitro.* J Virol, 1996. **70**(4): p. 2360-8.

14. Digard, P., V.C. Blok, and S.C. Inglis, *Complex formation between influenza virus polymerase proteins expressed in Xenopus oocytes.* Virology, 1989. **171**(1): p. 162-9.

15. Poch, O., et al., *Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains.* J Gen Virol, 1990. **71 (Pt 5)**: p. 1153-62.

16. Gonzalez, S., T. Zurcher, and J. Ortin, *Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure.* Nucleic Acids Res, 1996. **24**(22): p. 4456-63.

17. Asano, Y. and A. Ishihama, *Identification of two nucleotide-binding domains on the PB1 subunit of influenza virus RNA polymerase.* J Biochem, 1997. **122**(3): p. 627-34.

18. Nath, S.T. and D.P. Nayak, *Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1).* Mol Cell Biol, 1990. **10**(8): p. 4139-45.

19. Ghedin, E., et al., *Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution.* Nature, 2005. **437**(7062): p. 1162-6.

20. Bruns, K., et al., *Structural characterization and oligomerization of PB1-F2, a proapoptotic influenza A virus protein.* J Biol Chem, 2007. **282**(1): p. 353-63.

21. Chen, W., et al., *A novel influenza A virus mitochondrial protein that induces cell death.* Nat Med, 2001. **7**(12): p. 1306-12.

22. Henklein, P., et al., *Membrane interactions and alignment of structures within the HIV-1 Vpu cytoplasmic domain: effect of phosphorylation of serines 52 and 56.* FEBS Lett, 2000. **482**(3): p. 220-4.

23. Zamarin, D., et al., *Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1.* PLoS Pathog, 2005. **1**(1): p. e4.

24. Hara, K., et al., *Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding.* J Virol, 2006. **80**(16): p. 7789-98.

25. Obayashi, E., et al., *The structural basis for an essential subunit interaction in influenza virus RNA polymerase.* Nature, 2008. **454**(7208): p. 1127-31.

26. Stevens, J., et al., *Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus.* Science, 2004. **303**(5665): p. 1866-70.

27. Lin, T., et al., *The hemagglutinin structure of an avian H1N1 influenza A virus.* Virology, 2009. **392**(1): p. 73-81.

28. Ekiert, D.C., et al., *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. **324**(5924): p. 246-51.

29. Bullough, P.A., et al., *Structure of influenza haemagglutinin at the pH of membrane fusion.* Nature, 1994. **371**(6492): p. 37-43.

30. Rosenthal, P.B., et al., *Structure of the haemagglutinin-esterase-fusion glycoprotein of influenza C virus.* Nature, 1998. **396**(6706): p. 92-6.

31. Stevens, J., et al., *Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus.* Science, 2006. **312**(5772): p. 404-10.

32. Skibbens, J.E., M.G. Roth, and K.S. Matlin, *Differential extractability of influenza virus hemagglutinin during intracellular transport in polarized epithelial cells and nonpolar fibroblasts.* J Cell Biol, 1989. **108**(3): p. 821-32.

33. Scholtissek, C., et al., *The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses.* Virology, 1985. **147**(2): p. 287-94.

34. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.

35. Ye, Q., R.M. Krug, and Y.J. Tao, *The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA.* Nature, 2006. **444**(7122): p. 1078-82.

36. Gammelin, M., J. Mandler, and C. Scholtissek, *Two subtypes of nucleoproteins (NP) of influenza A viruses.* Virology, 1989. **170**(1): p. 71-80.

37. Gorman, O.T., et al., *Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses.* J Virol, 1991. **65**(7): p. 3704-14.

38. Shu, L.L., W.J. Bean, and R.G. Webster, *Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990.* J Virol, 1993. **67**(5): p. 2723-9.

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

40. Kistner, O., K. Muller, and C. Scholtissek, *Differential phosphorylation of the nucleoprotein of influenza A viruses.* J Gen Virol, 1989. **70 (Pt 9)**: p. 2421-31.41

41. Palese, P., et al., *Development of novel influenza virus vaccines and vectors.* J Infect Dis, 1997. **176 Suppl 1**: p. S45-9.

42. Colman, P.M., *Influenza virus neuraminidase: structure, antibodies, and inhibitors.* Protein Sci, 1994. **3**(10): p. 1687-96.

43. Laver, W.G., *Crystallization and peptide maps of neuraminidase "heads" from H2N2 and H3N2 influenza virus strains.* Virology, 1978. **86**(1): p. 78-87.

44. Varghese, J.N., W.G. Laver, and P.M. Colman, *Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution.* Nature, 1983. **303**(5912): p. 35-40.

45. Russell, R.J., et al., *The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design.* Nature, 2006. **443**(7107): p. 45-9.

46. Allen, H., et al., *Influenza virus RNA segment 7 has the coding capacity for two polypeptides.* Virology, 1980. **107**(2): p. 548-51.

47. Schulze, I.T., *The structure of influenza virus. II. A model based on the morphology and composition of subviral particles.* Virology, 1972. **47**(1): p. 181-96.

48. Wakefield, L. and G.G. Brownlee, *RNA-binding properties of influenza A virus matrix protein M1*. Nucleic Acids Res, 1989. **17**(21): p. 8569-80.

49. O'Neill, R.E., J. Talon, and P. Palese, *The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins.* EMBO J, 1998. **17**(1): p. 288-96.

50. Gregoriades, A. and B. Frangione, *Insertion of influenza M protein into the viral lipid bilayer and localization of site of insertion.* J Virol, 1981. **40**(1): p. 323-8.

51. Ruigrok, R.W., et al., *Membrane interaction of influenza virus M1 protein.* Virology, 2000. **267**(2): p. 289-98.42

52. Akarsu, H., et al., *Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2).* EMBO J, 2003. **22**(18): p. 4646-55.

53. Lamb, R.A., C.J. Lai, and P.W. Choppin, *Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins.* Proc Natl Acad Sci U S A, 1981. **78**(7): p. 4170-4.

54. Holsinger, L.J. and R.A. Lamb, *Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds.* Virology, 1991. **183**(1): p. 32-43.

55. Iwatsuki-Horimoto, K., et al., *The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly.* J Virol, 2006. **80**(11): p. 5233-40.

56. Cady, S.D., et al., *Structure and function of the influenza A M2 proton channel.* Biochemistry, 2009. **48**(31): p. 7356-64.

57. Sugrue, R.J., et al., *Specific structural alteration of the influenza haemagglutinin by amantadine.* EMBO J, 1990. **9**(11): p. 3469-76.

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

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

60. Schnell, J.R. and J.J. Chou, *Structure and mechanism of the M2 proton channel of influenza A virus.* Nature, 2008. **451**(7178): p. 591-5.

61. Krug, R.M., et al., *Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein.* Virology, 2003. **309**(2): p. 181-9.43

62. Bornholdt, Z.A. and B.V. Prasad, *X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus.* Nature, 2008. **456**(7224): p. 985-8.

63. Chien, C.Y., et al., *A novel RNA-binding motif in influenza A virus non-structural protein 1.* Nat Struct Biol, 1997. **4**(11): p. 891-5.

64. Kochs, G., A. Garcia-Sastre, and L. Martinez-Sobrido, *Multiple anti-interferon actions of the influenza A virus NS1 protein.* J Virol, 2007. **81**(13): p. 7011-21.

65. Garcia-Sastre, A., *Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses.* Virology, 2001. **279**(2): p. 375-84.

66. Satterly, N., et al., *Influenza virus targets the mRNA export machinery and the nuclear pore complex.* Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1853-8.

67. Geiss, G.K., et al., *Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10736-41.

68. Hale, B.G., et al., *Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling.* Proc Natl Acad Sci U S A, 2006. **103**(38): p. 14194-9.

69. Burgui, I., et al., *PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes.* J Gen Virol, 2003. **84**(Pt 12): p. 3263-74.

70. Ward, A.C., et al., *Expression and analysis of the NS2 protein of influenza A virus.* Arch Virol, 1995. **140**(11): p. 2067-73.

71. Elton, D., et al., *Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway.* J Virol, 2001. **75**(1): p. 408-19.44

72. Bizebard, T., et al., *Structure of influenza virus haemagglutinin complexed with a neutralizing antibody.* Nature, 1995. **376**(6535): p. 92-4.

73. Durrer, P., et al., *H+-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region.* J Biol Chem, 1996. **271**(23): p. 13417-21.

74. Nayak, D.P., E.K. Hui, and S. Barman, *Assembly and budding of influenza virus.* Virus Res, 2004. **106**(2): p. 147-65.

75. Neumann, G., M.T. Hughes, and Y. Kawaoka, *Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1.* EMBO J, 2000. **19**(24): p. 6751-8.

76. Chen, Z. and R.M. Krug, *Selective nuclear export of viral mRNAs in influenza-virus-infected cells.* Trends Microbiol, 2000. **8**(8): p. 376-83.

77. Bui, M., et al., *Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins.* J Virol, 2000. **74**(4): p. 1781-6.

78. Baudin, F., et al., *In vitro dissection of the membrane and RNP binding activities of influenza virus M1 protein.* Virology, 2001. **281**(1): p. 102-8.

79. Hughey, P.G., et al., *Expression of the influenza A virus M2 protein is restricted to apical surfaces of polarized epithelial cells.* J Virol, 1992. **66**(9): p. 5542-52.

80. Kundu, A., et al., *Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells.* J Virol, 1996. **70**(9): p. 6508-15.

81. Palese, P., et al., *Characterization of temperature sensitive influenza virus mutants defective in neuraminidase.* Virology, 1974. **61**(2): p. 397-410.45

82. Latham, T. and J.M. Galarza, *Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins.* J Virol, 2001. **75**(13): p. 6154-65.

83. Mora, R., et al., *Apical budding of a recombinant influenza A virus expressing a hemagglutinin protein with a basolateral localization signal.* J Virol, 2002. **76**(7): p. 3544-53.

84. Ali, A., et al., *Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein.* J Virol, 2000. **74**(18): p. 8709-19.

85. Avalos, R.T., Z. Yu, and D.P. Nayak, *Association of influenza virus NP and M1 proteins with cellular cytoskeletal elements in influenza virus-infected cells.* J Virol, 1997. **71**(4): p. 2947-58.

86. Harris, A., et al., *The crystal structure of the influenza matrix protein M1 at neutral pH: M1-M1 protein interfaces can rotate in the oligomeric structures of M1.* Virology, 2001. **289**(1): p. 34-44.

87. Zhao, H., M. Ekstrom, and H. Garoff, *The M1 and NP proteins of influenza A virus form homo- but not heterooligomeric complexes when coexpressed in BHK-21 cells.* J Gen Virol, 1998. **79 (Pt 10)**: p. 2435-46.

88. Farsad, K. and P. De Camilli, *Mechanisms of membrane deformation.* Curr Opin Cell Biol, 2003. **15**(4): p. 372-81.

89. Hui, E.K. and D.P. Nayak, *Role of G protein and protein kinase signalling in influenza virus budding in MDCK cells.* J Gen Virol, 2002. **83**(Pt 12): p. 3055-66.

90. Luban, J., *HIV-1 and Ebola virus: the getaway driver nabbed.* Nat Med, 2001. **7**(12): p. 1278-80.46

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

92. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.

93. Barman, S., et al., *Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses.* Virus Res, 2001. **77**(1): p. 61-9.

94. Ludwig, S., S. Pleschka, and T. Wolff, *A fatal relationship--influenza virus interactions with the host cell.* Viral Immunol, 1999. **12**(3): p. 175-96.

95. Martin-Serrano, J., D. Perez-Caballero, and P.D. Bieniasz, *Context-dependent effects of L domains and ubiquitination on viral budding.* J Virol, 2004. **78**(11): p. 5554-63.

96. Elena, S.F., et al., *Mechanisms of genetic robustness in RNA viruses.* EMBO Rep, 2006. **7**(2): p. 168-73.

97. Swayne, D.E., et al., *Pathogenicity and diagnosis of H5N2 Mexican avian influenza viruses in chickens.* Avian Dis, 1997. **41**(2): p. 335-46.

98. Horimoto, T. and Y. Kawaoka, *Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus.* J Virol, 1994. **68**(5): p. 3120-8.

99. Bottcher, E., et al., *Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium.* J Virol, 2006. **80**(19): p. 9896-8.

100. Klenk, H.D. and R. Rott, *The molecular biology of influenza virus pathogenicity.* Adv Virus Res, 1988. **34**: p. 247-81.47

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

102. Obenauer, J.C., et al., *Large-scale sequence analysis of avian influenza isolates.* Science, 2006. **311**(5767): p. 1576-80.

103. Garcia, M., et al., *Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico.* J Gen Virol, 1996. **77 (Pt 7)**: p. 1493-504.

104. Perdue, M.L., et al., *Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses.* Virus Res, 1997. **49**(2): p. 173-86.

105. Matrosovich, M., et al., *The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties.* J Virol, 1999. **73**(2): p. 1146-55.

106. Liu, J., et al., *Highly pathogenic H5N1 influenza virus infection in migratory birds.* Science, 2005. **309**(5738): p. 1206.

107. Hulse, D.J., et al., *Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens.* J Virol, 2004. **78**(18): p. 9954-64.

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

109. Wright, P.F., R.G. Webster, ed. *Orthomyxoviruses*. 4th ed. Fields Virology, ed. D.M.K.a.P.M. Howley. 2001, Lippincott Williams and Wilkins: Philadelphia. 1533-1579.48

110. Perdue, M.L., *Naturally occurring NS gene variants in an avian influenza virus isolate.* Virus Res, 1992. **23**(3): p. 223-40.

111. Li, Z., et al., *The NS1 gene contributes to the virulence of H5N1 avian influenza viruses.* J Virol, 2006. **80**(22): p. 11115-23.

112. Fanning, A.S. and J.M. Anderson, *PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane.* J Clin Invest, 1999. **103**(6): p. 767-72.

113. Salomon, R., et al., *The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04.* J Exp Med, 2006. **203**(3): p. 689-97.

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

115. Smith, G.J., et al., *Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam.* Virology, 2006. **350**(2): p. 258-68.

116. Charlton, B., B. Crossley, and S. Hietala, *Conventional and future diagnostics for avian influenza.* Comp Immunol Microbiol Infect Dis, 2009. **32**(4): p. 341-50.

117. Woolcock, P.R., et al., *Enhanced recovery of avian influenza virus isolates by a combination of chicken embryo inoculation methods.* Avian Dis, 2001. **45**(4): p. 1030-5.

118. Ryan-Poirier, K.A., et al., *Application of Directigen FLU-A for the detection of influenza A virus in human and nonhuman specimens.* J Clin Microbiol, 1992. **30**(5): p. 1072-5.

119. Fouchier, R.A., et al., *Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene.* J Clin Microbiol, 2000. **38**(11): p. 4096-101.49

120. Suarez, D.L., E. Spackman, and D.A. Senne, *Update on molecular epidemiology of H1, H5, and H7 influenza virus infections in poultry in North America.* Avian Dis, 2003. **47**(3 Suppl): p. 888-97.

121. Amano, Y. and Q. Cheng, *Detection of influenza virus: traditional approaches and development of biosensors.* Anal Bioanal Chem, 2005. **381**(1): p. 156-64.

122. Wright, K.E., et al., *Typing and subtyping of influenza viruses in clinical samples by PCR.* J Clin Microbiol, 1995. **33**(5): p. 1180-4.

123. Hoffmann, E., et al., *Universal primer set for the full-length amplification of all influenza A viruses.* Arch Virol, 2001. **146**(12): p. 2275-89.

124. Xie, Z., et al., *A multiplex RT-PCR for detection of type A influenza virus and differentiation of avian H5, H7, and H9 hemagglutinin subtypes.* Mol Cell Probes, 2006. **20**(3-4): p. 245-9.

125. Shan, S., et al., *Comparison of nucleic acid-based detection of avian influenza H5N1 with virus isolation.* Biochem Biophys Res Commun, 2003. **302**(2): p. 377-83.

126. Imai, M., et al., *Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method.* J Virol Methods, 2007. **141**(2): p. 173-80.

127. Pourmand, N., et al., *Rapid and highly informative diagnostic assay for H5N1 influenza viruses.* PLoS One, 2006. **1**: p. e95.

128. Li, J., S. Chen, and D.H. Evans, *Typing and subtyping influenza virus using DNA microarrays and multiplex reverse transcriptase PCR.* J Clin Microbiol, 2001. **39**(2): p. 696-704.50

129. Starick, E., et al., *Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus nucleoprotein antibodies and its application to field sera from different species.* J Vet Med B Infect Dis Vet Public Health, 2006. **53**(8): p. 370-5.

130. Lipatov, A.S., et al., *Influenza: emergence and control.* J Virol, 2004. **78**(17): p. 8951-9.

131. Wood, J.M., *Developing vaccines against pandemic influenza.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1416): p. 1953-60.

132. Ellebedy, A.H. and R.J. Webby, *Influenza vaccines.* Vaccine, 2009. **27 Suppl 4**: p. D65-8.

133. Demicheli, V., et al., *Vaccines for preventing influenza in healthy adults.* Cochrane Database Syst Rev, 2000(2): p. CD001269.

134. Jefferson, T., et al., *Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review.* Lancet, 2005. **366**(9492): p. 1165-74.

135. Peck, F.B., Jr., *Purified influenza virus vaccine. A study of viral reactivity and antigenicity.* JAMA, 1968. **206**(10): p. 2277-82.

136. Bresson, J.L., et al., *Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial.* Lancet, 2006. **367**(9523): p. 1657-64.

137. Atmar, R.L., et al., *Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations.* Clin Infect Dis, 2006. **43**(9): p. 1135-42.

138. Fedson, D.S. and K.L. Nichol, *Influenza vaccination: policy versus evidence: no gap between policy and evidence.* BMJ, 2006. **333**(7576): p. 1020.

139. Fedson, D.S., *Pandemic influenza: a potential role for statins in treatment and prophylaxis.* Clin Infect Dis, 2006. **43**(2): p. 199-205.51

140. Lipniacki, T., et al., *Stochastic regulation in early immune response.* Biophys J, 2006. **90**(3): p. 725-42.

141. Maassab, H.F., C.A. Heilman, and M.L. Herlocher, *Cold-adapted influenza viruses for use as live vaccines for man.* Adv Biotechnol Processes, 1990. **14**: p. 203-42.

142. Murphy, B.R., et al., *An influenza A live attenuated reassortant virus possessing three temperature-sensitive mutations in the PB2 polymerase gene rapidly loses temperature sensitivity following replication in hamsters.* Vaccine, 1997. **15**(12-13): p. 1372-8.

143. Thomas, P.G., et al., *Cell-mediated protection in influenza infection.* Emerg Infect Dis, 2006. **12**(1): p. 48-54.

144. Shinya, K. and Y. Kawaoka, *[Influenza virus receptors in the human airway]*. Uirusu, 2006. **56**(1): p. 85-9.

145. Hoffmann, E., et al., *A DNA transfection system for generation of influenza A virus from eight plasmids.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6108-13.

146. Epstein, S.L., et al., *DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice.* Emerg Infect Dis, 2002. **8**(8): p. 796-801.

147. Drape, R.J., et al., *Epidermal DNA vaccine for influenza is immunogenic in humans.* Vaccine, 2006. **24**(21): p. 4475-81.

148. Doherty, P.C., et al., *Recombinant vaccinia viruses and the development of immunization strategies using influenza virus.* J Infect Dis, 1989. **159**(6): p. 1119-22.

149. Lindblad, E.B., *Aluminium compounds for use in vaccines.* Immunol Cell Biol, 2004. **82**(5): p. 497-505.52

150. Stephenson, I., et al., *Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial.* Lancet, 2003. **362**(9400): p. 1959-66.

151. Nicholson, K.G., et al., *Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza.* Lancet, 2001. **357**(9272): p. 1937-43.

152. Banzhoff, A., et al., *MF59-adjuvanted vaccines for seasonal and pandemic influenza prophylaxis.* Influenza Other Respi Viruses, 2008. **2**(6): p. 243-9.

153. Wang, C., et al., *Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block.* J Virol, 1993. **67**(9): p. 5585-94.

154. Sidwell, R.W., et al., *Inhibition of influenza virus infections in mice by GS4104, an orally effective influenza virus neuraminidase inhibitor.* Antiviral Res, 1998. **37**(2): p. 107-20.

155. Hayden, F.G. and A.J. Hay, *Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine.* Curr Top Microbiol Immunol, 1992. **176**: p. 119-30.

156. Iwahashi, J., et al., *Isolation of amantadine-resistant influenza a viruses (H3N2) from patients following administration of amantadine in Japan.* J Clin Microbiol, 2001. **39**(4): p. 1652-3.

157. McKimm-Breschkin, J.L., *Resistance of influenza viruses to neuraminidase inhibitors--a review.* Antiviral Res, 2000. **47**(1): p. 1-17.

158. *Avian strain of influenza A virus isolated from humans in Hong Kong.* Commun Dis Rep CDR Wkly, 1999. **9**(15): p. 131, 134.

CHAPTER 3

PRODUCTION OF H5 SPECIFIC MONOCLONAL ANTIBODIES AND THE DEVELOPMENT OF A COMPETITIVE ELISA FOR DETECTION OF H5 ANTIBODIES IN MULTIPLE SPECIES

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Keywords : avian influenza, ELISA, monoclonal antibodies, species independent

Abbreviations :

AGPT : agar gel precipitation test

AIV: avian influenza virus

cELISA : competetive Enzyme-Linked ImmunoSorbent Assay

HA: hemagglutinin

HI: hemagglutination inhibition

HA5-Bac: H5 recombinant antigen expressed in the baculovirus system

HA5-mam: H5 recombinant antigen expressed in HEK293T cells.

HPAI: highly pathogenic avian influenza

HPH5N1: highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype

LPAI: low pathogenic avian influenza

mAb : monoclonal antibody

MALDI-TOF/MS: Matrix-assisted laser desorption/ionization- time of flight/mass spectrometry

NA: neuraminidase

NP: ribonucleoprotein

PBS: phosphate buffered **saline**

OD : optical density

OIE: World **organization** for animal health

RT-PCR: reverse transcription –polymerase chain reaction

rBV-HA5: recombinant baculovirus expressing H5 protein

SDS-PAGE: sodiumdodecylsulphate- polyacrylamide gel electrophoresis

SNT: Serum neutralization test

Abstract

The hemagglutinin gene of an avian influenza virus [AIV; A/duck/NC/674964/07 (H5N2)] was cloned and expressed in a baculovirus system (H5-Bac). In parallel a recombinant hemagglutinin of A/Vietnam/1203/04 (H5N1) was expressed in mammalian cells, purified and used for generation of H5 specific monoclonal antibodies (mAb). The purified H5-Bac was used to develop a competitive ELISA (cELISA) to detect H5 antibodies in a species-independent approach using one of the established H5-specific mAb as the competitor antibody. The cELISA performed with influenza antibody free sera or sera of animals infected with other than H5 encoding AIV showed no significant inhibition of H5-mAb binding, indicating a high test specificity. In contrast, sera of poultry (chickens, turkeys, ducks) experimentally infected with H5-encoding AIV were able to significantly inhibit the binding of the mAb in a species independent approach. Comparison of the results of the cELISA with results obtained by hemagglutination inhibition (HI) assay showed a gradient of the sensitivity (turkeys>ducks>chicken). The described results show that H5 specific antibodies in sera can be detected in a species-independent approach by using a recombinant protein.

Introduction

Influenza A viruses belong to the virus family *Orthomyxoviridae*. According to their hemagglutinin (HA) and neuraminidase (N) surface glycoproteins, they are classified into subtypes. Currently, 16 HA and 9 N subtypes are described (Palese and Shaw 2007). In the past ten years a highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype (HPH5N1) has caused severe outbreaks in poultry. Starting in Guangdong in China in 1996 (23) it spread over East and Southeast Asia to Europe and Africa (Cauthen, Swayne et al. 2000; 2007). Wild resident and migratory birds are probably one cause for the circulation and spread of HPH5N1 (Weber and Stilianakis 2007). In several countries where outbreaks of HPH5N1 in poultry occurred, infections of birds of prey, felids, and humans with fatal outcomes were also reported (Webster, Peiris et al. 2006). In addition, HPH5N1 caused neurotropic disease and death in ferrets and mice after experimental infection (7). Other low pathogenic avian influenza (LPAI) viruses of the subtype H5 may evolve to HPAI virus, if they circulate unrecognized in poultry flocks (11).

The key tool in identification and control of the threat caused by influenza A viruses is a constant and global AIV surveillance (Fouchier, Osterhaus et al. 2003). AIV surveillance is performed by three methods, first virus isolation in embryonated chicken eggs, second detection of viral RNA by RT-PCR, and third analysis of serum samples for the presence of antibodies against AIV proteins (Alexander 2004). Infectious influenza virus in animals can be detected for approximately 1 week (12, 18). The presence of AIV antibodies lasts longer and thus the epidemiology, and thus the ecology of the virus can be investigated without presence of replicating virus or viral RNA.

For surveillance, sera are investigated for the presence of antibodies against the group-specific viral ribonucleoprotein (NP) by either agar gel precipitation test (AGPT) or in enzyme-linked immunosorbent assay (ELISA) systems (Shafer, Katz et al. 1998; Zhou, Chan et al. 1998; Alexander 2004; Wu, Hu et al. 2007). Positive sera are investigated for HA subtypes antibodies by the serological standard method, the hemagglutination inhibition (HI) assays (Alexander 2004). The HI assay is considered to be highly specific. Serum neutralization tests (SNT) also primarily detect antibodies against HA (Webster, Cox et al. 2002). Both tests, HI and SNT are laborious and not well suited for automation and need the handling of infectious virus before inactivation with an inherent risk of accidental release into the environment. In contrast, indirect ELISA systems are easy to conduct and can readily be automated, but depend on the availability of secondary antibodies against immunoglobulin of the investigated species.

With the spread of HPAI H5N1 to other species, the importance of a broad range of species other than birds was recognized in the epidemiology of AIV. However, many species-specific conjugates that would be necessary for comprehensive surveillance are not available. Three cELISA for the detection of antibodies against H5 has been described (De Marco, Foni et al. 2005) We developed a H5-specifc cELISA based on recombinant antigen and evaluated its sensitivity and specificity in detecting antibodies against H5 in serum samples from multiple species with the goal to detect for the H5 LPAI and H5 HPAI circulation in several species. The presented data show that H5 antibodies can be detected in different birds species.

Materials and Methods

Generation of recombinant Baculovirus.

Viral RNA of avian influenza virus strain A/duck/NC/674964/07 (H5N2) was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA) from 200 µl of allantoic fluid. The virus was an isolate from a wild duck obtained as a result of a surveillance study and was kindly provided by Dr. David Stallknecht (University of Georgia, USA). Segment 4 cDNA of the viral influenza genome was amplified by RT-PCR using primer Bm-HA-1 and Bm-NS-890R as previously described (Hoffmann, Stech et al. 2001). The resulting PCR product was gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), cloned blunt ended into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced. After sequence analysis of the cloned viral cDNA two oligonucleotides (H5pfFP, CCACTAGTAAATGGAAAGAATAGTGATTGCCCTCG; H5pfRP, GGAAGCTTCTAATGGTGATGGTGATGGTGAGATCCCCTGATGCAAATTCTGCACTGC AATGATC*C*) were designed to amplify the open reading frame (ORF) encoding the HA. The primer H5pfRP contained a nucleotide sequence encoding an RGS-6xHis amino acid sequence (underlined) at its C-terminus prior the stop codon. The obtained PCR product was cleaved with *Spe* I and *Hind* III, and ligated into the appropriately cleaved baculo transfer vector pFastBac Dual (Invitrogen, Carlsbad, CA, USA). The sequence of the H5-enconding sequence of the resulting plasmid pfastD-H5 was confirmed by sequencing. The plasmid pfastD-H5 was used to generate a recombinant baculovirus using the Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA) following manufacturers instructions. The cell culture supernatant of transfected cells was harvested and used for two subsequent passages in Sf9 cells. The supernatant of the second passage was used as viral stock for infection of Sf9 cells for protein purification.

Detection of recombinant protein.

Cells were analyzed for the presence of recombinant protein by Western blot. Protein samples were separated by sodiumdodecylsulphate-12% polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot using standard conditions. The dilution of all antibodies was performed in TBST (150 mM NaCl, 10 mM Tris, pH 8.0, 0.05% Tween 20). For Western blot analysis, two different monoclonal antibodies (mAb) were used: anti H5 mAb VN1203/02 (NR-2730, BEI Resources, Manassas, VA), anti RGS-His mAb (Qiagen, Valencia, CA, USA). The binding of the mAb was monitored after incubation with a peroxidase-labeled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) by using chemoluminescence substrate Immobilon Western (Millipore, Billerca, MA, USA) and Gel Logic 2200 (Carestream Health, New Haven, CT, USA).

Preparation of H5-antigen from insect cells. For production of recombinant antigen (HA5-Bac), 80% confluent Sf9 cells were infected with rBV-HA5 at a MOI between 1 and 2. Three days after infection the recombinant protein was purified by affinity chromatography as described previously (Letzel, Mundt et al. 2007). Eluates were tested by SDS-PAGE as described above followed by staining with Commassie Brilliant Blue G-250. The protein containing samples were pooled and dialyzed in phosphate buffered saline (PBS) for 18 h using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, Rockford, IL, USA) with a 10 kDa molecular weight cut-off. For storage at -20°C, the antigen was diluted 1:2 with glycerol.

Production of H5 HA recombinant protein using mammal cells

The ORF of the H5 antigen encoding for the HA of A/Vietnam/1203/04 (H5N1) was codon optimized for human cells and synthesized (Genscript Inc., Piscataway, NJ, USA). The nucleotide sequence encoding the polybasic HA cleavage site was not included. To obtain a soluble protein that can be purified from the supernatant of transfected cells the C-terminal located transmembrane domain and the C-terminal located cytoplasmic tail encoding sequence was truncated by PCR using two oligonucleotides (HA5 -FP, CCAAG CTTGCCACCATGGAAAAAATCGTGCTGCTGTTTGC, HA5TMD-RP, ggGATA*TC*AGCTATAGATAGACAGGATCTGGTAGATGCC) and Deep Vent polymerase (New England Biolabs, Ipswich, MA, USA). The resulting PCR product was ligated into the Hind III/ EcoRV-cleaved eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) to obtain pcDNA3-HA5□TMD. After verification of the sequence suspension-adapted 100 ml of HEK293T cells (Invitrogen, Carlsbad, CA, USA) cultivated in protein free GIBCO FreeStyle 293 Expression Medium (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO2 were transfected with 300 □g of pcDNA3-HA5□TMD using 293fectin transfection reagent (Invitrogen, Carlsbad, CA, USA). Three days after transfection recombinant H5-mam was purified by immunoaffinity chromatography using purified VN1203/04 HA5-specific monoclonal antibody (NR-2728, BEI Resources, Manassas, VA, USA) coupled to a HiTrap NHS-activated sepharose 5 ml column (GE Healthcare Piscataway, NJ, USA) and a AKTA FLPC instrument (GE Healthcare Piscataway, NJ, USA). After elution with 3M glycine buffer, H5-mam was dialyzed against 200 volumes of PBS.

Generation of H5 HA-specific monoclonal antibodies

Purified H5-mam was used to immunize three female BALB C mice (Harlan Laboratories, Inc. Indianapolis, IN, USA) three times at 4 week intervals with 200 ug of protein via subcutaneous injection following standard procedures. Serum samples of the mice were monitored by an indirect ELISA using the purified protein H5-mam as antigen. The mouse with the highest ELISA titer was immunized via intraperitoneal injection with 200 ug of H5-mam protein in the absence of adjuvant three days prior to fusion. Splenocytes from immunized mice were fused with SP2/0 cells, and plated in methylcellulose medium containing HAT using a ClonaCell HY kit per manufacturer's specifications (Stemcell Technologies, Vancouver, BC, Canada). Hybridoma cells secreting antibodies specific for the H5-mam protein were determined by standard ELISA assay, using a goat anti-mouse IgG specific HRP conjugate (Thermo Fisher Scientific, Rockford, IL, USA). Selected hybridomas were adapted to growth in serum-free medium (PFHM II, Invitrogen, Carlsbad, CA, USA). The antibodies were concentrated from the cell-free hybridoma culture supernatants using ProPur protein G columns (Nunc, Roskilde, Denmark) following the instruction of the manufacturer.

Determination of mAb reactivity by immunofluorescence

In order to select a broad reacting H5 mAb, cells of the chicken fibroblast cell line DF 1 (8) cultivated in DMEM containing 10% fetal bovine serum were infected in parallel with three different H5 expressing LPAIV kindly provided by Dr David Suarez (South East Poultry Research Laboratory, Athens, GA, USA) and the donor LPAIV for the recombinant H5-Bac [A/duck/NC/674964/07 (H5N2)]. Twelve hours p.i., infected and non infected cells were ethanol fixed and processed for immunofluorescence using the newly generated H5 mAb. The presence

of mAb binding was visualized by using FITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) and an inverted microscope (Axiovert 40 CFL, Carl Zeiss Ltd Scientific, Jena, Germany).

Source of serums.

Sera from chickens, turkeys, and ducks infected with different H5 and H3 influenza strains were kindly provided by Dr. C.-W. Lee (The Ohio State University, Wooster, OH, USA). Sera from chickens with antibodies against H1, H3, H6, H9, H10, H11, H13, and H15 were kindly provided by Dr. D. Suarez (SEPRL, Athens, GA, USA). Further, sera of ducks and chickens infected with three different influenza strains [A/Ck/PA/13609/93 (H5N2), A/Ck/TX/167280-4/02 (H5N3), A/Mute Swan/MI/ 451072/06 (H5N1)] were obtained from animal experiments performed at the Poultry Diagnostic and Research Center (PDRC, Athens, GA, E. Mundt, unpublished results). Hyperimmune sera prepared in goats or chickens against H1, H3, H4, H5, H6, H7, H8, H10, and H11 were obtained from American Type Culture Collection (Manassas, VA, USA). Furthermore, 184 sera from chicken flocks from the field known to be free of antibodies against influenza as tested by agar gel precipitation test were provided by Dr. S. Thayer (PDRC, Athens, GA, USA).

cELISA.

For the cELISA, the protein detector ELISA kit (KPL, Gaithersburg, MD, USA) was used. The antigen was diluted in coating buffer to a final concentration of 500 ng/ml and 50 μl were added in each well of a 96-well flat bottom plate (FisherBrand, Santa Clara, CA, USA). After incubation over night at 4°C, antigen was removed, wells were rinsed three times with

wash solution and 100 µl blocking solution were added (KPL, Gaithersburg, MD, USA). The plate was incubated for 45 min at 37°C. For all subsequent steps appropriate sera and the competing H5 mAb were diluted in dilution buffer (Synbiotics, San Diego, CA, USA). The appropriate dilutions of sera and mAb were determined by checkerboard titration. Following the washing procedure, 50 µl of diluted sera were pipetted into the wells and incubated at 37°C for one hour. After adding 50 µl of the diluted H5 mAb the plate was incubated for 1 h and rinsed three times with wash solution. Finally 50 µl of 1:500 diluted peroxidase conjugated anti-mouse goat serum (KPL, Gaithersburg, MD, USA) were added and incubated for 1 h at 37°C. After three wash steps, 50 µl of peroxidase substrate solution (KPL, Gaithersburg, MD, USA) were added and the plate incubated for 15 min at room temperature. The enzymatic reaction was stopped by adding 50 µl of stop solution per well (KPL, Gaithersburg, MD, USA). The optical density (OD) values were measured at 405 nm using an ELISA reader (ELx 808, BioTek, Winooski, VT, USA). For the determination of the unimpaired binding of the H5 mAb (100% binding value) buffer without serum was added to four wells. The inhibition of the mAb binding for each serum was calculated according to the formula 100 - $OD_{serum}/OD_{mAb} \cdot 100$ = % inhibition of the mAb binding. One positive standard control serum was included in each 96-well plate. Over the course of the study a chicken serum obtained from a SPF bird infected with A/Ck/PA/13609/93 (H5N2) was used as positive control sera (E. Mundt, unpublished results).

Hemagglutination inhibition test.

The HI test was performed following the technique as described by OIE recommendations using 4 hemagglutinating units of the parental A/duck/NC/674964/07 (H5N2)

virus as antigen and 1 % chicken erythrocytes diluted in PBS. HI titers ≥ 16 were considered as positive.

Results

Expression and purification of recombinant H5 antigen.

To develop a tool for the detection of H5-specific antibodies in a species independent approach a recombinant baculovirus encoding the HA protein of A/duck/NC/674964/07 (H5N2) was generated. After purification of the recombinant H5-Bac protein two single bands with a molecular mass of approximately 62 kD were detected in the Coomassie brilliant blue stained gel (Fig. 1A). The identity of the recombinant protein was confirmed by using an anti-RGS-His mAb and the H5-specific mAb VN1203/02. In order to obtain a possible broader applicable cELISA system, a H5-antigen based on a HPAI H5N1 virus [here A/Vietnam/1203/2004 (H5N1)] was used for the generation of the mAb. Expression and purification of the H5-mam was performed as described in material and methods. The purification using the cell free supernatant in immunoaffinity chromatography resulted in approximately 1 mg of purified recombinant protein (Fig. 1B). The identity of H5-mam was confirmed by Western blot using an H5-specific mAb VN1203/04.

Generation and selection of the H5 monoclonal antibody

After repeated immunizations of female BALB C mice with the recombinant H5-mam, 6 different mAb were selected (JH1-JH6) which reacted positive in an indirect ELISA using H5-mam as antigen. To select a broad reacting mAb, DF1 cells were infected with four LPAI

encoding for different H5 [A/Ck/PA/13609/93 (H5N2), A/Ck/TX/167280-4/02 (H5N3), A/Mute Swan/MI/ 451072/06 (H5N1), A/duck/NC/674964/07 (H5N2)] and immunofluorescence was performed. Three mAb (JH1, JH2, JH4) reacted with all four H5-expressing viruses indicating a broad reactivity. Next, the three antibodies were used as competing antibodies in a cELISA using the recombinant H5-Bac either undiluted or in four different dilutions (1:10, 1:100. 1:1000, 1:10000). Sera obtained from chicken infected with H5N1, H5N2, and H5N3 with an HI > 16 were used in three different dilutions (1:5, 1:10, and 1:100). Only JH1 showed inhibition in binding to the antigen in the cELISA. Furthermore JH1 tested negative for its ability to inhibit hemagglutination of the recombinant H5-Bac and also of inactivated A/Vietnam/1203/04 (H5N1) (kindly provided by Dr. Webby, St Jude Children's Research Hospital, Memphis, TN, USA) using chicken red blood cells. Likely, JH1 does not bind to the protein structure of HA which is responsible for receptor binding of chicken erythrocytes.

Development of the cELISA

For the establishment of the cELISA, the optimal dilutions of test sera and the H5-mAb JH1 were determined by checkerboard titration. H5-mAb JH1 was used as the competing antibody. The highest inhibition was observed at a serum dilution of 1:10 and a dilution of the H5-mAb of 1:100. For the following investigations, the sera were diluted 1:10 and the mAb JH1 was diluted 1:100. In next experiments the cut-off value of the cELISA was evaluated by testing 184 field sera from chickens known to have no antibodies against influenza as tested by AGPT following standard procedures (personal communication, Dr. S. Thayer). The inhibition of the H5 mAb JH1 binding ranged between 0.02 and 24.02%, with a mean of 7.5 and a standard deviation of 5.4. 25% inhibition of the OD of the mAb binding was chosen as the cut-off value

for the subsequent experiments. The next series of experiments was performed to test if the cELISA was able to detect H5 antibodies in sera of different species. To this end, sera of chickens, ducks, and turkeys experimentally infected with H5-encoding viruses [A/Mute swan/MI/451072-2/06 (H5N1), A/PS/CA/406032/04 (H5N2), H5N2 A/Emu/NY/12716/94 (H5N2), A/Avian/NY/31588/00 (H5N2), A/TK/MN/10734-2/95 (H5N2), A/PH/MD/4457/93 (H5N2), A/CK/PA/13609/93 (H5N2), A/CK/TX/167280-4/02 (H5N3), A/Mallard/WI/42/75 (H5N3), A/TK/WI/68 (H5N9)]. The results showed that in all species H5 specific antibodies could be detected by the cELISA system (Table 1). Furthermore, there were no significant differences observed in the detection of antibodies between sera obtained after infection with different H5 viruses. In parallel, all sera were tested using 4 HA units of A/duck/NC/674964/07 (H5N2) antigen in an HI following the OIE recommendations. Sera with a HI titer >8 were considered as positive. This antigen was chosen since this virus isolate encoded the donor sequence for the recombinant baculovirus used in the cELISA. Thus the sera were assayed using very similar antigens. The analytical sensitivity and specificity of the ELISA was determined as previously described (4). If the HI test is considered the gold standard, the sensitivity of the cELISA system was 66%, 99%, and 97% for chickens, ducks and turkeys, respectively (Table 2). The specificities of the tests were 63%, 14%, and 83% for chickens, ducks and turkeys, respectively (Table 2). In addition, the overall sensitivity and specificity of the cELISA, including all sera in the calculation, was 85 % and 32%, respectively (Table 2). To address the result that sera reacted positive in the cELISA but showed an HI negative reactivity additional experiments were performed. To this end, the competitive NP ELISA (FlockCheck*AIMultiS-Screen, IDEXX, Westbrook, MA, USA) was used following the instructions of the manufacturer. All HI negative but cELISA positive sera were analyzed. It was observed that nine out of 14

chicken sera, 22 out of 41 duck sera, and eight out of ten turkey sera were positive in the NP ELISA but negative in the HI. This finding needs to be further addressed but indicates a higher sensitivity of the NP ELISA than the HI.

To assay the repeatability of the assay two plates were tested either at the same day or on two different days. In both cases sera determined positive or negative by the cELISA were randomly selected and tested. The repeatability at the same day was 98% whereas between two different days we observed 100% repeatability. Intra-assay variation was assessed on one ELISA plate with only the mAb control which resulted in an average OD of 1.14 with a standard deviation of 0.04 (maximal OD 1.25, minimal OD 1.0). To further test the repeatability of the cELISA in respect to the inhibition of the binding of the mAb, one positive serum from a duck and a chicken were used on one plate for each serum. The results showed a high repeatability and a low variation within each plate. The mean inhibition of the positive duck serum was 93.55 % (standard deviation 1.02 %, maximum inhibition 97%, minimum inhibition 87%) and the mean inhibition of the positive chicken serum was 47% (standard deviation 3.84%, maximum inhibition 56%, minimum inhibition 39%). Furthermore, 105 sera produced under controlled experimental conditions containing antibodies directed against influenza H1, H3, H4, H6, H7, H8, H9, H10, H11, H13, and H15 were investigated in the cELISA. The results were similar to the investigation of the field sera with the inhibition of mAb binding ranging between 0 and 22.3% with an average of 5.2 (standard deviation of 7.3).

Discussion

Over the past few years, extensive surveillance programs have become necessary and are conducted worldwide to obtain more information on the occurrence of AIV in poultry and wild birds. For the development of the cELISA, the combination of a random chosen LPAI H5N2, isolated from a wild bird, and a monoclonal antibody directed against the Asian H5N1 HPAI lineage was chosen to obtain a broad applicability of the cELISA. The homology of the amino acid sequences between both HA was 88%. This indicated a sufficient similarity between both viruses. Despite the description of a comparable cELISA based on full virus (De Marco, Foni et al. 2005), the system has not found a broader application. In addition, its sensitivity and specificity has not been described. Furthermore, a H5-cELISA has been described recently using infectious virus as antigen (3).

The use of recombinant antigen in the cELISA described here was chosen to eliminate the necessity to work with infectious AIV for the generation of antigen for the test at any stage for the preparation of the antigen. Also, a similar approach using a recombinant baculovirus-expressed protein as antigen in a cELSA has been described by Prabakaran et al. (15). The recent papers show that there is a need for such assays. The presented data indicate that the cELISA is able to detect H5 antibodies from a variety of H5-encoding LPAIV. This stays in contrast to the recent published data (15) where in the homologue system (HAIV H5N1) a high sensitivity was shown but the epitope is likely not present on approximately 50% of LPAIV. Thus 50% of H5 LPAIV infected would not be detected by this serological assay.

The disadvantage of the presented cELISA is the low sensitivity in chicken sera. On the other hand the sensitivity in duck and turkey sera was significantly higher. This lower sensitivity

in the assay in the chicken sera might be due to the fact that H5 specific antibodies in a number of chicken sera did not recognize this epitope characterized by the mAb JH1, but the antibodies in the sera of ducks and turkeys did. The cELISA using infectious virus as antigen showed a significant higher sensitivity in chicken sera 96% (3) but was likely caused by property of the used mAb to cause HI, thus blocking HI antibodies. One puzzling result was the comparable high number of serum samples which were positive by the cELISA but negative by HI resulting in a low specificity of the cELISA. The data obtained by using the competitive NP ELISA indicates that the cELISA provides a higher specificity in all species investigated. But these findings needs to be further addressed.

In summary, the major advantage of the cELISA is the possibility to screen a large number of sera within considerable less time in comparison to the HI assay. Therefore, the cELISA allows a rapid serological and species-independent detection of antibodies against H5 in a variety of species. Further experiments are necessary to increase the sensitivity and specificity including a NP cELISA system.

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References

(2007). "Avian influenza update: recent outbreaks of H5N1 in poultry worldwide." Euro Surveill **12**(1): E070125 070122.

Alexander, D. J. (2004). Avian Influenza. Manual of diagnostic tests and vaccines for terrestrial animals**:** 2.7.12.

Cauthen, A. N., D. E. Swayne, et al. (2000). "Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans." J Virol **74**(14): 6592-6599.

De Marco, M. A., E. Foni, et al. (2005). "Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 poultry epidemic periods (1998 to 2000)." Avian Pathol **34**(6): 480-485.

Fouchier, R. A., A. D. Osterhaus, et al. (2003). "Animal influenza virus surveillance." Vaccine **21**(16): 1754-1757.

Hoffmann, E., J. Stech, et al. (2001). "Universal primer set for the full-length amplification of all influenza A viruses." Arch Virol **146**(12): 2275-2289.

Letzel, T., E. Mundt, et al. (2007). "Evidence for functional significance of the permuted C motif in Co2+-stimulated RNA-dependent RNA polymerase of infectious bursal disease virus." J Gen Virol **88**(Pt 10): 2824-2833.

Palese, P. and M. L. Shaw (2007). Orthomyxoviridae: The viruses and their replication. Fields Virology. D. M. Knipe, D. E. Griffin, R. A. Lambet al. Philadelphia, PA, Lippincott Williams & Wilkins. **2:** 1647-1689.

Shafer, A. L., J. B. Katz, et al. (1998). "Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera." Avian Dis **42**(1): 28-34.

Weber, T. P. and N. I. Stilianakis (2007). "Ecologic immunology of avian influenza (H5N1) in migratory birds." Emerg Infect Dis **13**(8): 1139-1143.

Webster, R., N. Cox, et al. (2002). WHO manual on influenza diagnosis and surveillance. Geneva, Switzerland, World Health Organization.

Webster, R. G., M. Peiris, et al. (2006). "H5N1 outbreaks and enzootic influenza." Emerg Infect Dis **12**(1): 3-8.

Wu, R., S. Hu, et al. (2007). "Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus." Vet Res Commun **31**(5): 631-641.

Zhou, E. M., M. Chan, et al. (1998). "Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein." Avian Dis **42**(3): 517-522.

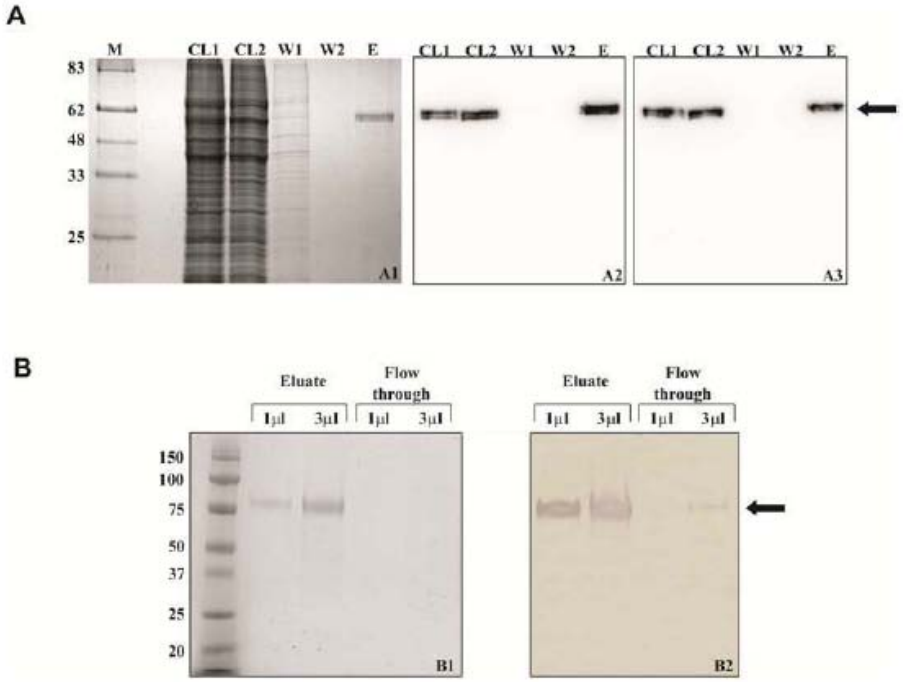

Figure 3.1 Expression and purification of H5 antigen in two different cell systems. (A) Samples of lysed cells (CL1), of a cell lysate after centrifugation (CL2), of two washing steps (W1, W2), and of a protein sample obtained after elution (E) were separated in 12% polyacrylamide gel by SDS-PAGE. The gels were either stained with Coomassie brilliant blue (A1) or a Western blot analysis using the H5-specific monoclonal antibody (mAb) NR2730 (A2) and an anti RGS-His mAb (c) was performed. The binding of the mAbs was visualised by chemoluminescence using a peroxidase-labeled anti-mouse goat serum. (B) The H5 protein expressed in mammal cells were purified using immunoaffinity chromatography. The eluate and the wash steps were analysed for the presence of the protein after separation by SDS-PAGE on a 12% polyacrylamide gel by protein stain (B1) and Western blot (B2) using an H5 specific mAb (VN1203/04). A molecular mass marker (M) is shown at the left side and the position of the recombinant protein is marked by an arrow at both panels.

Table 3.1: Comparison of HI and cELISA using sera of different species

Species	Test system		Avian influenza subtype strains				Total
	cELISA	HI	H5N1	H5N2	H5N3	H5N9	
Chicken	+	-	1/30[a]	12/88	1/46	0	14/172
	-	+	4/30	13/88	17/46	5/8	39/172
	+	+	16/30	52/88	24/46	3/8	95/172
	-	-	9/30	11/88	4/46	0	24/172
Duck	+	-	5/32	30/64	5/41	1/9	41/143
	-	+	0	0	1/41	0	1/143
	+	+	26/32	34/64	27/41	8/9	95/143
	-	-	1/32	0	5/41	0	6/143
Turkey	+	-	0	10/63	0	0	10/94
	-	+	0	2/63	0	0	2/94
	+	+	8/8	47/63	16/16	9/9	80/94
	-	-	0	2/63	0	0	2/94

[a]Number of sera which belonged to the appropriate category out of the total number of sera tested

Table 3.2: Determination of analytical sensitivity and specificity of the cELISA

		chicken		duck		turkey		All sera	
		HI test		HI test		HI test		HI test	
		+	-	+	-	+	-	+	-
cELISA	+	95	14	80	41	95	10	270	65
	-	39	24	2	6	1	2	42	32
Se[a]		66%		99%		97%		86%	
Sp[b]		63%		14%		83%		32%	

[a] sensitivity

[b] specificity

CHAPTER 4

HOST ADAPTATION ASSOCIATED WITH THE GLYCOPROTEIN GENES OF A LOW PATHOGENIC AVIAN INFLUENZA WILD BIRD ISOLATE WHEN PASSAGED IN CHICKENS

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To be submitted

Summary

Chicken origin and wild bird origin low pathogenic avian influenza virus (LPAI) strains were used to study viral adaptation and transmission in chickens with emphasis on possible changes in the glycoprotein genes. Six serial passages were performed and sequences of the hemagglutinin (HA) and neuraminidase (NA) genes were determined for the stock viruses and for each virus isolate during passage. Transmission was observed during passage of the LPAI chicken isolate as evidenced by serology, but no transmission was observed during passage of the LPAI wild bird isolate. Sequence analysis of the HA and NA coding sequences revealed mutations for both isolates. A majority of the observed mutations occurred early on during passage and remained constant with continuous passages. Interestingly, a number of mutations observed in later passages were already present in the virus population of both viruses as indicated by the presence of single nucleotide polymorphisms in the sequence chromatogram of the stock virus. Furthermore, the number of mutations observed during passage of the wild bird origin LPAI was higher than that observed in the chicken origin LPAI. This indicated a higher mutation rate likely contributed by selective pressure of the host during passage of the LPAI wild bird isolate. In the NA gene of the wild bird origin isolate, a 20 amino acid deletion was observed during the first passage and remained present with continuous passages. Further investigations by PCR analysis showed that the truncated NA gene was already present as a minority in the stock virus population and was not the result of a mutation. Rather, it was the result of selection during passage. Interestingly, the full length NA gene was still present in the virus population after six consecutive passages in chickens. This provided the first evidence that the truncated form of the NA gene of a wild bird origin LPAI that has been passaged in chickens

was already part of the virus population. Thus, selection of pre-existing minor viral populations during passage potentially plays an important role in virus evolution.

Introduction

Avian influenza virus (AIV) is a member of the family *Orthomyxoviridae* which belongs to the large group of enveloped viruses. The virus particle contains 8 segments of viral negative sense single-stranded RNA. The virus genome encodes for 11 proteins. Most of the proteins are part of the virus particle except the nonstructural protein 1 (NS1) as described previously [1, 2]. AIV belongs to the genus Influenza A virus. The classification of influenza viruses follows a combination of hemagglutinin (HA) and neuraminidase (NA) subtypes. As of today, 16 HA (H 1-16) and nine NA subtypes (N 1-9) of AIV have been identified [3, 4]

Wild birds are the natural reservoir for AIV, but most wild birds yield an asymptomatic infection allowing the virus to circulate in the environment without notice [5, 6]. Most of the AIV belong to the low pathogenic type (LPAI) whereas some LPAI in particular those belonging to the HA5 (H5) and HA7 (H7) subtype can become highly pathogenic AIV (HPAI) mainly due to introduction of a stretch of multiple basic amino acids into the proteolytic cleavage site between the HA1 and HA2 subunits of the HA protein [7-9] [10, 11]

It has been recently described that the introduction of a polybasic cleavage site in an H3 subtype of AIV does not necessarily confer high pathogenicty [12]. The latter report indicates that other factors may contribute to the HPAI phenotype. A different strategy was chosen by Brugh (1988) and Brugh and Perdue (1991) [13, 14]. Based on their experiments, they concluded that unrecognized subpopulations of HPAI may have been present in circulating populations for several months before emerging as the major proportion of the virus population. The described results indicate that the virus population per se already existed and was selected during the process. Li et al. (2010) showed that another hallmark for the adaptation of LPAI from

wild birds to chickens might be the truncation in the NA stalk region [15]. Banks et al. (2001) found that all examined viruses examined from the H7N1 Italian outbreaks contained a deletion in the NA stalk which was not present in the N1 genes of the wild bird virus isolates [16]. A similar observation was described by Pei et al. (2009), only a LPAI H5N1 turkey isolate contained the deletion in the stalk region but an H5N1 isolate from a mallard encoded for a full length NA gene [17]. Li et al. (2010) described that a deletion in the NA gene was only observed after passage of an H11N9 wild bird LPAI isolate in chicken whereas after passage in ducks only the full length NA sequence was detected [15]. These findings indicate that the truncation of the NA gene occurred in a species-specific manner. Hossain et al (2009) showed that after 23 serial passages in quail an H9N2 isolate acquired the ability to transmit from chicken to chicken whereas after ten additional passages in chickens the transmission rate increased [18]. The analysis of the sequence revealed a number of mutations in the viral genes including the truncation of the stalk region of the N2 protein.

The occurrence of sequence changes during passage of viruses is an event which is frequently observed in RNA viruses. The RNA polymerase of influenza virus has low intrinsic fidelity which results in a high error rate during genome replication [19]. Thus, changes in the viral genome are expected. On the other hand, the selection process from a minority to a majority due to better fitness of the minor population in a new host might result in the emergence of a virus with a different phenotype. This suggests that mutations first occur, then sequentially followed by selection of a distinct viral population during the appropriate propagation in the culturing system, e.g. tissue culture or animal.

Our study was aimed to investigate possible changes in genotype and probably viral phenotype during passage of an LPAI isolated from a wild bird [A/Mute Swan/ MI/ 451072/06 (H5N1)] and a chicken isolate [A/Ck/TX/167280-4/02 (H5N3)] in chicken.

Materials and Methods

Propagation of virus

Two H5 isolates, one isolate from a chicken [A/Ck/TX/167280-4/02 (H5N3)] and one isolate from a swan [A/Mute Swan/ MI/ 451072/06 (H5N1)] were kindly provided by Dr. David Suarez (South East Poultry Research Laboratory, Athens, GA, USA). After propagation of both viruses in 9 day-old embryonated chicken eggs (ECE) obtained from a specific-pathogen-free (SPF) leghorn chicken flock (Sunrise Farms, Catskill, NY, USA), the obtained allantoic fluid was tested for hemagglutinating (HA) activity. HA positive allantoic fluid was 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 using embryonated SPF eggs. The titer was calculated following the method as described by Reed and Muench [20]

Infection experiments

Three-week-old SPF leghorn chickens (Merial Select, Gainsville, GA, USA) were used for the infection experiments. All animal studies were conducted in BSL-$2Ag^{+}$ approved animal facilities at the Poultry Diagnostic and Research Center at the University of Georgia as approved by the Institutional Animal Care and Use Committee of the University of Georgia. The experiments were performed in isolator caging with HEPA-filtered supply and exhaust air.Water

and food were supplied ad libitum. Five three week-old SPF chickens were infected with a dose equivalent to 10^6 EID_{50}. Fifty percent of the dose was given via the nasal route and the remaining 50 % of the virus dose were applied via the choanal route. 24 hours post infection, five 22-day-old SPF birds were introduced. At day 2 (1 day for contact birds), 4 (3), 7 (6) , and 9 (8) pi a tracheal-pharyngeal and cloacal swab was taken from each bird and transferred to 2 ml of viral transport medium (1x minimal essential medium, 7.5% sodium bicarbonate, 15 mM HEPES, 1% fetal bovine serum, 4,000 U/ml penicillin, 400 µg/ml gentamycin, 8 µg/ml amphotericin B, 4,000 µg/ml streptomycin, 1000 µg/ml kanamycin sulfate). The birds were bled before infection, and at day 21 pi via the wing vein. At day 21 pi, the birds were euthanized according to the protocol as approved by the Institutional Animal Care and Use Committee of the University of Georgia.

The tracheal-pharyngeal and cloacal swab samples were centrifuged at 16000 x g, and 0.1 ml of the supernatant was used for immediate inoculation into 9-day-old embryonated SPF eggs that were incubated for 4 days at 37.5°C. The remaining swab samples were aliquoted and stored at -80C. The eggs were candled daily and embryo mortality observed within 24 h after inoculation was counted as nonspecific. The remaining eggs were investigated for presence of influenza virus using the hemagglutination (HA) assay as described by OIE after they were incubated for at least 4h at 4C. Serum samples were investigated for the presence of hemagglutination inhibiting (HI) antibodies by applying the HI test as described by the OIE using the homologue virus as antigen [21].

Positive allantoic fluid samples were pooled, aliquoted, and stored again at 80C. One sample was used for the determination of the EID50 following the method as described by Reed and Muench [20]. This mixture was used for the next infection experiment as described above.

Sequence analysis of the HA and NA genes

To analyze the sequences of viruses present in swab samples and in allantoic fluids of back isolated viruses the RNA was purified using the RNeasy purification kit (Qiagen) following the manufacturers protocol. To amplify the appropriate cDNA fragments encompassing the open reading frame (ORF) of the HA and NA proteins, reverse transcription polymerase chain reactions (RT-PCR) were performed using the One-step RT-PCR Kit (Qiagen) per the manufacturer's suggested protocol. The amplification of the HA ORF was performed using the primer pair UGAHA-F/ UGAHA-R (see table 1). For the amplification of N1 and N3 sequences, two previously published NA specific primer pairs were used [22]. The obtained RT-PCR fragments were separated on a 1% agarose gel and gel eluted using QIAquick Gel extraction kit (Qiagen, Germantown, MD, USA). The PCR fragments were directly sequenced using several oligonucleotides (see table 1) to obtain two fold coverage for each nucleotide sequence. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequence reactions were purified using the Cleanseq magnetic bead system (Agencourt Bioscience Corporation, Beverly, MA, USA) and analyzed on an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City , CA, USA) using an 80cm capillary array. The obtained sequences were analyzed using the Sequencher software (Genecodes Corporation, Ann Arbor, MI, USA).

The location of the observed mutations in the HA protein was analyzed using the Pymol program (The PyMOL Molecular Graphics System, Version 1.2, Schrödinger, LLC) based on the protein data bank code 2IBX which has been previously published by (Yamada et al., 2006) [23].

Analysis of the truncation of the N1 gene

For the investigation for the truncation of the N1 gene of the A/Mute Swan/ MI/ 451072/06 (H5N1), a number of reverse transcription-polymerase chain reaction (RT-PCR) experiments were performed. RNA from swab samples which resulted in positive virus isolation and RNA from the appropriate allantoic fluid samples were purified using High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The resulting RNA was used for single step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA, USA). For the analysis of the NA truncation, three primer pairs were used. The first primer pair was located upstream (N1-FP) and downstream (N1-RP) of the deleted nucleotide sequence (Figure 5) which resulted in a cDNA fragment of 392 bp for the full length N1 sequence and 334 bp for the truncated fragment. A third oligonucleotide located within the truncated part of the N1 sequence (N1-INT) was used in combination with N1-RP which results in the amplification of an RT-PCR fragment only in presence of the full length N1 sequence (209 bp). A fourth oligonucleotide (N1-bridge) was designed to amplify only the truncated version of the N1 nucleotide sequence in combination with N1-RP. The first 20 nucleotides were located at the 5'end before and the last 6 nucleotides 3' end after the deleted sequence. The expected RT-PCR fragment size was 194 bp. Amplified RT-PCR fragments were gel eluted as described above and cloned into the pCR2.1 plasmid using the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA). Plasmids containing an appropriate fragment were sequenced in both directions with M13 and M13R oligonucleotides using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence reactions were purified using Performa® DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD, USA). Sequence analysis was performed using the DNAStar

Lasergene software (DNAStar Inc, Madison, WI, USA). The location of the primers used for this experiment are depicted in figure 4A.

Passage in embryonated eggs

The stock virus of A/Mute Swan/ MI/ 451072/06 (H5N1) was used for a serial passage in embryonated chicken eggs. For each passage five eggs were used. The initial passage was performed by infecting five nine-day-old embryonated eggs with an EID50 of 10^5 were incubated at 37C for 4 days or until embryo mortality was observed. Allantoic fluid was obtained and tested for HA activity. Allantoic fluids tested positive were pooled and inoculated for a subsequent passage. Allantoic fluid of the fourth passage was individually taken and used for RT-PCR using the N1-FP/N1-RP (see above).

Results

Passage experiments in chickens

The six passage experiments (pas1 –pas6) in chickens using the wild bird isolate [A/Mute Swan/ MI/ 451072/06 (H5N1), H5-WB] and isolate from a chicken [A/Ck/TX/167280-4/02 (H5N3), H5-CK) were performed in parallel (Figure 1). The death of birds infected with pas2 and pas3 (H5-WB) and during the initial passage of the H5-CK was not likely related to influenza infection since no gross lesions were observed during necropsy of the birds. Virus was back isolated from birds infected with H5-CK at day 2 and 4 p.i during all passages. The number of birds where the virus was back isolated varied between 1/5 and 4/5 of the infected birds. A similar pattern was observed in chickens which have been infected with H5-WB except that virus

was back isolated from two out of five infected birds from day 7 p.i. during pas1. The results showed that both viruses replicated in all infected chickens as indicated by seroconversion.

In average, it was observed that the HI titers of chickens infected with H5-Ck was higher than the HI titers of chickens infected with the H5-WB. Virus was back isolated from a number of birds from both groups of infected chickens with different frequencies, but no differences in frequencies of virus back isolation was observed between both groups and between the different passages within the groups. Although seroconversion was observed during pas1, pas3, and pas4 of chickens infected with H5-CK, no virus was back isolated from these chickens. The seroconversion can be counted as an indicator of transmission but transmission was not consistent throughout passagging of H5-CK and not present during the passage of H5-WB. In addition, shedding of virus was only detected in the oro-pharyngial swabs and never with the cloacal swabs which showed that both viruses replicated more efficiently in the respiratory tract.

Analysis of the sequences of the viral glycoproteins

The analysis of changes in the glycoprotein genes during the passage in chickens was performed. To this end, all allantoic fluids which tested positive for hemagglutination activity and their respective swab samples were used for the amplification of the HA and NA ORF. The obtained RT-PCR fragments were directly sequenced in both directions which resulted in at least two reads for each nucleotide.

The passages of H5-WB showed that most of the observed exchanges of the amino acids (aa) occurred very early during passage in both glycoproteins (Figure 2). The aa exchanges I73V, D99N, T341K, and K393T in the HA protein were observed during pas1 and became established

in the viral population as evidenced by the sequence data. The amino acid exchange at position 172 (Ala to Thr) was first observed during pas5 but some samples still encoded for alanine at position 172. The same was observed with the aa exchange at position 403 (T403N). One aa exchange at position 394 (V394I) was only observed during pas2. None of the observed exchanges resulted in the loss or addition of a potential glycosylation site as analyzed with online tools available at http://www.expasy.org. The mutation at position 341 (Thr to Lys) resulted in a substitution of a hydrophilic aa with a basic aa which might be an indication for further changes at the HA0 cleavage site to a more basic amino acid sequence.

The observed changes in the NA protein were more dramatic. During pas1, a 20 aa deletion in the stalk region was observed. This deletion was accompanied with an aa exchange at position 52 (Ser to Asn). These mutations were observed in two out of four samples during pas1 and were present in all samples of subsequent passages. The deletion resulted in the loss of four potential N- glycosylation sites. In addition, during pas2 (1/4), pas 4 (2/4), and 5(4/8) the amino acid exchanged at position 49 was observed (Cys to Tyr) but was not observed in any sample at pas6. In total, 10 aa exchanges were observed when the deletion of the stalk region was counted as a single event.

Amino acid exchanges were also observed during passage of the H5-Ck but with a relatively lower frequency (Figure 2). Amino acid exchanges in the HA protein were observed during pas1 (G155D, N170D, Q174R, K177E, N260D) and pas6 (S216F). G155D (1/4), K177E (1/4). N260D (1/4) was only observed during pas1 whereas the remaining aa exchanges became established in the virus population. In addition, during pas6 in one sample an aa exchange S216F was detected in one out of six samples analyzed. In the NA ORF, only one amino acid exchange at postion100 was observed. During pas1 and pas2 the mutations D100E D100G were observed

respectively.. With passage 3,D100E became established in the virus population whereas the exchange D100G was not observed in any further samples. In total, six amino acids were exchanged during the course of the experiments.

In order to investigate whether the observed amino acid exchanges were either due to mutation or due to selection in the chicken of an already existing virus, the chromatograms of the fully sequenced HA and NA ORF of the appropriate stock virus was analyzed (Figure 3). For the HA gene of H5-WB isolate, it was observed that three out of the seven aa exchanges were likely already present in the viral population due to the presence of appropriate minor peaks in the chromatograms (I73V, D99N, K393T). The chromatograms for the mutations in the NA gene of H5-WB did not show minor peaks for any observed mutations. The chromatograms of the H5-Ck HA revealed that two of the six mutations already exhibited minor peaks (Q174R, K177E) in the sequence of the stock virus; whereas for the NA gene the observed mutation D100E was already present in the chromatogram. One additional position in H5-Ck stock sequence in the HA gene was observed at nucleotide position 652 (A/G) which would result in a T218A substitution, but this subpopulation was not selected for during passage in chickens. In addition in the NA gene of the H5-Ck, a synonymous nucleotide substitution at position 981 (A/G) was observed.

Analysis of the aa exchanges in the HA gene using the Pymol program revealed that most of the aa substitutions occurred in the globular head region of the protein. For H5-WB, aa substitutions at position 73, 99, and 172 were localized at the globular head whereas aa 393 and 395 were localized at the stalk region of the HA2 protein localized near the globular head. The late and inconsistent exchange at position 403 (T403N) was located in the major helix spanning the HA2 stalk region. The exchanges observed in the HA of the H5-Ck were localized almost

exclusively in the globular head region of the protein (aa 155, 170, 174, 177, and 216) whereas the aa exchange at position 260 was localized at the lower part of the globular head.

Deletion in the stalk region of the N1 protein was already present

In order to analyze the deletion/mutations in the stalk region of the N1 gene of H5-WB, we performed a variety of RT-PCR experiments. The question which needs to be addressed was whether the observed mutations were due to aa substitution/deletion or were they the result of host selection when passaged in chickens. To this end, we used three primer pairs which would generate RT-PCR fragments of different sizes (see Material and Methods). Samples from each passage and also the stock virus were analyzed for the presence of the appropriate RT-PCR fragments (Figure 4 B). The RT-PCR's were performed in parallel. Only in the stock virus of H5-WB, the long RT-PCR representing the full length N1 sequence was detected (N1Frag-Full). As expected also the RT-PCR fragment which was generated using the internal primer (N1Frag-Int) was generated. Also surprising, the primer pair which would generate the RT-PCR fragment representing the deleted form of the N1 gene (N1Frag-Del) was positive using the stock virus. This result indicates that the stock virus population contained viral particles which encoded for the deleted version of the N1 gene. Using pas1 samples, the same picture was observed as before, N1Frag-Full, N1Frag-Int, and N1Frag-del were observed. The picture changed with samples from pas2. The N1-FP/N1-RP primer pair generated only the short version (N1Frag-Short) of the N1 portion of the appropriate N1 gene being 60 bp shorter due to the deletion. Although the N1Frag-Int was still present, the amount of the amplified fragment was reduced. Also, the N1Frag-Del fragment was present confirming the results of the first (stock virus) and

second (pas1) RT-PCR. All fragments of each lane were gel eluted, cloned into pCR2.1, and sequenced. Sequence analysis confirmed the sequences. Finally, it needed to be ruled out that the primer pair used for the detection of the N1Frag-Del would be able to amplify on a full length N1 sequence. Since the last six nucleotides could be able to bind to the viral sequence, A PCR was performed using a plasmid containing the N1Frag-Full The PCR was performed using the same parameters as the RT-PCR except that for the annealing temperature a gradient was used (Figure 4C). The results showed that with a high probability this primer pair was unable to amplify a fragment under these conditions.

Further experiments were performed to investigate whether the truncated form of the N1-protein could be selected during passage in embryonated eggs. To this end, four consecutive passages were performed in embryonated eggs. HA positive samples from each passage were pooled and used for the subsequent passage to prevent artificial selection of viral subpopulations. At the forth passage, allantoic fluid from each egg was used to investigate if a switch from the majority (full length N1) to the minority (truncated N1) occurred (Figure 5). The results from the RT-PCR showed clearly that only the 392bp fragment was amplified which indicates that during four passages in embryonated eggs no change in the distribution of the N1 populations occurred. This showed that the selection to the majority encoding for the N1 truncation occurred in the chicken by an unknown selection mechanism.

Discussion

Genetic changes of AIV during passage in animals is not predicable and may result in random changes due to the dynamic and unpredictable nature of AIV. The passage of an LPAI

isolates belonging to the H5 or H7 subtype might result in the generation of an HPAI, since H5 as well as H7 encoding LPAI viruses are the only AIV which are known for this highly pathogenic phenotype. To the authors knowledge, an HPAI was only established so far by either pre-selection of large plaques in cell culture [7, 13, 14], subsequent infection in naïve chickens by reverse genetics [11], and by brain to brain passage of LPAI [24]. It is known that presence of a polybasic cleavage site between the HA1/HA2 subunits of the HA protein is a key contributor to pathogenicity of AIV [7, 11]. Alone the presence of a polybasic cleavage site in the HA does not warrant for an HPAI phenotype [12, 25-28]. Thus, the change in the viral phenotype from LPAI to HPAI might also be polygenic and in addition a gradual process.

Our study was designed to investigate changes in the viral genotype of the viral glycoproteins and probably the phenotype of H5 isolates from a wild bird and a chicken during passage in chickens. The results showed that after six passages the H5-WB isolate did not transmit from bird to bird as evidenced by virus isolation and serology although a number of mutations in the glycoprotein genes including the truncation in the N1 gene were present. This suggests that the observed mutations were not sufficient to establish bird-to-bird transmission of the virus. H5-WB initially replicated in infected birds at a rate as previously described by Spackman et al. (2007) for the same virus with the exception that virus was not isolated from cloacal swabs. But, the back isolation rate from cloacal swabs by Spackman et al (2007) was low indicating a preferred replication of this virus in the respiratory tract. In later passages (pas2, pas5, pas6), it was observed that four out of five birds shed virus which might be an indication for adaptation to the new host which resulted in a better replication; the virus did not transmit from bird to bird [29].

In contrast, H5-Ck was able to transmit during the first, third, and fourth passage as evidence by seroconversion of the contact birds. Isolation of virus from infected birds was expected, but we were unable to isolate virus from contact birds likely due to a very low rate of virus shedding if any. In summation, the observed sequence changes in the viruses were not sufficient to warrant a constant bird-to-bird transmission. Transmission of LPAI from chicken to chicken may vary since replication of these viruses differs tremendously between virus isolates. Morales et al (2009) showed that only five out of 16 LPAI isolates from wild birds were able to infect chickens as indicated by the serological results [30]. On the other hand, one H11N9 isolate from a wild bird replicated in chickens and ducks and transmitted to naïve hatch mates of both species [15]. Similar to our results, Hossain et al (2008) observed that the original H9N2 isolate from a duck was not able to transmit from chicken to chicken [18]. Only after 23 passages in quail using lung homogenate for each subsequent infection, chicken to chicken transmission was observed. This suggests that there is a great deal of variability between LPAI virus isolates in respect to their ability to infect chickens and transmit from chicken to chicken [18].

As already mentioned above, observed mutations were obviously not sufficient to confer transmission between chickens. But, it is notable that most of the observed mutations occurred in an early stage of infection and remained relatively stable during passage prompted us to the question whether these amino acid exchanges were indeed mutations during passage or the result of a selection process of an already present virus population. Ramakrishnan et al. (2009) described single nucleotide polymorphisms (SNP) in LPAI isolates and also showed that identical SNP's were detected by both methods Sanger sequencing and pyrosequencing [31]. Similarly, we found SNP's at positions which were selected during passage in chickens, but on the other hand, we also found SNP's which were present in the stock sequence which were not

selected for during passage. We found mutations which occurred during passage but were not present as SNP in the master sequence. This led us to the conclusion that minor virus populations as presented by the SNP's became selected for during passage; also, mutations occur during passage which were later selected during passage likely due to better fitness of the mutated virus. This finding confirms assumptions from Brugh (1988) and Brugh and Perdue (1991) that certain viral subpopulations are already present, but these need to be selected for during passage [13, 14].

One good example for this process of positive selection of a minority was the detection of the deletion in the N1 gene of H5-WB. We clearly showed that the N1-deletion containing viral population was already present in the stock virus and the passage in chickens resulted in the selection of this population. The selection of the truncated version of the neuraminidase gene occurred at a very early time point in chickens. On the other hand, the full length sequence was still present in the virus population even after the sixth passage. Obviously, there was a switch from minority to majority (N1 full length to N1 deletion) and from majority to minority (N1 deletion to N1 full length) during passage in chicken. This provides evidence in contrast to findings of Li et al (2010) where the preexistence of the stalk deletions was not found by using primers located outside of the deletions region[15]. We found the same phenotype during RT-PCR by using only a set of flanking primers since only the majority of the RNA template was amplified. By using a more sophisticated approach, we were able to unravel this genotype Based on our data, this selection was due to passage in chickens and not the result of a passage itself since after four passages in embryonated eggs the full length form of the N1 protein was still the majority in the viral population.

Our study shows that the mutation rate of a LPAI H5 isolate from a wild bird resulted in more amino acid exchanges compared to the passage of a LPAI H5 isolate from chickens. Most of the observed mutations were already present in the virus population before the infection experiments. The presented data, although restricted to the analysis of the glycoprotein genes, show that the observed changes in the amino acid sequences were rather the result of a selection of existing virus populations than occurred mutations during the passage in chickens.

References

1. Inglis, S.C., et al., *Polypeptides specified by the influenza virus genome I. Evidence for eight distinct gene products specified by fowl plague virus.* Virology, 1976. **74**(2): p. 489-503.
2. Lazarowitz, S.G., R.W. Compans, and P.W. Choppin, *Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes.* Virology, 1971. **46**(3): p. 830-43.
3. Fouchier, R.A., et al., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* J Virol, 2005. **79**(5): p. 2814-22.
4. Lamb, R.A., ed. *Genes and proteins of the influenza virus*. The Influenza Viruses, ed. R.M. Krug, Fraenfel-Conrat, H., Wagner, R.R. 1989, Plenum Press: New York. 1-88.
5. Stallknecht, D.E. and S.M. Shane, *Host range of avian influenza virus in free-living birds.* Vet Res Commun, 1988. **12**(2-3): p. 125-41.
6. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.

7. Li, S.Q., M. Orlich, and R. Rott, *Generation of seal influenza virus variants pathogenic for chickens, because of hemagglutinin cleavage site changes.* J Virol, 1990. **64**(7): p. 3297-303.

8. Klenk, H.D., R. Rott, and M. Orlich, *Further studies on the activation of influenza virus by proteolytic cleavage of the haemagglutinin.* J Gen Virol, 1977. **36**(1): p. 151-61.

9. Bosch, F.X., et al., *Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses.* Virology, 1981. **113**(2): p. 725-35.

10. Perdue, M.L., et al., *Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses.* Virus Res, 1997. **49**(2): p. 173-86.

11. Horimoto, T. and Y. Kawaoka, *Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus.* J Virol, 1994. **68**(5): p. 3120-8.

12. Stech, O., et al., *Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain.* J Virol, 2009. **83**(11): p. 5864-8.

13. Brugh, M., *Highly pathogenic virus recovered from chickens infected with mildly pathogenic 1986 isolates of H5N2 avian influenza virus.* Avian Dis, 1988. **32**(4): p. 695-703.

14. Brugh, M. and M.L. Perdue, *Emergence of highly pathogenic virus during selective chicken passage of the prototype mildly pathogenic chicken/Pennsylvania/83 (H5N2) influenza virus.* Avian Dis, 1991. **35**(4): p. 824-33.

15. Li, J., et al., *Adaptation and transmission of a duck-origin avian influenza virus in poultry species.* Virus Res. **147**(1): p. 40-6.

16. Banks, J., et al., *Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy.* Arch Virol, 2001. **146**(5): p. 963-73.

17. Pei, Y., et al., *Genetic characterization of two low pathogenic avian influenza virus H5N1 isolates from Ontario, Canada.* Virus Genes, 2009. **38**(1): p. 149-54.

18. Hossain, M.J., D. Hickman, and D.R. Perez, *Evidence of expanded host range and mammalian-associated genetic changes in a duck H9N2 influenza virus following adaptation in quail and chickens.* PLoS One, 2008. **3**(9): p. e3170.

19. Stech, J., et al., *Independence of evolutionary and mutational rates after transmission of avian influenza viruses to swine.* J Virol, 1999. **73**(3): p. 1878-84.

20. Reed, L.J. and H. Muench, *A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS.* Am. J. Epidemiol., 1938. **27**(3): p. 493-497.

21. *OIE*. 2009 [cited 2010; May 2009:[Available from: http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf.

22. Hoffmann, E., et al., *Universal primer set for the full-length amplification of all influenza A viruses.* Arch Virol, 2001. **146**(12): p. 2275-89.

23. Yamada, S., et al., *Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors.* Nature, 2006. **444**(7117): p. 378-82.

24. Londt, B.Z., J. Banks, and D.J. Alexander, *Highly pathogenic avian influenza viruses with low virulence for chickens in in vivo tests.* Avian Pathol, 2007. **36**(5): p. 347-50.

25. Kawaoka, Y., C.W. Naeve, and R.G. Webster, *Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin?* Virology, 1984. **139**(2): p. 303-16.

26. Deshpande, K.L., et al., *Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence.* Proc Natl Acad Sci U S A, 1987. **84**(1): p. 36-40.

27. Ohuchi, M., et al., *Mutations at the cleavage site of the hemagglutinin after the pathogenicity of influenza virus A/chick/Penn/83 (H5N2).* Virology, 1989. **168**(2): p. 274-80.

28. Suarez, D.L., et al., *Recombination resulting in virulence shift in avian influenza outbreak, Chile.* Emerg Infect Dis, 2004. **10**(4): p. 693-9.

29. Spackman, E., et al., *Characterization of low-pathogenicity H5N1 avian influenza viruses from North America.* J Virol, 2007. **81**(21): p. 11612-9.

30. Morales, A.C., Jr., et al., *Biologic characterization of H4, H6, and H9 type low pathogenicity avian influenza viruses from wild birds in chickens and turkeys.* Avian Dis, 2009. **53**(4): p. 552-62.

31. Ramakrishnan, M.A., et al., *The feasibility of using high resolution genome sequencing of influenza A viruses to detect mixed infections and quasispecies.* PLoS One, 2009. **4**(9): p. e7105.

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Table 4.1: Oligonucleotides for the analysis of the HA and NA genes of two LPAI

Name	Sequence	Gene[a]	Reaction[b]	Orientation	Location[c]	Genbank Accession No.
UGAHA-F	AGCAAAAGCAGGGGTCYAWACTATSAAA	HA-H5N1 HA-H5N3	RT-PCR,seq	sense	1-28	CY034679
UGAHA-R	AGTAGAAACAAGGGTGTTTTTAATTATAATCTG	HA-H5N1 HA-H5N3	RT-PCR,seq	antisense	1735-1767	CY034679
MIHA-302F	TGGTCATACATCGTGGAAAAA	HA-H5N1	seq	sense	302-322	CY034679
MIHA-620R	TGTTTGTTCAGCTGCATCATT	HA-H5N1	seq	antisense	620-640	CY034679
MIHA-821F	GCTCCTGAATATGCGTACAAAA	HA-H5N1	seq	sense	821-842	CY034679
MIHA-1135R	ATCCACTTCCCTGCTCATTG	HA-H5N1	seq	antisense	1135-1454	CY034679
MINA-425F	TCAAGGGGCCTTATTGAATG	NA-H5N1	seq	sense	425-444	CY034680
MINA-898F	GAGACAACTGGCATGGTTCA	NA-H5N1	seq	sense	898-917	CY034680
MINA-751R	CTGGCCATTACTTGGTCCAT	NA-H5N1	seq	antisense	751-770	CY034680
MINA-1250R	AATCCAACCCCGTCAGTTCT	NA-H5N1	seq	antisense	1250-1269	CY034680
TXHA-334F	GTGCTATCCAGGAGGCTTCA	HA-H5N3	seq	sense	334-362	CY034683
TXHA-694R	TGACCGGGATTGACCTCTTA	HA-H5N3	seq	antisense	694-713	CY034683
TXHA-863F	GCAATCATGAAAAGTGAACTGG	HA-H5N3	seq	sense	863-884	CY034683
TXHA-1359F	TGGAAAATGAAAGAACTCTGGA	HA-H5N3	seq	sense	1359-1380	CY034683
TXHA-1177R	TCCCATCAATTGCTTTCTGG	HA-H5N3	seq	antisense	1177-1196	CY034683
TXNA-345F	GGGAGCACTGCTAGGGACTA	NA-H5N3	seq	sense	345-364	CY034684
TXNA-688R	TCCAGTAAATCCTGTGATCTGC	NA-H5N3	seq	antisense	688-709	CY034684
TXNA-873F	GGAAACAGGGTATGTTTGCAG	NA-H5N3	seq	sense	873-893	CY034684
TXNA-1176R	AGGGCTGAAAACAGTCCTTG	NA-H5N3	seq	antisense	1176-1195	CY034684
N1-FP	CCAAATCAAAAGATAATAACTATTGG	NA-H5N1	RT-PCR	sense	27-52	CY034680
N1-RP	AAAGAAGGTCCTGCATTCCAAGTGAGAGC	NA-H5N1	RT-PCR	antisense	391-419	CY034680
N1-INT	CAGACATACATCAACATAAGTAATACC	NA-H5N1	RT-PCR	sense	210-236	CY034680
N1-Bridge	gcaatcaaagtgtcattacctgcagg	NA-H5N1	RT-PCR	sense	166-185, 245-250	CY034680

[a] The used viruses were A/Mute Swan/ MI/ 451072/06 (H5N1) and A/Ck/TX/167280-4/02 (H5N3)

[b] The oligonucleotides were used for sequencing (seq) and/or for reverse transcription-polymerase chain reaction (RT-PCR)

[c] The location of the oligonucleotides is in accordance with the appropriate full length sequence as indicated by the genbank accession number

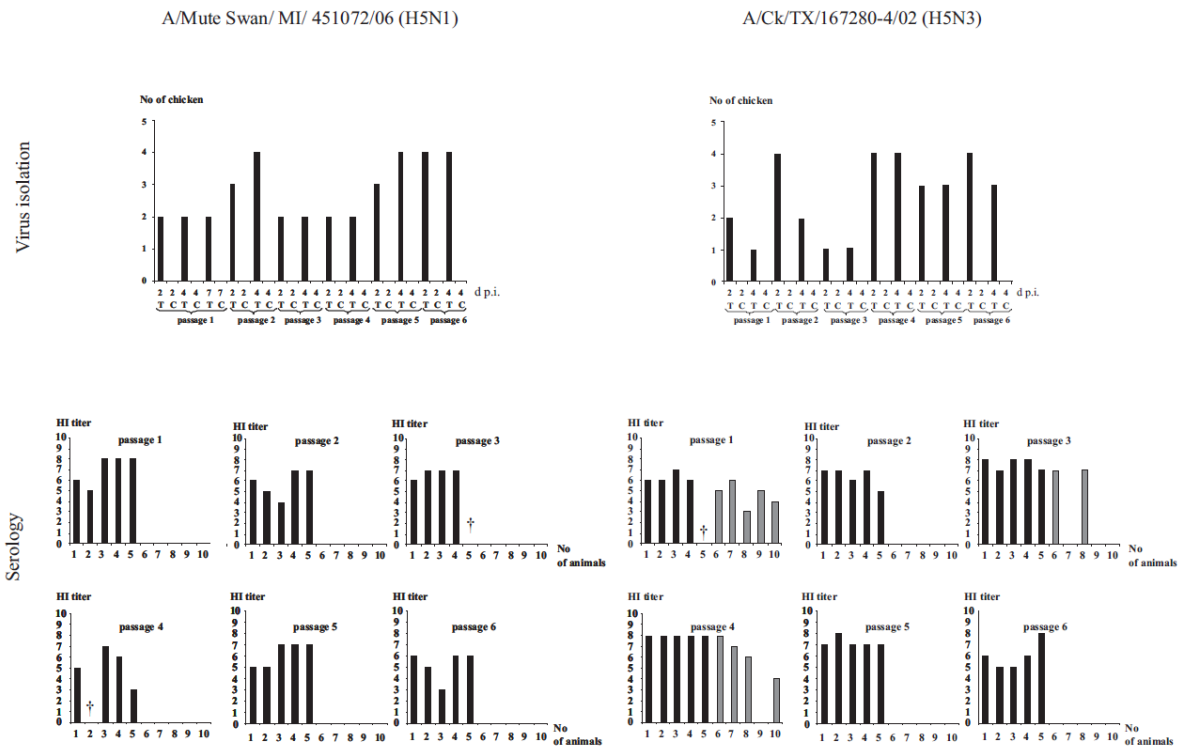

Figure 4.1: Replication of A/Mute Swan/ MI/ 451072/06 (H5N1) and A/Ck/TX/167280-4/02 (H5N3) during passage in chicken. Five 21-day-old chickens (1-5) were infected with a LPAI wild bird isolate [A/Mute Swan/ MI/ 451072/06 (H5N1)] and a LPAI isolate from a chicken [A/Ck/TX/167280-4/02 (H5N3)]. 24 h post infection five 22-day-old hatch mates were added as contact birds (6-10). Virus isolation was performed as described in Material and Methods from tracheal/oropharygal swabs (T) and cloacal swabs (C). Virus was isolated only from infected birds (number of birds is represented by a bar) from passage 1 through passage 6. Serology was performed from all birds from serum samples taken at 21 d p.i. by HI test using the homologue antigen. The HI titer are shown by the reciprocal value of log 2. Birds which died during the study are indicated with a cross. HI titer of infected birds are shown by black bars whereas the HI titer of the contact birds are shown by grey bars.

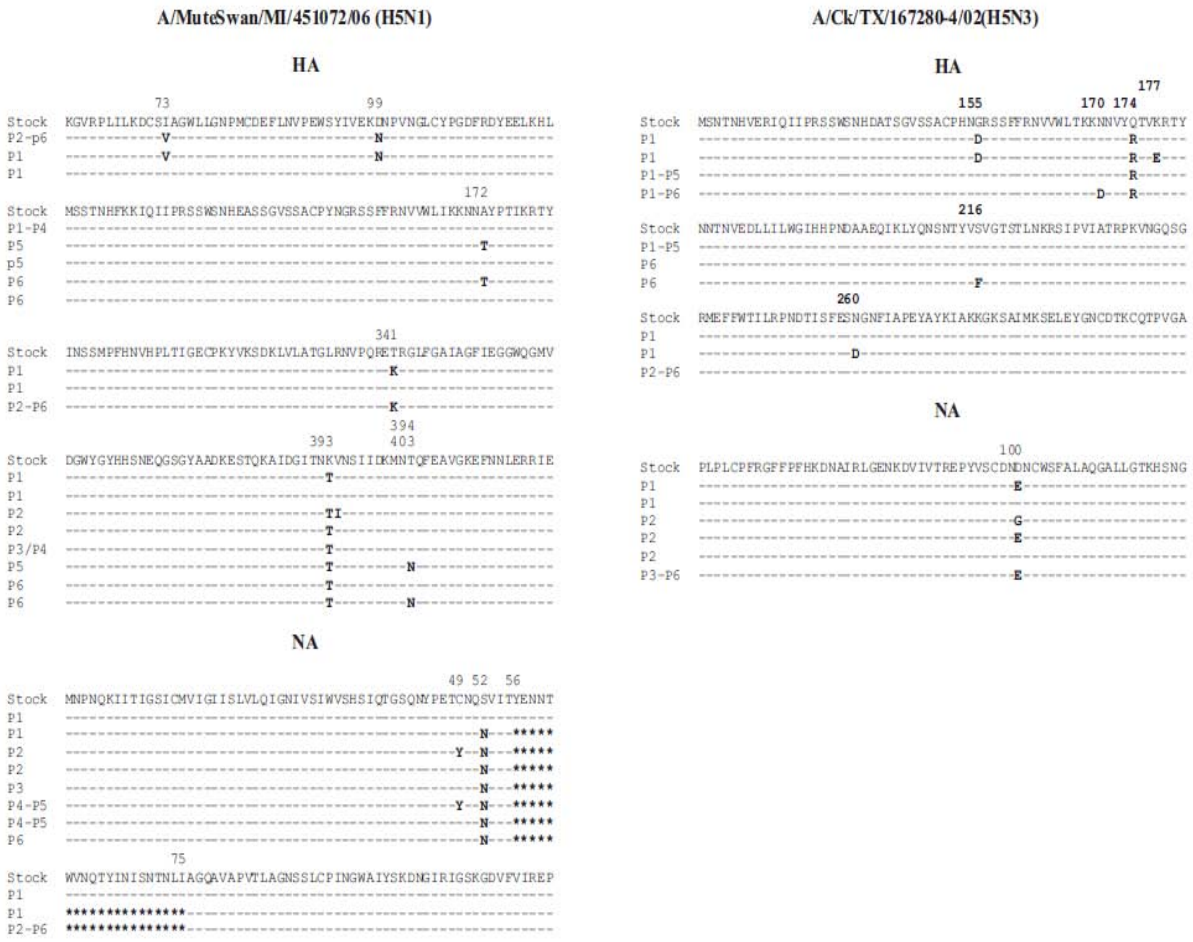

Figure 4.2: Analysis of amino acid sequences of the HA and NA genes during passage. Obtained samples (swab samples, allantoic fluid of re-isolated virus) from chickens during passage (p) of LPAI strains A/Mute Swan/ MI/ 451072/06 (H5N1) and A/Ck/TX/167280-4/02 (H5N3) were amplified by RT-PCR and directly sequenced. Sequences from the stock viruses were used as reference sequence (see table 1 for genbank accession number). Amino acids which did not change during passage are shown by a dash whereas the exchanged amino acids are shown in single letter code. Deleted amino acids are shown by an asterisk. The position of exchanged amino acids is shown above of the respective reference sequence.

Figure 3

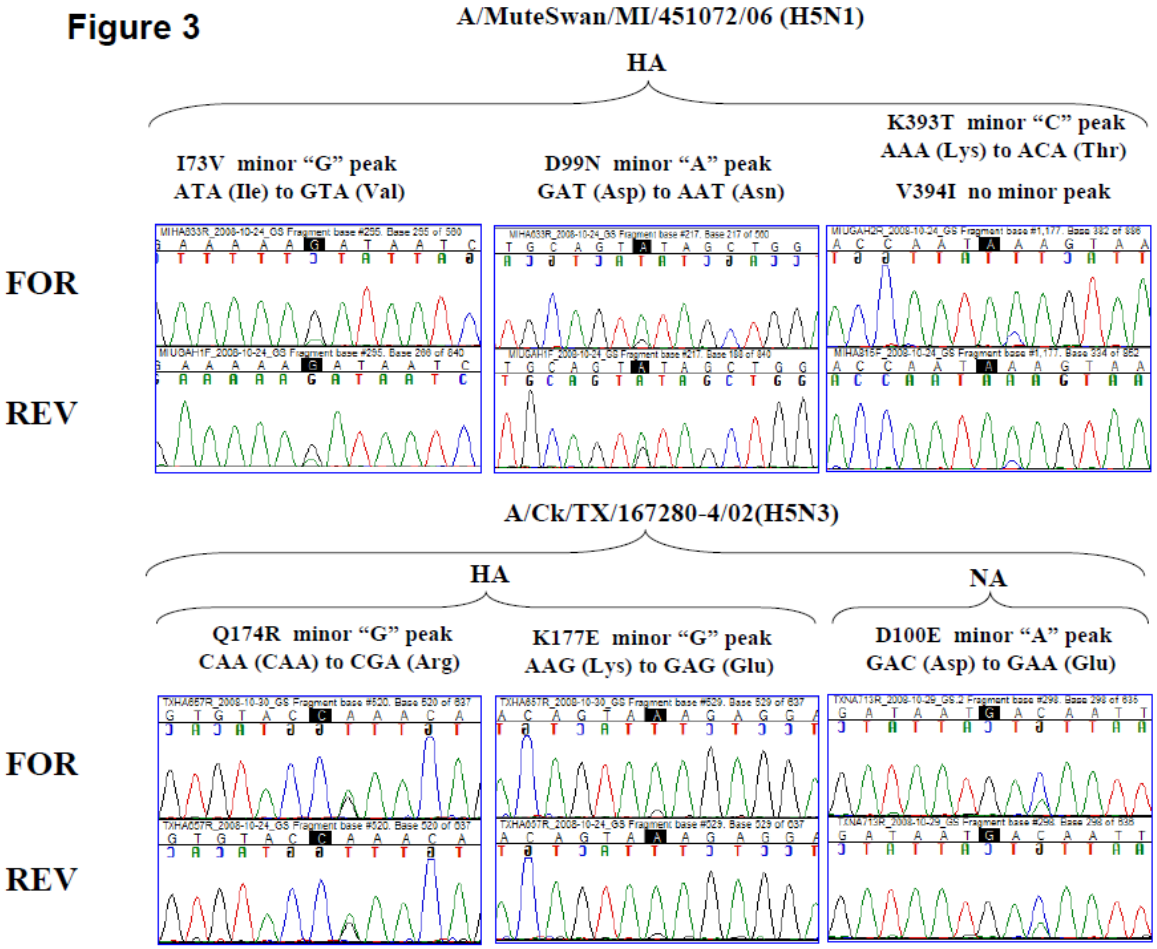

Figure 4.3. Analysis of sequences using chromatographs. Allantoic fluid of both viruses [A/Mute Swan/ MI/ 451072/06 (H5N1), A/Ck/TX/167280-4/02 (H5N3)] was used for the determination of the sequences of the respective open reading frames of the HA and NA genes. The places were amino acid exchanges were observed during passage were analyzed for the presence of minor peaks present in the chromatograms. The chromatograms of the forward (FOR) and reverse (REV) sequencing is shown. The observed minor sequencing peak representing the appropriate change in the codon is shown in the head of each chromatogram. The first nucleotide of the appropriate codon is marked by a black box.

Figure 4

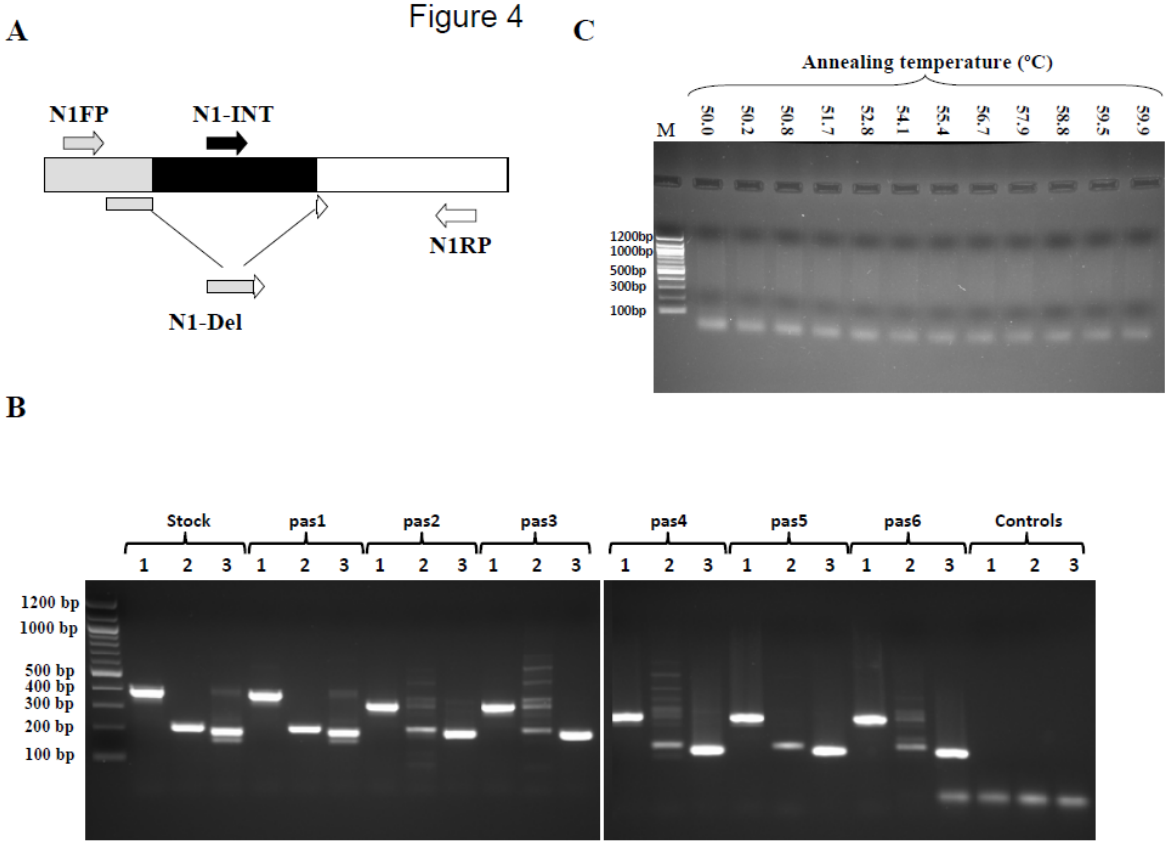

Figure 4.4. Analysis of the viral population of H5-WB for the presence of different N1 sequences by RT-PCR. (A) The location of used oligonucleotides is depicted in a schematic drawing. (B) Allantoic fluid samples from the stock virus and of each passage were analyzed by using three pairs of primer, N1FP/N1RP (1), N1-INT/N1RP (2), N1-Del/N1RP (3). Allantoic fluid of a non-infected embryonated egg was used as negative control. (C) A PCR with a temperature gradient in the annealing step was performed using the primer pair N1-Del/N1RP on a plasmid containing a DNA fragment representing the full length sequence. The reaction products in B and C were analyzed on a 1.5% agarose gel along with a 100 bp ladder.

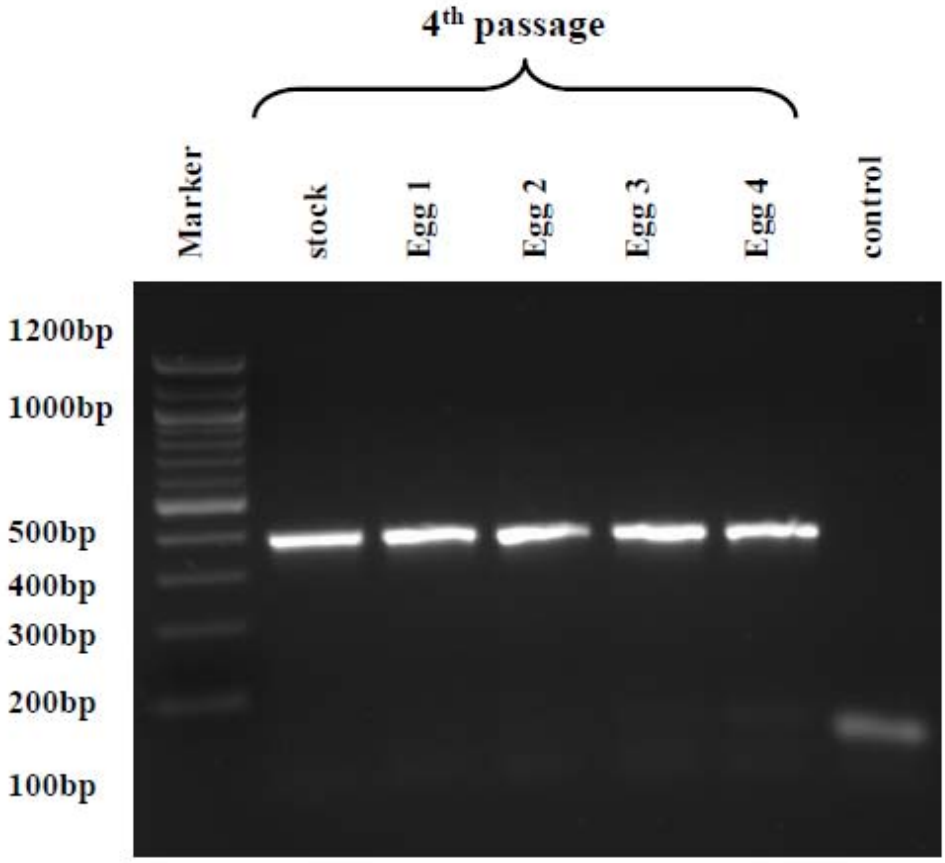

Figure 4.5. The N1 deletion is not selected during passage in embryonated eggs. Allantoic fluid samples from the stock virus and the four eggs of the 4th passage were analyzed using primer pair N1FP/N1RP. Allantoic fluid of a non infected embryonated egg was used as negative control (control). The reaction products were analyzed on a 1.5% agarose gel. A 100 bp DNA ladder was used as a marker.

CHAPTER 5

DISCUSSION

Influenza A is an extremely unstable virus due to the error prone RNA dependent RNA polymerase and possible viral reassortments that can occur due to the segmented nature of the influenza genome. Viral reassortment and mutations that readily occur in the glycoproteins, HA and NA, present major problems when it comes to influenza diagnostics. Also, changes have been experimentally observed that occur in the HA and NA genes when wild bird isolates are forced to undergo selection and adaptation.

With the emergence of high pathogenic H5N1 outbreaks in the past 10 years, the need for extensive surveillance programs for avian influenza is ever present to fully understand the occurrence of avian influenza in wild birds and domestic poultry. In this study, we developed an H5 specific species independent ELISA system using the combination of a low pathogenic H5N2 wild bird isolate along with a high pathogenic H5N1 Asian isolate to establish a broad assay for detection of all H5 isolates. There was approximately 88% homology between the H5N2 and H5N1 isolates between the HA amino acid sequences. Using our described cELISA system we eliminate the need for use of infectious live virus. Also, the use of the baculovirus expression system for expression of recombinant antigen in this test produces high yields of protein and rapid generation of antigen when stocks run relatively low. Using our H5 cELISA, we were able to detect the presence of antibodies from serum isolated from multiple species that have been experimentally or naturally infected with multiple low pathogenic H5 strains. Our H5 cELISA,

displayed high specificity for all H5 species when compared to the NP indirect ELISA but low specificity compared to the HI assay. The H5 cELISA has high sensitivity with turkey and duck serum, but relatively lower sensitivity in chickens. In conclusion, our H5 cELISA provides a highly specific rapid serological test when compared to a general NP ELISA. Additionally, it provides a species independent approach to detection of H5 specific antibodies in serum.

To obtain a better understanding of the mechanisms involved in low pathogenic avian influenza transmission and adaptation when passaged through chickens we serial passaged two low pathogenic strains of avian influenza first of which was a H5N1 wild bird isolated from a mute swan and then an H5N2 chicken isolate. We investigated viral shed using tracheal and cloacal swabs. Additionally, we looked at seroconversion using the HI test and analyzed amino acid exchanges that occurred during serial passaging of each virus. We were able to back isolate virus from multiple infected birds in both studies but we were not able to back isolate virus from contact birds in either study. No transmission occurred during passaging of the H5N1 virus as supported by virus isolation results and serological results of day 21 serum. On the other hand, transmission was observed when the H5N3 chicken isolate was serially passaged and this was supported by the serological results obtained from day 21 serum. During serial passaging of H5N1 and H5N3 in chickens certain amino acid mutations in HA and NA became established. One amino acid exchange (T343K) resulted in the addition of a basic amino acid in proximity to the proteolytic HA cleavage site during passage of the H5N1 wild bird isolate. Additionally, the amino acid exchange A174T resulted in the introduction of a potential N-glycosylation site at the globular head of the HA protein. Additionally, very few mutations occurred in the HA and NA protein of the H5N3 when serially passaged in chickens. So, the wild bird isolate has a higher

mutation rate in comparison to the chicken isolate during passaging in chickens. This result indicates an adaptive process of the wild bird isolate.

The observed deletion in the neuraminidase protein of the H5N1 results in the truncation of the stalk region of the NA structure. The deletion in the stalk region appears to already exist as a minority in the stock viral population and appears to become selected for during passaging. The full length NA gene was still present in the virus population after six consecutive passages in chickens. This provided the first evidence that the truncated form of the NA gene of a wild bird origin LPAI that has been passaged in chickens was already part of the virus population. Thus, selection of pre-existing minor viral populations during passage potentially plays an important role in virus evolution.