MOLECULAR CHANGES IN THE SURFACE GENES OF H9 INFLUENZA VIRUS AND THE EFFECTS ON VIRUS PHENOTYPIC CHARACTERISTICS: RECEPTOR SPECIFICITY, VIRUS REPLICATION AND FITNESS

by

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(Under the Direction of Daniel R. Perez)

ABSTRACT

Influenza A (IAV) virus remains a major pathogen of humans and animals. H9N2 viruses are low pathogenic avian influenza viruses and are currently the most widespread influenza virus in land-based poultry. In recent years, H9 viruses have begun to acquire molecular characteristics that make them more human like as well as contribute to the evolution of influenza viruses by donating genes to emerging IAVs. In the first part of this thesis, we characterized the effects of ferret adapted molecular changes on the replication and transmission of reassortant viruses in natural hosts of influenza. We found that molecular changes associated with mammalian transmission in ferrets are viable in pigs and are not detrimental in quails. We further identified a role for the internal gene constellation for reassortant virus replication in different species.

The entry of influenza virus is initiated by the HA (Hemagglutinin) binding to sialic acid (SA) cellular receptor via its receptor-binding site (RBS). In general, avian influenza viruses bind α2,3SA while human viruses prefer α2,6SA in concert with the abundance of these SAs in these hosts. Changes in the HA can alter SA preference and modulate host range of influenza viruses. In the final part of this thesis, we focused on determining the plasticity of amino acid at position 226, a position associated with a shift in receptor recognition. We identified a diverse set of amino acids that were tolerated at this position, some of which have yet to be found in natural isolates and determined by in-vitro studies the fitness and receptor specificity of these alternative variants. Taken together this body of work expands our understanding of the effects of molecular changes on the surface proteins of influenza viruses and broadens existing knowledge on the effects of single amino acid changes in the HA.

INDEX WORDS: Influenza virus, H9N2, 226, Influenza hemagglutinin, HA, Receptor binding site, sialic acid.

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

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DEDICATION

This work is dedicated to the following people in my life who have made sacrifices for my success and have given more than I could ever repay. Lanre, Teri, my parents and parents-in-law. Thank you.

And to the One who never leaves me nor abandons me, the One who knows me and loves me beyond measure!

ACKNOWLEDGEMENTS

I want to start by thanking my advisor Dr. Daniel R. Perez. Thank you for your guidance and support, for letting me learn and grow in your lab, for the opportunity and resources you made available to me. Thank you for all your great insights into influenza in particular and life in general, I am most grateful. I would also like to thank members of my committee, Drs. Claudio Afonso, Maricarmen Garcia, Dave Stallknecht and S. Mark Tompkins. Thank you for your support and feedback.

I would like to thank all past Perez lab members. Drs. Troy Sutton and Weizhong Li, you both took me under your wings as a new graduate student. Thank you for teaching me and for always being available to answer whatever questions I had. To other Perez lab-Maryland members, thank you for the fun times!

My thanks also go to current members of the Rajao-Perez lab—Dr. Daniela Rajao, Lucas, Stivalis, Silvia, Ginger, Zhimin, John and new members Brittany, Jojo and Joaquin. You guys made the lab a great place to be and I wish you all the very best. To Jefferson, thank you for everything and don’t forget ‘you owe me’! My thanks also go to Andrea Ferrero-Perez for your kindness through the years.

To my mother-in-law, Modupe Williams, I literally could not have done this without you. Thank you. To my parents, Ben and Ronke Obadan, thank you for your unwavering and continued support through the years. I love you.

To my siblings, thank you for your understanding, for being fine with me missing so many family occasions and for being there for me. To my church family, RCCG-AG, thank you for your support and encouragement.

To Teri, thank you for loving mummy even when she has to go to the lab and can't play soccer with you! Finally, to the LOML, Lanre Williams, thank you for believing in me, for encouraging me, trusting me and for all the sacrifices you've made. I could not have completed this journey without you and would not have wanted to. Thank you for always seeing the best in me and for always being there for me. I love you!

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CHAPTER 1

INTRODUCTION

Infections caused by influenza A viruses (IAVs) are reported in humans and animals worldwide despite the use of vaccines and/or biosecurity measures. The likelihood that human and/or animal infections with IAVs can be eradicated is nearly impossible due to characteristics of the virus and its numerous hosts.

Influenza A virus belongs to the family Orthomyxoviridae. Included in this family are viruses of the genus Influenzavirus B, Influenzavirus C, Thogotovirus, Isavirus and Quaranjavirus. Members of this family are enveloped, characterized by their segmented genome composed of single stranded, negative sense RNA (1). IAVs genome contains 8 segments that encode for at least 10 viral proteins. IAVs have a broad host range including birds, pigs, horses, dogs, sea mammals and humans. Due to the high genetic variability of IAVs, they are responsible for seasonal epidemics and occasional pandemics. Influenza B mainly cause human infections especially in children and have also been isolated from marine mammals. Like IAVs they can cause epidemics. Influenza C cause mild infections in humans and there are also reports of swine infections. A new genus, Influenza D, was recently proposed for viruses that were identified in swine (2). Since then, these viruses have also been isolated from cattle and small ruminants (3). In addition to differences in host range, the number of genome segments differ among influenza A, B, C and D with influenza A and B having 8 segments and influenza C and D having only 7.

The influenza A viral genome is encapsidated by a lipid envelope derived from the host cell during budding. Embedded in the envelope are 3 viral proteins, the hemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2). Another viral protein, the matrix 1 (M1), underlies the lipid envelope giving structure to the virion. The HA and NA surface glycoproteins form spikes on the virion surface visible by electron microscopy and are important for virus subtype distinction. Based on the genetic and antigenic properties of these surface glycoproteins, IAVs are classified into 16 HA and 9 NA subtypes (4, 5). The number of influenza A subtypes increased to 18 HA and 11 NA subtypes with the identification of 2 viruses from bats in Central and South America (6, 7). The new viruses are currently referred to as 'influenza A-like' as the HA and NA lack canonical functions associated with these surface proteins (8, 9).

Wild aquatic birds of the orders Anseriformes and Charadriiformes are the natural reservoir hosts for IAVs and all 16 HA and 9 NA subtypes have been detected in aquatic birds. Due to co-evolution and adaptation of influenza viruses in aquatic birds, infection is usually asymptomatic (10). Viral replication in the epithelial cells of the intestinal tract of infected birds is followed by shedding in fecal material into aquatic habitat and eventual transmission to naive birds via the fecal-oral route (4). Avian IAVs can be further classified into low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) viruses, based on their molecular characteristics and pathogenicity in chickens (11). LPAI viruses cause mild respiratory and or gastrointestinal (GI) disease in affected land-based birds usually with low mortality. In contrast, HPAI infections are not limited to the respiratory or GI tracts but rather present as a systemic infection with high mortality in infected flocks. Most influenza subtype combinations correspond to LPAI and to date only viruses of the subtype H5 and H7 have been associated with

HPAI infections. The increased incidence of HPAI human infections with high mortality rates have led to intensified research into these avian influenza viruses.

Avian influenza of the H9 subtype are LPAI and while most poultry infections with H9N2 viruses are characteristically mild, the presence of secondary bacterial infections result in high morbidity, decrease in egg production and mortality (12-14). H9N2 viruses have also been the cause of human infections as a result of direct contact with infected poultry in areas where H9N2 viruses are endemic (15, 16). However, serological surveys suggest that H9N2 human infections are more prevalent (17-19).

The constant evolution of influenza viruses either by the exchange of genetic material (reassortment or genetic shift) or the accumulation of mutations in the viral genome (genetic drift) contribute to the emergence of novel viruses. These novel viruses can go on to cause zoonotic infections as was the case with nearly all influenza pandemics, where reassortment yielded a virus to which there was no pre-existing immunity in the human population.

Research objectives and specific aims

The main goal of my research is to further expand the current understanding of the molecular changes in the HA of H9N2 viruses and what impact such changes will have on the phenotype of H9 viruses. Though several adaptive mutations in influenza gene segments have been described as necessary for transmission of an avian influenza virus to humans, a better understanding of these changes is necessary due to the potential public health impact (20). In particular, the role of the HA in the early stages of the virus-host interaction, through its function in virus entry and fusion, make changes in this surface protein important for the outcome of viral infection. Virus entry is initiated by the interaction between the HA and the sialic acid (SA) receptor through the receptor binding site (RBS) of the HA gene. Conformational changes arising from the change in the pH leads to fusion with the endosomal membrane and release of the viral genome into the cytoplasm (21). These functions of HA contribute to the antigenicity, viral-host restriction, replication potential, pathogenicity and transmissibility of influenza viruses. The RBS is located in the membrane distal tip of the HA1 subunit and is formed by amino acids that comprise the 130 loop, 190 helix and 220 loop (22). Amino acid residues in the RBS interact with the cellular receptor and changes in this region have been associated with receptor switching, particularly at positions 226 and 228 (H3 numbering). This thesis project focuses on identifying and characterizing the effect of molecular changes on the HA of a prototype H9N2 virus of the G1 lineage particularly in the receptor binding site with the following aims:

Specific Aims

Specific aim 1: To determine the effect of mammalian adaptation on the replication and transmission of reassortant H9 Influenza virus in natural hosts of influenza.

- Determine the replication of mammalian adapted reassortant H9 Influenza virus in a natural host of influenza using the Japanese quail (Coturnix japonica) as a model.
- Evaluate the transmission potential of these mammalian adapted reassortant H9 viruses in quails.

Specific aim 2: To determine the plasticity of amino acid residues in the receptor-binding site at amino acid position 226 (H3 numbering) in the HA of a prototypic H9 subtype IAV.

- To design a degenerate PCR library targeting position 226 (216 in H9) of the receptor-binding site of the H9 HA and attempt generation of an H9 virus by partial PCR based reverse genetics.
- To isolate and identify alternative single amino acid mutants not found in nature thus far.

Specific aim 3: To determine the effects of single amino acid mutation at position 226 on phenotypic, antigenic and fitness properties of mutant viruses.

- To assess and compare receptor binding preference and avidity of H9 influenza mutant viruses.
- To determine tissue tropism and growth kinetics of H9 influenza mutant viruses in *in vitro* and *ex vivo* systems.
- To determine the replication and transmission fitness of H9 influenza mutant viruses *in vivo* in avian models of influenza infection.

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CHAPTER 2

LITERATURE REVIEW

Influenza virus, history and pandemics.

Influenza A viruses (IAVs) are members of the family *Orthomyxoviridae*. Influenza B, C, D, Thogotovirus, Isavirus and Quaranjavirus are the other members of this family. With the exception of the Isavirus and Influenzavirus D genera, all members of this family are known to cause human infections. Isavirus is the causative agent of Infectious Salmon anemia, a contagious disease of the Atlantic salmon while Thogotovirus and Quaranjavirus are tick borne diseases that have been associated with occasional human infections. Influenza B viruses cause mainly human infections but have also been associated with infections of seals. Influenza C virus on the other hand cause human and swine infections. Unlike other members of the Orthomyxoviridae family, Influenza A viruses infect a broader host range including humans, swine, horses, marine mammals, domestic and wild birds all around the world.

Based on serological assays, IAVs are classified into subtypes denoted by a combination of the HA and NA glycoprotein (1, 2). Currently, there are 17 HA and 11 NA subtypes. Wild aquatic birds of the order Anseriformes and Charadriiformes make up the natural reservoirs of influenza viruses and almost all subtype combinations are found in these reservoirs except the H17N10 and H18N11 viruses, which were recently isolated from fruit bats in Central America (3-5). These bat derived viruses have diverged extensively from other known subtypes, as evidenced by the surface genes lacking canonical functions associated with HA and NA, such that they are currently referred to as influenza-like viruses (6, 7).

The first record of IAV infection in poultry was probably the outbreak in chickens in Northern Italy in 1878 which was described as a contagious disease of poultry with high mortality (8). In humans, the distinct clinical symptoms of IAV infection can allow for retrospective identification of possible influenza epidemics. The first clear description of an influenza epidemic occurred in 1173-1174 while the first pandemic is accepted to have occurred in 1580, arising from Asia, spreading through Africa to Europe (9). Several other pandemics are believed to have occurred during the 18th and 19th century (10, 11).

The deadliest influenza pandemic so far recorded occurred in the 20th century. Often referred to as ‘Spanish Influenza’, this pandemic of 1918 caused by an avian-origin H1N1 influenza virus, led to an estimated 50 million deaths mostly among younger people -- an unusual age distribution for influenza related deaths (11). Three waves of the pandemic were reported. The first wave being mild with deaths among the elderly. A second wave which occurred in the autumn, with what appears to be a more virulent virus, led to the hallmark of the 1918 pandemic i.e. the ‘W’ shaped mortality curve due to deaths in healthy adults between 20 and 40 years of age (12-14). The third wave in early 1919 was mild, with virus eventually becoming endemic in the population (13). The origin of the pandemic remains debated, but evidence suggests either an origin from China due to migrant workforce movement to the USA and Europe or from the United States where the first outbreak occurred in March 1918 (15).

The pandemic of 1957 also known as the ‘Asian flu’ originated from the Yunan province of China (9). Within six months, the virus had spread to all continents mainly along sea routes, arriving at ports and spreading inland, first to urban areas and then to more rural areas (9, 16). Overall, 40-50% of people became infected with about half exhibiting clinical signs, leading to approximately 1 million deaths. This pandemic was caused by an H2N2 influenza virus which was antigenically different from any previously circulating virus. The HA, NA and the Polymerase basic protein 1 (PB1) genes of the human H1N1 virus were replaced by avian origin genes, indicating a reassortment event between an avian H2N2 virus and human virus possibly in swine (17, 18). This H2N2 virus would displace the previously circulating H1N1 virus from 1918 and would remain in the human population for 11 years.

In 1968, a novel virus subtype would emerge from China to cause the third pandemic of the 20th century. This virus, an H3N2 virus, obtained genetically distinct HA and PB1 genes from a Eurasian avian H3 virus by reassortment and retained the NA and other internal genes from the previously circulating H2N2 virus. The 1968 ‘Hong Kong Flu’ pandemic was relatively mild, a feature that has been related to the presence of antibodies to the N2 neuraminidase. While antibodies to the neuraminidase cannot prevent infection, such antibodies can confer partial protection against clinical disease (19). This H3N2 virus continues to circulate as a seasonal virus in the human population.

The most recent pandemic began in February 2009 in Mexico and caused an estimated 60 million infections around the world and about 30,000 deaths in the United States (20). Surprisingly, the pandemic was caused by an H1N1 virus, a subtype that had been circulating in the human population since 1977. This virus which originated from swine was antigenically distinct from previously circulating H1N1 viruses and was given the name ‘pandemic H1N1’ to distinguish it from the seasonal H1N1 virus. Analysis of the viral gene segments showed that the virus was a result of several reassortment events. The HA (H1) was of the classical swine lineage, which had been circulating since 1918, the NA and M genes from Eurasian swine lineage while the PB2, PB1, PA, NP and NS genes were from a triple reassortant H3N2 North American swine virus (21). This pandemic was characterized by lower infection rates in people older than 60y due to the presence of preexisting cross-reactive immunity in adults (22). The 2009 virus has since displaced the 1977 H1N1 virus and is currently the seasonal H1N1 virus.

Influenza A virus structure and genome organization

IAVs are enveloped, single stranded, negative sense, RNA viruses with a segmented genome consisting of 8 segments. These 8 segments code for between 10 and 16 viral proteins. The major viral proteins are the PB2, PB1, PA, HA, NP, NA, M1, M2, NS and NEP. Other accessory proteins not found in the virion but expressed in infected cells are PB1-F2, PB1-N40, PA-X, PA-N155 and PA-N182. Visible by electron microscopy (EM) as spherical and/or filamentous particles, the virion surface has distinctive characteristic spikes that correspond to the surface glycoproteins, hemagglutinin, and neuraminidase which are embedded in the viral envelope. The virion envelope is obtained from the host cell plasma membrane on budding and contains cholesterol lipid and non-lipid rafts. Also embedded in the envelope, but not visible by EM, is the M2 protein which functions as an ion channel. Inside the virion, each segment of the RNA genome is encapsidated by the nucleoprotein NP and the heterotrimeric viral RNA dependent RNA polymerase consisting of the PB2, PB1 and PA polymerase genes to form the viral ribonucleoprotein (vRNP) (23). Inside the core is the NEP protein though in very small amounts (24, 25). Beneath the viral envelope lies the Matrix 1 (M1) protein which is in contact with the underlying viral ribonucleoprotein giving a scaffold to the virion content and contributing to virion morphology (24).

Influenza virus proteins: Structure and function

RNA dependent RNA Polymerase complex:

The IAV polymerase complex is a 270 kDa heterotrimeric protein complex comprised of the PB2, PB1 and PA genes. The PB2 protein is responsible for binding the 5’,7-methylguanosine capped structure of cellular pre-mRNA prior to cleavage by the endonuclease PA (26). This cap binding domain is found between residues 318-483 of the PB2 (27). This process generates the primer necessary for viral replication. PB2 is reported to localize to the mitochondria for some viruses. The presence of the mitochondrial targeting sequence (MTS) in the N-terminal portion of the PB2 protein is responsible for this localization which is prevalent in seasonal human subtypes (sH1N1, H3N2) and less so in avian viruses (28). Recent studies have associated the mitochondrial localization of the PB2 protein to increased replication, a result of decreased IFN-β production. In addition to mitochondrial targeting, PB2 interacts with importin, a member of the cellular importin family (29). PB2 is also involved in host range determination via the 627-domain. The presence of a glutamic acid (E) at position 627 restricts avian influenza polymerase replication in mammalian cells, while the acquisition of a lysine (K) at this position overcomes this restriction (30). Recent work by Long et al, attribute this restriction to a host protein ANP32A which differs by 33 amino acids in avian species compared to human analog (31). The exact mechanism by which the gene rescues replication is yet to be solved but ANP32A increases the levels of vRNA, cRNA and mRNA transcripts in infected cells (31).

The PB1 subunit is responsible for the polymerase activity of the replication complex, catalyzing nucleotide addition during RNA elongation (32). The functional domain of PB1 is made up of 4 conserved motifs (motifs 1-4) with the active site confined to the S-D-D motif (aa 444-446) (33, 34). Interaction with other members of the polymerase is mediated by its C terminus to the PB2 protein (amino acids 678-757) and the N terminus to the C terminus of the PA protein (35-37). PB1 is also involved in binding the viral RNA (vRNA) and complementary RNA (cRNA) during transcription and replication.

The endonuclease function of polymerase complex resides in the N terminus of the PA subunit (38). This function was for many years attributed to PB1 but recent structural studies provide evidence that PA has the an active site similar to PD-(D/E)XK family of nucleases (39, 40). PA cleaves capped cellular pre-mRNA 13-15 nucleotides from the capped structure to generate a primer for viral mRNA synthesis. The amino acid residues H41, Q80, D108, Q119 and K134 make up the catalytic site of PA (38).

Accessory proteins from the polymerase genes

From PB1, two other protein products have been recently identified. The PB1-F2 protein is a small pro-apoptotic protein encoded by a +1 reading frame of the PB1 (41). PB1-F2 localizes to the mitochondria and via its C terminus induce pore formation in membranes and cytotoxic effects (42). Its contribution to virulence appears to be strain and host specific (43, 44). PB1 N40 is a third transcript generated from codon 40 of PB1 due to leaky scanning by the ribosomes thus lacking the PA binding motif. While not essential for viral replication in-vitro, its expression is dependent on the other PB1 transcripts (45).

In 2012, a transcript from PA was identified (46). Referred to as PA-X, this protein is translated as a +1 frameshift open reading frame extension of PA and is involved in host cell shut off and the inhibition of host cell antiviral response (46, 47). PA-N155 and PA-N182 are transcripts from the PA gene originating from the 11th and 13th AUG codon of the PA (48)

Nucleoprotein (NP)

A product of segment 5, the NP protein is a major structural protein made early in infection and one of the most conserved genes of the influenza virus. Having a net positive charge, NP interacts with the negatively charged phosphate backbone of the viral RNA through basic amino acids in the RNA binding groove as well as several cellular macromolecules (49, 50). NP has been shown to interact with ssRNA, PB1, PB2 and M1 proteins as well as cellular proteins such as actin and importin α. Each of the segmented genomes of influenza is coated with NP proteins in a distinct pattern with 1 NP for every 24 nucleotides (51). The presence of nuclear localization signals on the NP protein is sufficient for the nuclear import of each genome segment necessary for virus replication (52). Furthermore, NP associates with itself, forming large oligomeric complexes which aids to maintain the RNP structure (53). NP functions in viral replication and during RNA synthesis, NP has been shown to play a role in the switch from mRNA transcription to genome replication.

Non-structural (NS) protein

The smallest of the 8 gene segments encode the Non-structural protein 1 (NS1) protein and the Nuclear Export Protein (NEP). NS1 is the major product while the NEP is produced via a splicing event. The main function of the NS1 protein is the inhibition of the host innate immune responses particularly the interferon pathway (54). NS1 also inhibits cellular mRNA processing by sequestering to cellular protein CPSF-30 and preventing mRNA polyadenylation (55). Phylogenetically, 2 alleles of the NS1 proteins termed A and B exist. Allele B is comprised exclusively of avian NS1 proteins while allele A includes some avian and all human, swine and equine NS1 genes. It appears that a replicative advantage exists for allele A in mammalian context as the introduction of an allele B NS1 gene yielded a poorly replicating virus in mammalian models (56). Unlike NS1, NEP is a structural protein and as its name suggests, is involved in the export of vRNPs from the nucleus into the cytoplasm. NEP also acts as a bridge between the vRNPs and the nuclear pore complex (57). In addition, interaction between the NEP and M1 may serve to regulate gene expression by masking the nuclear localization signal on M1 thus preventing re-entry of the RNPs into the nucleus.

Matrix gene

This gene codes for the M1 and M2 proteins. M1 forms a rigid structure beneath the lipid envelope and interacts with the surface glycoproteins and the internal RNPs acting as a link between the outer virion surface and the inner components (58). The location of the M1 protein makes it important in virion morphology (59). M1 functions in virion assembly by directing the exit of newly formed RNPs out of the nucleus and also preventing re-entry (60). The M2 protein is a tetrameric membrane transmembrane protein whose transmembrane domain acts as a proton pump. The low pH of the endosome triggers the M2 proton pump to move hydrogen ions into the virion core causing dissociation of the vRNP and release into the cytoplasm (61).

Surface glycoproteins

Neuraminidase

The NA is a type II integral membrane protein found on the surface of influenza virions. Structurally the NA is composed of a head domain, a transmembrane region and a conserved cytoplasmic tail (62). The enzymatic active site responsible for cleaving glycosidic linkages of neuraminic acid is located in the homo-tetrameric head. NA functions in the early stages of infection by cleaving sialic acid on respiratory tract mucin to facilitate efficient virus entry (63, 64). Furthermore, cleavage of sialic acid on the cellular surface is necessary for virus budding. EM studies reveal aggregation of newly formed virions on cellular surface in viruses with temperature sensitive NA mutant (65). NA is also an antigenic determinant of influenza infection. Along with HA, antibodies to NA are made following influenza infection and can serve to ameliorate disease as was seen in the pandemic of 1968 (66). Compounds targeting the enzymatic site of NA such as oseltamivir and zanamivir are currently used as antiviral against influenza infections (67, 68). NA is also important in the adaptation of avian influenza viruses from aquatic birds to domestic poultry. This adaptation is associated with truncation in the NA stalk which can result in the increased pathogenicity of adapted viruses (69-72).

Hemagglutinin

The hemagglutinin (HA) is a rod-shaped, homo-trimeric, type 1 transmembrane protein found on the surface of influenza virions (73, 74). It is the most abundant protein on the surface of the virion and found in a 4-6:1 ratio compared to neuraminidase (75) (76). Based on cryoelectron tomographic studies there are approximately 300 HA on each virion. HA exists as a precursor protein HA0 which must be cleaved into its subunits HA1 and HA2 for it to function (74). The main function of HA is host cellular receptor recognition, binding and fusion. It has also been suggested that HA plays a role in budding and the formation of the viral particle (77, 78). Structurally, the HA consists of a globular head domain (part of the HA1 subunit), the fibrous stem (HA2 and the cytoplasmic tail. The fibrous stem is made up of triple stranded coiled coil alpha helices from each of the HA monomers. While the amino acid sequence similarity between HA subtypes may vary up to 50%, all HAs retain a very conserved structure, including the more distantly related influenza B virus (79). Phylogenetically HA can be grouped into 2 groups. Group 1 consisting of the H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 subtypes while H3, H4, H7, H10, H14 and H15 subtypes make up group 2.

Recognition of the cellular receptor is mediated by the receptor binding site (RBS) located at the membrane distal tip of each HA monomer. This shallow depression is formed by 4 structures—the 130 loop, the 190 helix, the 220 loop as well as a group of conserved amino acid residues at position 98, 153, 183 and 195 that form a network of hydrogen bonds which makes up the bottom of the receptor binding site (74, 80). The HA2 portion is involved in the second basic function of HA—fusion. Similar to other enveloped viruses, the fusion of the viral and endosomal membranes following endocytosis is required for the release of the viral genome. For influenza, this process is triggered by the low pH encountered in the endosome. Following cleavage of the precursor HA0 protein (81), the acidic pH of the endosome induces an irreversible structural change in the HA leading to a separation of the HA1 globular portion from the HA2 stem. This exposes the N-terminal fusion peptide of the HA2 portion, culminating in its insertion into the endosomal membrane and eventual juxtaposition of the endosomal and viral membranes leading to the fusion (82). The presence of multiple HA units leads to the formation of a pore through which the viral genome is released into the cytoplasm.

Influenza HA: Role in pathogenicity and immunogenicity

In addition to classification based on antigenic properties of the HA and NA, avian influenza viruses (AI) are classified based on their pathogenicity in chickens into highly pathogenic (HPAI) and low pathogenicity avian influenza (LPAI). LPAI cause mild disease in birds associated with decreased egg production and depression. Most AI subtypes are classified as LPAI. In contrast, HPAI cause severe infection in birds usually with a high mortality due to systemic infections caused by HPAI viruses. According to the OIE, viruses capable of causing ≥75% mortality following intravenous inoculation of 0.2ml (1:10 dilution of virus stock) and have an intravenous pathogenicity index (IVPI) of greater than 1.2 are considered HPAI. Only members of H5 and H7 subtypes have been identified as HPAI (83). The genetic signature of HPAI is characterized by the presence of multiple basic amino acids in the cleavage site of the HA (84). The number of basic amino acids at the cleavage site differs among HPAI. The presence of these basic amino acids arginine and lysine (R, K) increases the types of proteases able to cleave the precursor HA_0. LPAI viruses have only 1 R at the cleavage site and are cleaved by trypsin like proteases, which are found in the respiratory tract, limiting infection to the respiratory tract. The acquisition of more basic residues allows cleavage by furin, a protease found in most organs leading to systemic infections (85). While HPAI viruses have the polybasic cleavage site motif, some viruses with multiple basic amino acids do not satisfy the IVPI index requirement of HPAI as defined by the OIE. So far, HPAI have only been associated with some strains of the H5 and H7 subtypes. HPAI H5 and H7 viruses evolve from LPAI H5 and H7 viruses through insertion of nucleotides, a result of slippage of the viral polymerase or recombination events to be highly pathogenic (86-89)

The key role of HA in the viral life cycle as well as its abundance on the surface of the influenza virion makes it a target of humoral response following infection. Antibodies against the HA mainly target the globular head portion of the HA gene. These antibodies are able to neutralize incoming influenza viruses by preventing binding to cellular receptors and provide protection in mouse and human models of infection. Early studies using an H3 virus identified 5 antigenic sites on the HA (Sites A-E). For H1 viruses, antigenic sites are referred to as Sa, Sb, Ca1, Ca2 and Cb and 3-5 sites have been identified for other influenza subtypes. Accumulation of amino acids changes in one or more of these sites lead to antigenic drift of the influenza virus, a consequence of the host derived immune pressure on these sites. Antigenic drift is responsible for the need to update the influenza vaccine annually. Antibodies that target these antigenic sites while able to neutralize influenza viruses as measured by conventional hemagglutination inhibition and/or virus neutralization assays are strain specific. In recent years, broadly neutralizing antibodies (bnAbs) that target the RBS of HA have been identified (90-92). This class of antibodies has a long hypervariable loop region which is inserts into the RBS thus preventing virus-receptor interaction (93) and can be subtype specific (94, 95) or heterosubtypic in their action (90, 96). In contrast to the hypervariable HA head, the stalk region of the HA is conserved across all IAV subtypes (74). Antibodies directed against the stalk are mainly broadly neutralizing since they recognize conserved epitopes (97), however this region is immuno-subdominant compared to the HA head . These stalk bnAbs occur in natural infections and following vaccination, research into how to refocus the immune response to generate stalk antibodies is currently of great interest.

Influenza virus receptor recognition and interaction

As mentioned above, the globular head of the HA interacts with the cellular receptor through the RBS. Sialic acid was first recognized as the cellular receptor for influenza in 1941 by Hirst when he identified that influenza viruses could agglutinate red blood cells and that adsorbed cells were released following incubation at 37^0C (98). In 1955, it was identified that the substance released after incubation at 37^0C was a N-acetyl-D neuraminic acid (99), making this the receptor for influenza. This was the first biological function associated with sialic acid (100). It is now understood that sialic acid are terminal sugars found on glycoproteins and glycolipids located on the surface of many cells and are involved many physiologic processes including cell signaling, stabilization of protein and glycan structures and cellular differentiation (101, 102). Their location at the termini of N-glycans and abundance make sialic acid an available target as receptors for Influenza A viruses and other viruses that belong to the *Paramyxoviridae* and *Reoviridae* families (103, 104). Sialic acids (SA) are monomeric, nine carbon sugars derivatives of neuraminic acid which are N-acylated to form N-acetylneuraminic acid (Neu5Ac) or N-glycololyneuraminic acid (Neu5Gc). Other modifications can occur further increasing the diversity of sialic acids. Sialic acids can vary in the linkage formed between the C2 carbon and the underlying sugars, an alpha linkage to the C-3 or C-6 position of the galactose (Gal) being the most common (101). The nature of this linkage is important for influenza virus species specificity. In nature, sialic acids are α2,3- or α2,6-linked to galactose and N-acetyl galactosamine (GalNAc), α2-6-linked to N-acetyl glucosamine (GlcNAc), or α2-8-linked to the second Sia residue (105). Other factors important in receptor interaction include the affinity of the HA for the SA, the structure of oligosaccharides under the SA as well as abundance and accessibility of the SA receptors. Changes to amino acid residues in the RBS, around the rim of the RBS and even at locations distant to the RBS which alter the glycosylation or electrostatic charge of the HA head can affect the receptor binding of influenza viruses (106, 107).

Receptor specificity and host range restriction.

The type of linkage between the SA and the underlying sugar is important in virus host specificity. It is widely accepted that SA in a α2,3 linkage to the underlying galactose is preferred by avian influenza viruses while human viruses favor an α2,6 linkage. This specificity correlates broadly with the expression of mainly α2,3SA in the intestinal mucosa of birds in which influenza infection is gastrointestinal, while in humans, the upper respiratory tract expresses mainly α2,6SA and infection is respiratory (108-110). Pigs however were shown to possess both types of SA. This led to the 'mixing vessel' hypothesis which considered that pigs could be infected with both avian and human viruses such that 'mixing' of genomes could take place in pigs to yield novel viruses (111). It has now been shown however that this simplistic view of both SA linkage restriction and mixing vessel is not absolute. Recent studies using lectin staining have identified heterogeneity in the distribution of SA in human, avian, swine and other animal models of influenza infection. In humans, α2,6SA are more abundant in the upper respiratory tract (URT) compared to the lower respiratory tract (LRT). The alveoli stained more intensely with MAA-II, a marker for α2,3. In addition, an age dependent expression of α2,3SA was observed, with children having higher levels of α2,3SA compared to adult (110, 112). It has been suggested that presence of the avian like receptors in the LRT may account for the limited human to human transmission of the H5N1 influenza virus and the vulnerability of children to infection (112). In pigs, α2,6SA are predominant in the ciliated epithelium of the URT while α2,3SA expression increases towards the LRT and is highly expressed in the bronchioles and alveoli similar to human SA distribution (113). In ferrets, α2,6SA predominate in the trachea and hilium of the lungs while the submucosal glands in the trachea express both α2,3 and α2,6SA (114). In mice, Ibricevic et al identified only α2,3SA expression on primary mouse tracheal epithelial cells. In avian species, both α2,3 and α2,6SA are expressed in chicken trachea with higher α2,6 expression in the goblet cells of the trachea (115). For the quail, two independent studies confirm the expression of both α2,3 and α2,6SA in the trachea though differences in the relative abundance were observed. Result from Kimble et al showed no difference in the expression of α2,3 and α2,6 on ciliated epithelium of the quail trachea while α2,6SA expression was much higher in the Costa et al report (115, 116). Age dependent expression of α2,6SA in turkeys has also been observed (116). These differences in sialic acid tissue distribution in different species and in particular the presence of both α2,3 and α2,6SA in land-based poultry suggest that in addition to the 'classical' pig mixing vessel theory, chickens and quail are biologically equipped to act as intermediate hosts and mixing vessels.

Molecular determinants of receptor specificity

The role of the HA in the recognition and binding to the cellular receptor is the first step in virus-cell contact. Changes to the HA in and around the receptor binding site can modulate the ability of the virus to recognize cellular receptors. These changes are not universal and vary with different influenza HA subtypes. The link between amino acid residues in the HA and the sialic acid linkage recognized was first demonstrated by Rogers and Paulson (117). Using derivatized red blood cells with α2,3 or α2,6SA they observed binding of avian viruses to cells with α2,3 linked SA and the reverse for human viruses. Later work by this group identified position 226 as critical for the SA specificity described above (118). In H3 and H2 subtypes, two positions are responsible for switching the receptor preference. Position 226 and 228 both in the 220 loop of the HA receptor binding site (108, 119). A glutamine (Q) to leucine (L) mutation at position 226 and glycine (G) to serine (S) change at position 228 switch receptor preference from avian α2,3 to human α2,6 (108, 120). This Q226/G228 amino acid signature is synonymous with avian influenza viruses, as most avian influenza viruses possess these residues. A different set of amino acid positions is involved in receptor specificity for H1 viruses. A change from glutamic acid (E) to aspartic acid (D) at position 190 and a glycine (G) to aspartic acid (D) at position 225 is needed to switch the receptor preference from α2,3 to α2,6 binding (121, 122). Studies using a 1918 pandemic H1N1 virus revealed that the D190/D225 mutations shifted virus specificity exclusively to α2,6 and contributed to efficient respiratory droplet transmission in ferrets compared to the avian like virus with E190/G225 motif. Interestingly an ‘intermediate’ virus with D190/G225, was able to bind both α2,3 and α2,6SA but was inefficient in respiratory droplet transmission (122). With HPAI H5N1 viruses, while the L226/G228 mutations in the context of the A/Vietnam/1203/2004 (H5N1) virus expanded the sialic acid preference to both α2,3 and α2,6 binding, the D190/D225 combination abrogated binding to both human and avian receptors (123) suggesting that the molecular changes required for receptor specificity varies with virus subtypes. Individual strain differences have also been observed with respect to receptor specificity. For the recent H7N9 viruses, while the A/Shanghai/1/2013 has the avian Q226 residue and prefers α2,3 sialic acids, the A/Shanghai/2/2013 with L226 has weak α2,6 binding while maintaining its preference for α2,3 sialic acid receptors (124). Another H7N9, A/Anhui/1/2013 binds both α2,3 and α2,6 sialic irrespective of the amino acid at position 226 (125).

Recent studies have argued that the structural topology of the glycans rather than the sialic acid linkage itself mediate changes in the sialic acid preference of influenza HA. Chandrasekaran and colleagues posit that the sialic acids bound in α2,3 linkage have a ‘cone like’ or ‘trans’ conformation based on the space occupied by the sugar moieties of the receptor. This restricts the HA contact to the trisaccharide motif of the receptor i.e. interaction is mainly with the sialic acid, the Gal-2 and GlcNAc-3 (126). In contrast, there is increased flexibility of the sugars in α2,6 linkage due to the C6-C5 bond making 2 conformations possible -- either the compact ‘cone’ like topology with short α2,6 glycans or a more widened ‘open umbrella-like’ topology with longer and branched α2,6 glycans. In the open umbrella conformation specific to α2,6 linkage, the HA has a more extended contact with the sialic acid receptor and this interaction is influenced by the branching and length of the sugars beyond the trisaccharide motif (126). It is surmised that depending of the topology of the glycans, the amino acid residues in the HA that make contact with the sugars in the receptor would vary (126, 127). In addition to the glycan structural conformations, other studies have identified the contribution of entropy to the protein-ligand interaction. These studies associate the increased flexibility of the α2,6 conformation with an increased entropic penalty such that the HA has to overcome this entropic penalty (which can be up to 5 times that of the α2,3 linkage) to bind in a α2,6 conformation (128, 129). Thus, for a virus to evolve to prefer α2,6 binding, it must acquire either mutations that can overcome the entropic penalty or that allow the HA to bind in a bi-dentate manner longer branched sugars found on the cell surface as has been shown for human H3 viruses (130-132).

Influenza HA and glycosylation

Glycosylation is a post translational modification of glycoproteins occurring in the golgi or endoplasmic reticulum whereby oligosaccharides are attached to glycoproteins. Glycosylation can be N-linked where the attachment occurs at an asparagine (Asn) residue or O-linked through a serine (Ser) or threonine (T) residue. N-linked glycosylation is characterized by the Asn-X-Ser/Thr-Y glycosylation motif where X/Y refers to any of the 20 amino acids except proline. For O-linked glycosylation, no consensus sequon has been identified. Both the HA and NA can be N-glycosylated in a manner similar to host proteins. Glycosylation is an important mechanism by which the HA evades the immune system. This has been observed in the head portion of the HA of H3N2 viruses where there has been an accumulation of N-glycosylation sites. Initial viruses circulating in 1968 had only 2 glycosylation sites while recent viruses have 6-7 sites (133, 134). By masking the antigenic sites, the virus is able to go unrecognized by antibodies present in the host (135) and persist in the population. It is hypothesized that the lack of accumulation of glycosylation by the H2N2 virus may have accounted for the short duration of circulation of this subtype in humans. Glycosylation may also come at a cost as these oligosaccharides block cellular receptors preventing virus binding to host cells or the ability of shorter sugars to access the RBS (134, 136) thus attenuating the viruses (137, 138)

Influenza H9N2 subtype

H9N2 in avian species

H9N2 viruses are low pathogenic influenza viruses. The first report of H9N2 infection was in 1966 in a turkey farm in the Wisconsin, United States (139). This outbreak was characterized by upper respiratory illness, decreased egg production and low mortality (140) though in the presence of secondary bacterial infections, mortality can be high (141).

Similar to other influenza viruses, H9N2 viruses can be classified into 2 lineages—the North American and Eurasian lineages (142). H9N2 viruses of the North American lineage have been associated with outbreaks in turkey farms in the US (143), and these viruses are yet to establish a stable lineage in land based poultry (144). In wild birds, the North American lineage H9N2 viruses are more frequently isolated from apparently healthy shorebirds and wild ducks. Unlike the Eurasian lineage H9N2 viruses, the diversity of North American lineage H9N2 viruses is limited with evidence suggesting 2 sub-lineages of the North American lineage H9N2 —one found in wild birds and the other in turkeys (145).

The first reported isolation of an H9 virus in poultry in Asia was in a quail farm in Hong Kong where birds presented with upper respiratory disease in 1988 (146). Since then, H9 viruses of the Eurasian lineage have been isolated from multiple species including land-based poultry, wild aquatic birds, swine and humans and have become the most prevalent influenza virus subtype in poultry (147, 148). Of particular importance is the establishment of stable lineages of these viruses in land-based poultry across Eurasia. Furthermore, H9N2 viruses have become enzootic in poultry populations in the Middle East, Africa and parts of Asia including Iran, Israel, Pakistan, Egypt, China, Bangladesh among others (149-152). In general, 3 main lineages have been described for Asian H9N2 viruses -- the G1, Y280 and Korean-like lineages having the following prototype viruses A/quail/Hong Kong/G1/1997, A/duck/Hong Kong/Y280/1997 and A/duck/Hong Kong/Y439/1997 respectively (153). More recent phylogenetic studies however call for more than 3 lineages. The 2011 study by Fusaro et al. focusing on Middle Eastern isolates identified 4 sub-lineages of the G1 lineage, (Group A-D) with extensive intra and inter subtype reassortment occurring among these sub-lineages. In their analysis, lineage A and B circulate extensively in Asia while lineages C and D are restricted geographically (154). In another study, H9N2 viruses are grouped into 7 main lineages representing 98 different genotypes (145). All these studies infer the increasing diversity and geographical spread of H9N2 viruses.

In regions where HPAI H5N1 is also enzootic, occasional cases of co-infection with both H9N2 and H5N1 viruses have been reported (155, 156). Co-infected birds displayed no signs of infection characteristic of the HPAI infection a feature that could allow for the dissemination of HPAI in poultry (157). The live bird markets (LBM) play an important role in the maintenance and transmission of avian influenza viruses including H9N2. The congregation of several avian and mammalian species in a confined space with little to no biosecurity measures, as well as the continuous introduction of immunologically naïve animals create an ideal environment that favors persistence of AIV infections as has been seen in China. Furthermore, the positive effect observed following the closure of LBM in reducing transmission events during the Asian H5N1 outbreak places emphasis on the role of LBMs in AIV ecology in Asia.

H9N2 in mammalian species

The enzootic nature of the viruses in live bird markets particularly in China coupled with the close human contact with potentially infected birds have raised concerns about the public health risks of these H9N2 viruses. The first human H9N2 cases occurred in China in 1998. Guo et al. identified 5 cases of H9N2 infections in 2 cities (Shantou and Shandong) of the Guangdong province (158). These cases were all characterized by acute respiratory disease and occurred in both male and female patients with an age range between 1 and 75 years (159). The next year in neighboring Hong Kong, 2 more cases were reported (160). A four-year old child was presented at a hospital with fever, anorexia, headache and sore throat with asthma as an underlying condition. The second case was of a one-year old girl also having fever and anorexia. In both cases, infection was mild and self-limiting, a feature that has been generally associated with H9N2 human infections (160). In the same year, a report of an H9N2 infection in Guangzhou, China was made (161). In 2003, Butt et al., reported the case of a 5yr old boy infected with H9N2 in Hong Kong and in 2008, 2 more cases were reported in Hong Kong (159, 162). While only a few human cases have been reported, most infections resolve following treatment, have been associated with direct contact with infected poultry, and are confined to countries where H9N2 is enzootic (163). Compromise of the immune system however, may lead to complications as was observed in a case where the patient had a history of a bone marrow transplant (159). Also, because infections with H9N2 viruses are relatively mild, the number of human infection with H9N2 viruses may be higher than published reports suggest (164). Serological evidence lend support to the higher than reported incidence of H9N2 infection in human populations. This is particularly the case among people who have contact with poultry especially for occupational reasons (165). Reports from China, show that the presence of antibodies against H9 viruses is much higher in poultry workers than the general population. One study in Shandong found 2.3% of poultry workers positive for H9 antibodies compared to no seroconversion in the general population (166). Similar studies in Shanghai and in Guangdong show that poultry workers and veterinarians had an increase incidence of H9 antibodies compared to populations without contact with poultry (167). Sero-epidemiological surveys in several other countries including Vietnam, Cambodia, Nigeria, India, Egypt, Pakistan and Iran all identify people with H9 specific antibodies (168-173) with an incidence as high as 47% in Pakistan (174). Some studies however caution that the high incidence of seroconversion may be due to the presence of antibodies against H2N2 which cross react with H9N2 viruses especially among individuals who may have been exposed to the seasonal H2N2 virus circulating prior to 1968 (175, 176).

In addition to humans, other mammalian species have been infected with H9N2 viruses. In swine, the first isolation of an H9N2 virus was in an abattoir in Hong Kong in 1998 during routine sampling of swine prior to slaughter (177). H9N2 infections characterized by influenza-like symptoms in infected swine have also been reported. Two outbreaks of H9N2 swine influenza outbreaks in China in 2003 were associated with clinical signs including fever, coughing as well as paralysis and low mortality among infected pigs (178, 179). In addition to swine infections, serological surveys in some swine production systems have identified pigs with antibodies to H9 viruses (180) . Recent serological evidence reveal swine infections in Egypt (181)

Given the close social relationship between domestic pets, reared avian species and humans, the evidence of dogs having antibodies to H9 viruses is not surprising. Serological evidence in China and Iran reveal that dogs can be infected with avian H9N2 virus (182, 183). In China, an increase in the number of dogs positive for H9 antibodies increased over the course of 3 years to about 45% of dogs tested (182). Laboratory infection of dogs and cats with H9N2 viruses also yield productive infection though with inefficient transmission (184) further suggesting that these species could act as intermediate host of influenza infection.

Influenza H9N2 and reassortment

The segmented nature of influenza viruses intrinsically favors the possibility of reassortment. Reassortment occurs when 2 strains of influenza viruses co-infect the same host cell leading to the exchange of gene segments giving rise to a progeny genetically and phenotypically distinct from either parental virus (185). With eight genome segments, influenza reassortment can theoretically give rise to 256 distinct progeny (186). The process of reassortment can confer beneficial properties to a virus population by increasing the viral diversity and fitness and can also lead to the generation of novel viruses that are capable of causing epidemics or pandemics.

Phylogenetic analyses of H9N2 viruses confirm that these viruses have undergone several reassortment events (187, 188). Interestingly, not only has there been intra-subtype reassortment, more importantly, inter-subtype reassortment events involving H9N2 viruses appear to be frequent. Several zoonotic viruses of great public health importance have originated from reassortment with H9N2 viruses. The currently circulating H7N9 virus that has caused more than 600 deaths in China was a product of genetic reassortment between H9N2 and H7 viruses (189). H9N2 viruses are also considered the donors of the internal gene cassette to the HPAI H5N1 viruses that emerged in China in the late 1990s (190). The H5N1 viruses have become endemic in poultry populations in North Africa and Asia and are responsible for sustained sporadic human infections especially in Egypt (191). An H10N8 Asian virus that resulted in a human mortality also has the internal gene segments from avian H9N2 virus (192).

In addition to naturally occurring H9N2 reassortants with other avian influenza viruses, laboratory generated reassortant viruses are viable and capable of transmission in animal models of influenza infection. Viruses carrying the surface genes of an avian H9N2 virus and internal genes from an H3N2 seasonal human virus replicated and efficiently transmitted in ferrets (193). Transmission was limited to naïve ferrets directly in contact with infected ferrets only with no transmission to respiratory contacts. The introduction of 5 amino acid changes during sequential passage of this reassortant virus in ferrets yielded a virus that acquired respiratory transmission phenotype (194). Furthermore, H9N2 and pandemic H1N1 virus reassortant viruses also show improved replication and transmission phenotype in ferrets.

Molecular determinants of specificity for H9N2 HA

Since the late 1990s, H9N2 viruses have become more prevalent in land-based poultry in Asia and the middle East. Interestingly, these viruses have naturally acquired the L226 mutation that was critical for the switch to human receptor preference in H3 and H2 viruses (195). In comparison to viruses with Q226, the L226 mutation allows H9N2 viruses to preferentially infect non-ciliated cells in an human airway epithelial cell culture a feature of human influenza viruses (195). More importantly, this mutation is biologically relevant as viruses with L226 bind preferentially the α2,6SA on glycan microarray similar to human H3 viruses and the Q226 virus is exclusively α2,3SA binding (193, 196). L226 residue is prevalent in H9N2 isolates from human infections (85% of human H9N2 isolates have L226) and in the ferret model this residue is associated with improved direct contact transmission (193). In addition to L226, other molecular changes have been identified in the H9 HA that have contributed to virus phenotype. In a study where reassortant H9:sH3N2 viruses were passaged in ferrets, the acquisition of a T189A (HA1) and G192R (HA2 numbering) yielded a virus able to transmit by airborne transmission to ferrets (194) and the T189A mutation has been shown to increase the binding specificity to human receptors (196) on epithelial cells of human tracheal tissues. Swapping the backbone to that of a pandemic H1N1 virus also resulted in efficient ferret to ferret aerosol transmission. The contribution of amino acid changes to the phenotype of influenza viruses in general and H9N2 viruses in particular, informed the studies in this thesis with a focus on position 226 (216 H9 numbering) of the H9 hemagglutinin.

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CHAPTER 3

REPLICATION AND TRANSMISSION OF MAMMALIAN-ADAPTED H9 SUBTYPE INFLUENZA VIRUS IN PIGS AND QUAIL[1]

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[1] Adebimpe O. Obadan, Brian J Kimble, Daniela Rajao, Kelly Lager, Jefferson J.S Santos, Amy Vincent and Daniel R. Perez. 2015. Journal of General Virology 96: 2411-2521

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Abstract

Influenza A virus is a major pathogen of birds, swine, and humans. Strains can jump between species in a process often requiring genetic mutation and genome reassortment resulting in outbreaks and, potentially, pandemics. H9N2 avian influenza is predominant in poultry across Asia and has occasionally infected humans and swine. Pandemic H1N1 (H1N1pdm) is endemic in humans and swine and has a history of reassortment in pigs. Previous studies have shown the compatibility of H9N2 and H1N1pdm for reassortment in ferrets, a model for human infection and transmission. Here, the effects of ferret adaptation of H9 subtype surface gene segments on the infectivity and transmission in at-risk natural hosts, specifically swine and quail, is analyzed. Reassortant H9N1 and H9N2 viruses, carrying 7 or 6 gene segments from H1N1pdm showed infectivity and transmissibility in swine, unlike the avian H9N2 virus with ferret-adapted surface genes. In quail, only the reassortant H9N2 with the 6 internal gene segments from H1N1pdm strain was able to infect and transmit, however, less efficiently than the avian H9N2 virus with ferret-adapted surface genes. These results highlight that ferret-adapted mutations on the hemagglutinin of H9 subtype virus do not restrict the virus ability to infect swine or quail and that the ability to transmit in these species depend on the context of the whole virus. As such, this study emphasizes the threat H9N2 reassortant viruses pose to humans and agricultural species, and the role swine and quail could play in the generation of novel viruses as possible mixing vessel.

Introduction

Influenza A viruses (IAV) are classified into subtypes based on the antigenic differences of the surface glycoproteins hemmaglutinin (HA) and neuraminidase (NA) into 18 HA and 11 NA subtypes with aquatic birds considered natural hosts of influenza and all subtypes except H17N10 and H18N11 viruses (Fouchier et al., 2005; Webster et al., 1992), (Gamblin and Skehel, 2010; Tong et al., 2012; Tong et al., 2013). IAVs of the H9N2 subtype are low pathogenic viruses, and two geographically distinct lineages have been described -- the North American and Eurasian lineages. Members of the Eurasian lineage jumped into poultry in Asia in the late 1980's causing outbreaks. These outbreaks have occurred in land-based poultry in many countries including China, Iran, Pakistan, Lebanon, Israel and Egypt (Barbour et al., 2006; Naeem et al., 1999; Nili and Asasi, 2003) and H9N2 viruses are currently endemic in poultry populations across much of Asia and the Middle East, mainly chickens, quail, and turkeys (Alexander, 2000; Guo et al., 2000; Naeem et al., 1999; Sun et al., 2010).

The H9N2 IAV host range is not restricted to birds. Human and swine infections with H9N2 have been reported. Human infections with H9N2 viruses are relatively few and have been associated with direct contact with infected birds (Uyeki et al., 2002). Studies showed that these isolates were of the Eurasian G1-like sub-lineage (Lin et al., 2000). Subsequent isolates from sporadic human cases have been from the G1- and Y280-like lineages (Butt et al., 2010; Butt KM, 2005; Cheng et al., 2011). In 1998, the first swine H9N2 of the Y280-like sub-lineage was isolated in Hong Kong. Unlike human infections, swine infections appear more common and are associated with all sub-lineages (Cong et al., 2007; Rui-Hua et al., 2011; Xu et al., 2008). Incidental and experimental infection of canines and felines with H9N2 have also been reported (Zhang et al., 2013)

H9N2 viruses are of particular interest as they have been identified along with H7 and H5 as being of pandemic concern. H9N2 viruses have also been identified as the donor, as well as recipient, of the internal genes to the highly pathogenic IAV H5N1 and the newly emerged H7N9 and H10N8 viruses that have infected humans in Asia (Chen et al., 2014; Liu et al., 2013; Wu et al., 2013). H9N2 reassortants have been isolated from both birds and swine (Abolnik et al., 2007; Cong et al., 2007; Wang et al., 2012; Yu et al., 2008; Yu et al., 2011). Under experimental conditions, H9N2 viruses also demonstrate compatibility for reassortment, especially with regards to human seasonal H3N2 and H1N1pdm viruses, with many reassortants showing increased infectivity, transmissibility and pathogenicity in mice and ferrets (Kimble et al., 2014; Kimble et al., 2011; Qiao et al., 2012; Sorrell et al., 2009; Sun Y, 2011; Wan et al., 2008).

We previously reported that a reassortant virus having surface genes from an avian H9N2 virus (A/guinea fowl/Hong Kong/WF10/1999) and internal genes from a human H3N2 virus (A/Memphis/14/1998) - herein referred to as 2WF10:6M98 -transmitted only to direct contact ferrets (Wan et al., 2008). We further showed that following mammalian adaptation of the 2WF10:6M98 virus in ferrets, a ferret-adapted virus emerged, 2P10:6M98, in which only 3 amino acid changes in the surface genes (T189A in HA1, G192R in HA2 and I28V in the NA) were sufficient for airborne transmission to indirect contact ferrets (Sorrell et al., 2009). Transferring the HA gene segment of the 2P10:6M98 virus (with or without the NA gene segment) into the H1N1pdm backbone (1P10:7pdm or 2P10:6pdm) also allowed for efficient airborne transmission in ferrets (Kimble et al., 2014; Kimble et al., 2011).

Whilst our previous results show that two mutations (T189A in HA1 and G192R in HA2) in the HA were essential for airborne transmission of H9:H1N1pdm reassortants in ferrets, it is unknown whether these mutations would restrict the host range of the reassortant viruses. In this regard it is important to note that the ferret is widely used as an animal model to study airborne transmission of influenza viruses, adaptive mutations that lead to such a phenotype have been identified in H9, H7, and H5 subtypes (Herfst et al., 2012; Imai et al., 2013; Ku et al., 2014; Li et al., 2014; Sorrell et al., 2009; Sutton et al., 2014; Wan et al., 2008). However, studies are lacking to demonstrate whether the ferret-adaptive mutations would affect the replication and transmission phenotype of these reassortants in pigs and quail. We chose the pig, an important agricultural species and intermediate host of influenza virus and quail, a land-based poultry species that has been shown to allow replication of multiple influenza viruses (Makarova et al., 2003). Our results showed that the replication and transmission of H9: H1N1pdm reassortant viruses differed in the swine and quail hosts and that molecular changes that confer efficient transmission can be virus and host specific.

Results

Effect of ferret-adapted mutations on infectivity, transmissibility, and pathogenicity of H9 subtype viruses in swine

To test the effect of mammalian-adapted (ferret-adapted) mutations of the H9 HA (Kimble et al., 2011; Sorrell et al., 2009) for replication and transmission in pigs and quail, five viruses were prepared (Table 3.1). Viruses containing the ferret-adapted H9 HA gene segment from the A/ferret/Maryland/P10_UMD/2008 (H9N2) virus (2P10:6WF10, 2P10:6pdm, and 1P10:7pdm) were rescued in the context of the avian-origin A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10), or the pandemic-origin A/Netherlands/602/2009 (H1N1) (H1N1pdm). Controls included reverse genetics versions of the wt WF10 and H1N1pdm viruses.

Pigs were screened for prior influenza exposure and were negative by an ELISA assay prior to the study. IAV was not detected in nasal secretions of any pig at 0 days post-inoculation (pi). Negative-control pigs remained negative for virus isolation throughout the course of the experiment. Pigs, regardless of group, showed no clinical signs of disease over the course of the study.

None of the 15 pigs directly inoculated (DI) with the 2P10:6WF10 virus showed viral shedding at any sampling time points nor did their direct contact (DC) pigs (data not shown). In contrast and as expected, all nasal swabs from the H1N1pdm DI group were positive from 2-6 dpi; and stopped shedding virus by 8 dpi (Fig 3.1). All 6 H1N1pdm DC pigs were positive for virus by 2 days post contact (dpc) and continued shedding through 6 dpc before beginning to clear the virus with low levels of virus shedding by 8 dpc (3/6 positive) and 10 dpc (1/6 positive). The 2P10:6pdm DI pigs shed virus from 2-6 dpi, although the amount of virus titer was, on average, ~1.5 $\log_{10}$ lower than the group infected with the H1N1pdm virus. DC pigs in the 2P10:6pdm group became infected later when compared to the H1N1pdm group. Additionally, there was an apparent second round of transmission within the infected DC pigs as two previously negative DC pigs began shedding at 10 dpc, well after the DI pigs had cleared the virus. Overall, 4 of the 6 DC pigs became infected with 2P10:6pdm virus. Finally, all 1P10:7pdm DI pigs shed virus from 2 dpi through 6 dpi but were negative by 8 dpi. The 1P10:7pdm virus was efficiently transmitted as 5 of 6 DC pigs began shedding by 4 dpc with clearance by 8-10dpc. There was also an apparent secondary transmission event in this group as the one previously negative DC pig began shedding virus at 10 dpc making 6 of 6 contact pigs positive for infection. Of note, nasal swabs collected from 5 DI pigs in the 1P10:7pdm virus group that were euthanized on 3 dpi were clearly positive for virus isolation but the titers were just above the limit of detection (indicated with an asterisk in Fig. 3.1) This observation is inconsistent with virus titers in nasal swabs collected from these and other DI pigs in this group at 2 and 4 dpi, but consistent with lower virus titers in BALFs at 3 dpi in the same subset of 5 pigs (Fig. 3.2c). The significance of such finding remains to be discerned, but it does not change the observation that the 1P10:7pdm virus replicated in the respiratory tract of DI pigs.

Pathological analysis revealed that pigs inoculated with the 2P10:6WF10 virus had very few visible lung lesions at 3 dpi and not different from the negative control group at 5 dpi. The H1N1pdm infected pigs showed the most significant macroscopic lung lesions (Fig. 3.2a and b), with average percentage of lung affected (average of 5 pigs/group) being 16.5% and 20.5% at 3 dpi and 5 dpi, respectively. Pigs inoculated with the 2P10:6pdm and 1P10:7pdm viruses showed an intermediate level of gross pathology. The 2P10:6pdm group averaged 5.6% and 3.3% on 3 dpi and 5 dpi, respectively. The 1P10:7pdm group had lesions covering 6.7% and 4.3% of visible lung surface at 3 dpi and 5 dpi, respectively (Fig. 3.2a and b).

The 2P10:6WF10 group had no detectable virus in the BALF on either 3 or 5 dpi, consistent with the nearly complete lack of pathology and lack of virus shedding at these time points (Fig 3.2c). The H1N1pdm group had the highest average titer of all 5 groups on both 3 dpi and 5 dpi. The 1P10:7pdm group had the lowest average titer for both days, however by 5 dpi the titer was nearly equivalent to the 2P10:6pdm virus group. Consistent with virus shedding in nasal swabs, BALF virus titers from the H1N1pdm infected pigs were significantly higher than for the 1P10:7pdm group on both 3 and 5 dpi.

Ferret-adapted mutations do not alter infectivity, transmissibility, and pathogenicity of H9 subtype viruses in quail

Unlike in swine, viral replication in quail respiratory samples was evident in the 2P10:6pdm group (Fig. 3.3), in which DI quail shed virus in trachea from 1 dpi to 5 dpi and became negative by 7 dpi. In contrast, DI quail in the 1P10:7pdm group were positive only at 1 dpi, quickly clearing the virus and remaining negative for the remainder of the experiment. DI quail in the 2P10:6WF10 group were positive for virus from 1 dpi through 5 dpi with the highest virus titers at 3 dpi. This was similar to the wt WF10 infected quail, although in this latter group, virus could be detected at 7 dpi. None of the DI quail showed clinical signs of disease, consistent with previous observations (Perez et al., 2003a). No virus was isolated from cloaca swabs in any of the groups except occasional shedding in the 2P10:6WF10 and wt WF10 groups. None of the PBS control quail shed virus (not shown).

In the DC groups, 2 of the 6 DC quail in the 2P10:6pdm group were positive for virus by 4 dpi and another began shedding on day 5, with these quail shedding for only 2-3 days. DC transmission did not occur in the 1P10:7pdm group as none of the DC quail became positive during the course of the experiment, consistent with the limited shedding in the corresponding DI group. All DC quail in the 2P10:6WF10 and wt WF10 groups became positive by 2 dpc, began to clear virus by 6 dpc and had stopped shedding by day 8, except for one DC quail in the 2P10:6WF10 group (Fig. 3.3). Virus titers in lungs of inoculated quail collected at 4 dpi and 6 dpi corresponded well with virus titers in tracheal swabs (Figs 3.3 and 3.4). Virus titers in the lungs of quail infected with viruses carrying the WF10 internal gene segments (2P10:6WF10 and wt WF10) were ~1.5$\log_{10}$ higher compared to those having the pdm backbone (2P10:6pdm and 1P10:7pdm, Fig. 3.4).

Seroconversion in swine and quail is consistent with H9 influenza virus infection

To measure seroconversion, all surviving pigs were bled at 14 dpi and 20 dpi and tested by NP ELISA for evidence of infection (Table 3.2). No negative PBS control pigs showed evidence of seroconversion (data not shown). From the 2P10:6WF10 group, only 3 of the remaining 5 DI pigs seroconverted by 20 dpi and none of the DC pigs seroconverted, consistent with poor virus replication in this group. It must be noted that one DI pig in the 2P10:6pdm group and one DI pig in the H1N1pdm were euthanized prior to serum collection due to unforeseen circumstances unrelated to the experiment. The DI H1N1pdm group showed seroconversion in 3 of 4 remaining DI pigs at 14 dpi and 4 of 4 at 20 dpi. All 6 DC pigs in the H1N1pdm group seroconverted by 14 dpi. The DI pigs in the 2P10:6pdm group had 2 of 4 seropositive at 14 dpi and 4 of 4 at 20 dpi. The two DC pigs that were positive by virus isolation by 6 dpc seroconverted by 20 dpi and were the only DC pigs to do so. The 5 remaining DI pigs in 1P10:7pdm virus group were seropositive by 20 dpi. And 4 of the 6 DC pigs in this latter group seroconverted at 14 dpi, but only 3 were positive by 20 dpi.

Quail (6 DI and 6 DC quail/group) serum samples were tested for seroconversion by HI assay (Table 3.2). No negative control quail seroconverted. Of the 6 DI quail in the 1P10:7pdm group, only 1 did not seroconvert; however, HI titers were low (≤80), which is consistent with virus replication below the limit of detection. Also consistent with this observation was the lack of seroconversion in the DC quail in this group indicating a lack of transmission. All DI quail in the other virus groups had significant HI titers (2P10:6pdm ≥80; 2P10:6WF10 and wt WF10 ≥320) against the respective homologous viruses, consistent with active virus replication. Likewise all DC quail in the 2P10:6WF10 and wt WF10 groups had HI titer of ≥320 consistent with efficient virus transmission. DC quail in the 2P10:6pdm group showed low levels of seroconversion (HI ≤80) in 4 of 6 quail. In this group, 1 bird that never shed detectable virus had low HI levels.

Discussion

H9N2 viruses in South-east Asia continue to play a pivotal role in the emergence and maintenance of other avian influenza viruses that affect poultry and are a threat to human health. H9N2 viruses have been the donors of broad-host-range genes through reassortment leading to the emergence of zoonotic viruses like the H5N1, H7N9, and H10N8 strains in China and, more recently, the introduction of Eurasian-lineage H5N8 into the USA and Canada, and subsequent reassortments generating H5N2 and H5N1 mixed-lineage viruses (Eurasian–American) (Chen et al., 2014; Guan et al., 1999; Lee et al., 2015; Liu et al., 2013; Pasick et al., 2015; Wu et al., 2013; Yu et al., 2011). Many of the recent H9N2 isolates from poultry in Asia and the Middle East possess hallmarks of human adaptation and have demonstrated the capacity to occasionally infect humans and swine. The typical mild nature of human H9N2 infections reported to date (Chen et al., 2011; Kimble et al., 2011) suggests that many occurrences may go unreported. Indeed, human serological studies suggest that human exposure is high in poultry workers and in individuals in contact with poultry where H9N2 viruses are present. Studies in Cambodia, Egypt, India, Nigeria, and Vietnam have revealed significant exposure of humans to H9N2 viruses (Huang et al., 2013; Liu et al., 2009; Okoye et al., 2013; Pawar et al., 2012; Uyeki et al., 2012; Zhou et al., 2014).

In this study, we tested the replication and transmission in pigs and quail of reassortant H9:pdm viruses having ferret-adaptive mutations. We reported previously that three amino acid changes in the surface genes of an avian H9N2 virus conferred efficient airborne transmission of a reassortant H9N2 : H3N2 virus in the ferret model (Sorrell et al., 2009). We further showed that, on the H1N1pdm backbone, the two changes in the HA alone were sufficient for airborne transmission in ferrets without further adaptation (Kimble et al., 2011). In this study, we sought to determine whether the ferret-adaptive mutations would restrict the host range of these viruses in other natural hosts of influenza. Swine carry both avian- (α-2,3SA) and human- (α-2,6SA) like influenza receptors in their respiratory tract (Ito et al., 1998; Kimble et al., 2010; Wan and Perez, 2006). This allows for potential infection with both avian- and human-origin IAVs and creates an ideal environment where two different viruses may reassort and lead to novel genotypes. Similar to our results in ferrets, both the 1P10:7pdm and 2P10:6pdm viruses replicated and were transmitted to contact pigs, with the number of transmission events (number of DC pigs that became virus positive) higher in the 1P10:7pdm (H9N1) group compared with the 2P10:6pdm (H9N2) group. In a similar study (Qiao et al., 2012), tested alternative H9N1 and H9N2 viruses having H1N1pdm internal gene segment in pigs and chickens and found that the H9N1 reassortant virus with a wholly avian-origin HA gene was also more efficient than a reassortant H9N2 virus in replication and transmission in swine compared, but not in chickens. It is important to emphasize that the studies in that report were consistent with the notion that the H9 HA and N1 NA combination favors the transmission of these viruses in ferrets and pigs but not in chickens or quail. This leaves the possibility of a swine H9N2:H1N1pdm reassortant adapting to the pig respiratory tract with the potential to transmit to humans. In a report by (He et al., 2014) where H9N2:H1N1pdm reassortants were tested in the guinea pig model, the results were somewhat different, with the (H9N2)2:6pdm reassortants transmitting more efficiently than the (H9N1)1:7pdm, although the (H9N1)1:7pdm viruses displayed enhanced pathogenicity. The animal origin of the H9N2 viruses (avian and swine) as well as different lineages (G1 and Y280) may account for these differences. A recent report from Li et al, showed that some naturally occurring H9N2 isolates have the capacity for respiratory droplet transmission in ferrets without adaptation (Li et al., 2014). At least one of these isolates was transmitted with apparently higher efficiency, although the mean peak titers were lower than those observed for the H9N2 and H9N1 avian/human reassortants presented previously. The HA of the natural isolates from the study by Li *et al*, have in common with our ferret-adapted P10 H9 the I155T mutation, which favors binding to α2,6SA, which may favor replication in mammals. However, P10 HA also differed from the viruses described by 21 other amino acid positions (compared with sites with consensus amino acids). It remains to be determined whether wholly avian H9N2 viruses from the Li *et al.* study are compatible for replication and transmission in pigs. It also remains to be determined whether the viruses that showed improved replication and transmission in ferrets in the Li *et al.* report will have a similar phenotype in pigs. Further detailed sequence analyses of viruses should shed light on the adaptive changes that take place during initial rounds of replication in different hosts.

A recent report concluded that the H1N1pdm matrix (M) and NA genes cooperate to allow improved replication and transmission of a triple reassortant swine virus in pigs (Ma et al., 2012). Another study showed that the inclusion of the H1N1pdm M and NA genes in the background of a laboratory-adapted A/Puerto Rico/8/1934 (PR8) virus increased the replication and transmission of the resultant reassortant virus compared with the wt PR8 virus in a guinea pig model (Campbell et al., 2014). Our present results in the pig as well as our previous finding in the ferret support this notion, as the efficiency of transmission was increased with the 1P10:7pdm virus in both ferrets and pigs.

Quail are another important agricultural species that are susceptible to infection with many influenza subtypes (Makarova et al., 2003; Thontiravong et al., 2012b) and could become mixing vessels for IAV reassortment (Perez et al., 2003a; Perez et al., 2003b; Thontiravong et al., 2012a). In the quail study, our results showed that reassortant viruses with both ferret-adapted H9 surface genes (HA and NA) on a wild-type H9 or H1N1pdm backbone replicated and were transmitted in quail. The role of the internal gene constellation appeared to be an important factor in the replication and transmission phenotype observed in quail. The 2P10:6pdm virus having ferret-adapted surface genes on an H1N1pdm backbone replicated in the quail and was transmitted to 50% of the infected birds. Replacing the H1N1pdm internal genes with the avian WF10 backbone increased the transmission efficiency of the 2P10:6WF10 virus to 100%, similar to that of wt WF10. This observation reflects the adaptation of the avian- and mammalian-origin backbones for their respective hosts. The importance of the origin of internal genes in the transmission event was also observed in the pigs. In the 2P10:6WF10 virus, despite having surface genes that allowed transmission in pigs, the presence of an avian-origin internal gene constellation obliterated replication and transmission in pigs. In the quail study, we also found that the molecular changes due to mammalian adaptation did not affect the ability of the virus to replicate when both ferret-adapted surface genes were present. It has been reported that a functional balance between HA and NA activity is important for virus replication and transmission (Sun et al., 2013; Yen et al., 2011). An imbalance caused by an avian-origin HA and a mammalian-origin NA may account for the lack of proper replication and transmission observed in quail with the 1P10:7pdm virus. This poor replication phenotype of an H9N1 reassortant has been reported previously in chickens (Qiao et al., 2012). Amino acids at positions 106 and 248 of the N1 NA of H1N1pdm viruses have been reported to play a role in the low-pH stability of H1N1pdm viruses (Takahashi et al., 2013). This low-pH stability has been shown to be important for N2 NA replication in ducks (Takahashi et al., 2003). A valine-to-isoleucine substitution at position 106 and asparagine-to-aspartic acid at position 248 increased the replication of A/California/04/9009 virus by 10-fold in Madin–Darby canine kidney (MDCK) cells. The N1 NA used in this study had V106 and N248, which may have contributed to the poor replication observed in quail. Further tests to confirm the effects of these mutations are needed but are beyond the scope of the present report.

This study highlighted the potential role that pigs and quail may play as amplifiers of reassortant influenza viruses. It also underscores the potential threat that H9N2 viruses could pose to humans as a future pandemic subtype. Swine can be host to both H9N2 and H1N1pdm viruses and both viruses have a history of reassortment in swine (Howard et al., 2011; Vijaykrishna et al., 2010; Yu et al., 2011). Pigs could also serve as suitable environment for the adaptation to the mammalian host of any potential reassortant virus. Similarly, quail can be infected with H9 viruses and H1N1pdm have been shown to reassort experimentally in quail following co-infection with other avian influenza viruses (Thontiravong et al., 2012a). The results in this report showed that quail are susceptible to and can transmit reassortant H9N2:H1N1pdm viruses and that transmission of these viruses depends on the compatibility of the surface and internal gene segments. These findings emphasize the need for continued surveillance of influenza in swine and quail and the need for additional research on how H9N2 influenza behaves in both species and how hosts can affect reassortment

Materials and methods

Viruses

Viruses were generated by reverse genetics using previously described plasmids (Kimble et al., 2011; Sorrell et al., 2009). Plasmids containing the genes for A/Netherlands/602/2009 (H1N1) (pdm), A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10), and the ferret-adapted surface genes from the A/ferret/Maryland/P10_UMD/2008 (H9N2) (P10) virus were used. Five viruses were prepared (Table 3.1): The wt pdm and WF10 viruses, the P10 HA and NA on the WF10 backbone (2P10:6WF10), the P10 HA with the 7 other genes from pdm (1P10:7pdm), and the P10 HA and NA with the 6 remaining genes from (2P10:6pdm).

Animal studies

Swine studies were performed in large animal BSL3-Ag facilities of the National Animal Disease Center (NADC), Ames, IA, following protocols approved by the NADC and the University of Maryland institutional animal care and use committees (IACUC). The study was done on 3-week-old crossbred pigs that were obtained from a high-health herd free of swine IAV and porcine reproductive and respiratory syndrome virus. Nucleoprotein (NP)-blocking enzyme-linked immunosorbent assay (ELISA; IDEXX, Westbrook, Maine) was used to confirm the absence of antibodies to IAV. Prior to the start of the study, pigs were treated with ceftiofur crystalline free acid (Pfizer Animal Health, New York, NY) and enrofloxacin (Bayer Animal Health, Shawnee Mission, KS) to reduce bacterial contaminants. The pigs were divided into 5 groups (n=15/group) and inoculated with 2 ml intratracheally and 1 ml intranasally of 10^5 TCID50 ml^{-1} of either 2P10:6pdm, 1P10:7pdm, H1N1pdm, or 2P10:6WF10 virus or mock inoculated with phosphate-buffered saline (PBS). Inoculation was performed under anesthesia, using an intramuscular injection of a cocktail of ketamine (8 mg kg^{-1} of body weight), xylazine (4 mg kg^{-1}), and tiletamine-zolazepam (Telazol; 6 mg kg^{-1}) (Fort Dodge Animal Health, Fort Dodge, IA). Two days post inoculation (dpi), 6 naïve pigs for each virus group were introduced to the 15 inoculated pigs as direct contacts, except in the PBS control group. Nasal swabs (Fisherbrand Dacron swabs, Fisher Scientific, Pittsburg, PA) were collected in 2 ml of minimal essential medium (MEM) on 2, 4, 6, 8, 10, and 12 dpi. On 3 and 5 dpi, 5 pigs from each group were swabbed, bled, and humanely euthanized with a lethal dose of pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). The lungs were scored for macroscopic lesions, followed by collection of bronchoalveolar lavage fluid (BALF) for virus titration. Nasal swabs were filtered and subjected to virus isolation by inoculating 200 µl aliquots and 200 µl of serum-free Opti-MEM (Gibco®, Life Technologies, Carlsbad, CA) supplemented with 1 µg ml^{-1} of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and antibiotics onto confluent phosphate-buffered saline (PBS)-washed MDCK cells in 24-well plates. Virus isolation-positive nasal swab and BALF samples were then titrated in MDCK cells as previously described (Kitikoon et al., 2006) and $TCID_{50}$ titers were calculated according to the method of Reed and Muench (Reed and Muench, 1938).

Quail studies were conducted in a BSL3+ facility at the Department of Veterinary Medicine, University of Maryland under protocols approved by the IACUC. Four-week old Japanese quail obtained from B&D Game Farm (Harrah, OK) were kept under observation for 1 week prior to start of experiment. Nucleoprotein (NP)-blocking ELISA (Synbiotics Co., San Diego, CA) was used to confirm the absence of antibodies to influenza prior to infection. Quail were randomly divided into 4 groups (n=12/group) and housed in HEPA-filtered isolators. Each quail was infected with 1 ml containing 10^6 TCID50 ml^{-1} of respective virus through the nares, trachea and cloaca (0.25 ml administered via the trachea and nares and 0.5 ml via the cloaca). A negative control group of 6 quail received 1 ml PBS through the same routes. Naïve quail (n=6/group) were introduced as direct contact quail at 1 dpi. Tracheal and cloacal swabs were collected from all quail daily until 14 dpi. Swabs were suspended in 1 ml 3.7% Brain Heart infusion media (BHI) (Becton Dickinson, Sparks, MD) containing 10,000 U Penicillin, 10 mg Streptomycin and 25 µL Amphotericin B and stored at -80ºC until used in virus titrations. At 4 and 6 dpi, 3 directly infected quail per group were euthanized (2 quail in the control group) and lung tissue collected for viral load titration. The left lung lobe was homogenized in 0.5 ml PBS using 3 mm tungsten carbide beads (Qiagen, Valencia, CA) in a Tissuelyser LT (Qiagen, Valencia, CA) at 50 cycles min^{-1} for 10 min. Samples were then clarified by centrifugation at 1000 *g* for 10 min and stored at -80ºC until used. Swabs and lung homogenate samples were titrated in MDCK as described above.

Serological analysis

Pig serum samples collected at 14 and 20 dpi were tested for anti-NP antibodies by ELISA as described above. Quail serum samples collected at 21 dpi were tested by Hemagglutination Inhibition (HI) assay. HI assays were performed following treatment of samples with receptor-destroying enzyme (RDE) (Denka Seiken, Japan), heat inactivation at 56°C for 30 min, to remove nonspecific hemagglutinin inhibitors and natural serum agglutinins. HI assays were performed with 8 HAU of each virus antigen and 0.5% chicken RBC according to standard techniques (**WHO**). Negative and positive controls were included in the assay. Seroconversion was considered positive when HI titers to homologous virus were ≥ 40.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 4.00 (GraphPad Software Inc., San Diego, CA). Comparison between 2 treatment means was done using a Students *t* test while multiple mean comparisons was done using two-way ANOVA followed by Bonferoni’s multiple comparison test. $P<0.05$ was considered significant.

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Table 3.1: Genetic content of viruses used for each experimental group in pigs and quail.

Group	HA subtype	NA subtype	Internal genes	Mutations (gene)
2P10:6WF10	H9 (ferret-adapted)	N2 (ferret-adapted)	Avian H9N2	T189A (HA1), G192R (HA2), I28V (NA)
2P10:6pdm	H9 (ferret-adapted)	N2 (ferret-adapted)	Pandemic H1N1	T189A (HA1), G192R (HA2), I28V (NA)
1P10:7pdm	H9 (ferret-adapted)	N1 (human-adapted)	Pandemic H1N1	T189A (HA1), G192R (HA2)
WF10	H9 (avian)	N2 (avian)	Avian H9N2	N/A
H1N1pdm	H1 (human-adapted)	N1 (human-adapted)	Pandemic H1N1	N/A

Table 3.2: Seroconversion in pigs and quail after exposure to different viruses.

Virus group	Pigs				Quail	
	NP ELISA positive serum [#]				HI titers 21 dpi [*]	
	DI 14dpi	DC 14dpi	DI 20dpi	DC 20dpi	DI	DC
2P10:6pdm	2/4	0/6	4/4	2/6	6/6 (226)[§]	4/6 (33)
1P10:7pdm	2/5	4/6	5/5	3/6	5/6 (57)	0/6 (0)
2P10:6WF10	2/5	0/6	3/5	0/6	6/6 (1140)	3/3 (320)
H1N1pdm	3/4	6/6	4/4	6/6	N/A	N/A
wt WF10	N/A	N/A	N/A	N/A	6/6 (507)	5/5 (735)

[#] Number of seropositive pigs out of total number of inoculated (DI) or contact (DC) pigs at 14 and 20 dpi, using a commercially available kit

[*] Number of seropositive quail out of total number of DI or DC birds at 21 dpi.

[§] Number in parentheses are geometric mean titers of seropositive samples ≥20

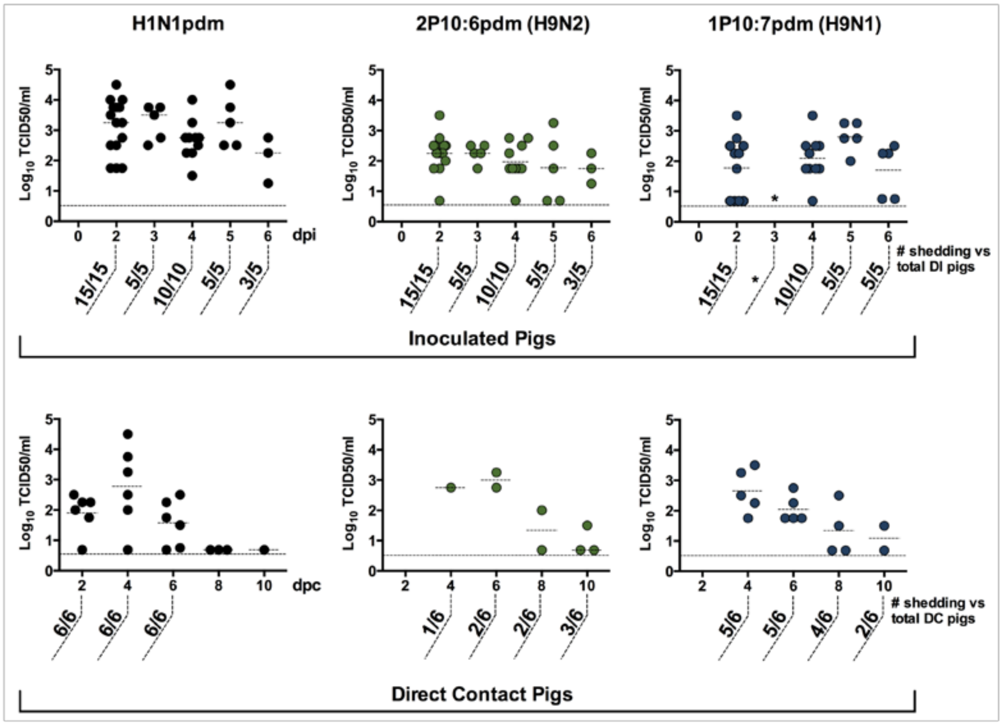

Figure 3.1: Reassortant H9 viruses transmit in swine. Pigs (n=15/group) were inoculated (DI) with the viruses indicated and nasal swabs collected at various days post-infection (dpi). The number of DI pigs positive for viral shedding in each group vs. the total number of pigs is listed under each time point. On 3 and 5 dpi, 5 DI pigs/group were sacrificed for virus titration in BALFs. Direct contact (DC) pigs (n=6/group) were introduced in the same pen housing the DI pigs at 1 dpi. Nasal swabs were collected from DC pigs and titrated as indicated above. The number of DC pigs positive for viral shedding in each group vs. the total number of pigs is listed under each time point. Note the DC graphs are measured in days post contact (dpc) not dpi.

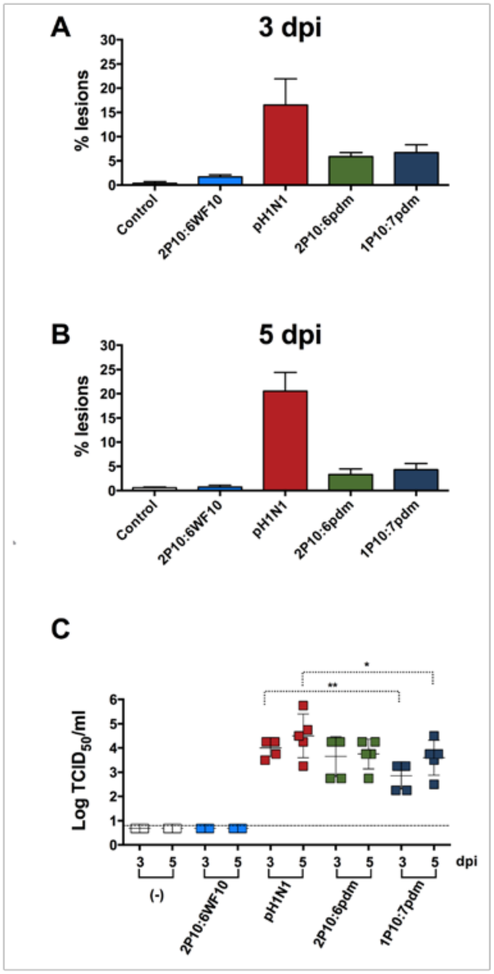

Figure 3.2: H9 reassortants result in intermediate pathology and replication in swine lungs. 5 DI pigs/group were euthanized on 3 (a) and 5 dpi (b). Lungs were scored for visible lesions and a total percentage of the lung surface affected was calculated as previously described (Halbur et al., 1995). (c) BALF samples were collected at the time of necropsy and then titrated for virus by $TCID_{50}$ in MDCK cells. Each square represents BALF titers per pig at different time points. A two way ANOVA was used to determine significant differences with virus and time as factors. Dashed line indicates statistically significant differences between groups (* denotes ($P<0.05$) and ** denotes ($P<0.01$).

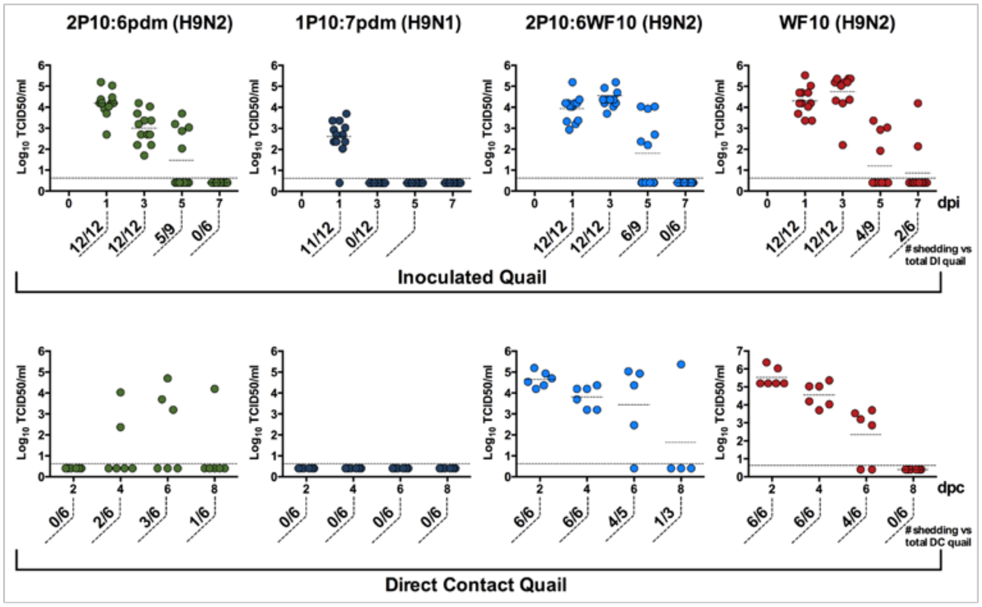

Figure 3.3: H9 reassortant viruses replicate and transmit in quail. Quail (n=12/group) were inoculated (DI) with the viruses indicated and tracheal and cloacal swabs collected at various days post-infection (dpi. The number of DI quail positive for viral shedding in each group vs. the total number of quail is listed under each time point. Direct contact (DC) quail (n=6/group) were introduced in the same pen housing the DI pigs at 1 dpi.. The number of DC quail positive for viral shedding in each group vs. the total number of quail is listed under each time point. Note the DC graphs are measured in days post contact (dpc) not dpi.

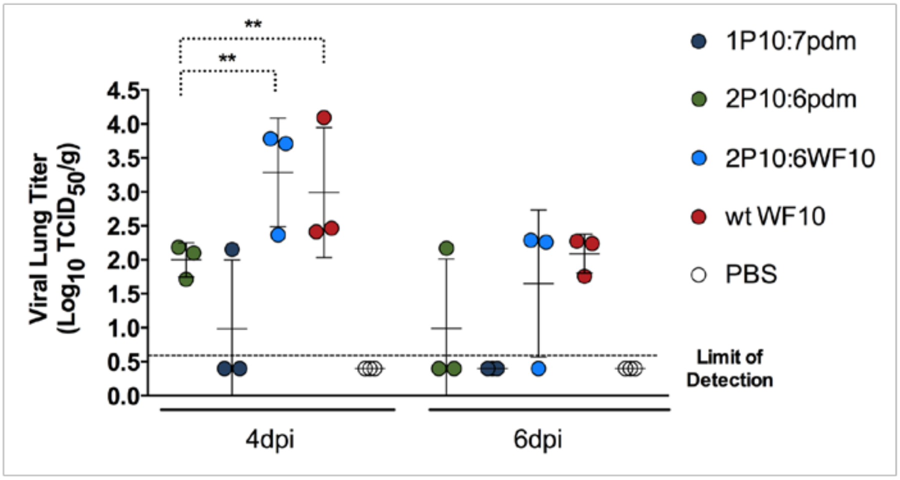

Figure 3.4: Reassortant viruses replicate in quail lungs. On 4 and 6 dpi, 3 DI quail/group were euthanized and lung samples collected for virus titration. Each circle represents lung titers per quail at different time points. A two way ANOVA was used to determine significant differences with virus and time as factors. Dashed line indicates statistically significant differences between groups (* denotes ($P<0.05$) and ** denotes ($P<0.01$).

CHAPTER 4

PLASTICITY OF AMINO ACID 226 IN THE RECEPTOR-BINDING SITE OF AN H9 SUBTYPE INFLUENZA A VIRUS AND ITS EFFECT ON VIRUS REPLICATION, TRANSMISSION, AND TROPISM.[2]

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To be submitted to Plos Pathogens

Abstract

Influenza A viruses (IAVs) remain a significant public health threat causing more than 300,000 hospitalizations in the United states during 2015-2016 season alone. While only a few IAVs of avian origin have been associated with human infections, the ability of these viruses to cause zoonotic infections further increases the public health risk of influenza. Of these, H9N2 viruses in Asia are of particular importance as they have contributed internal gene segments to other emerging zoonotic IAVs. Notably, recent H9N2 viruses have acquired molecular markers that allow for a transition from "avian-like" to "human-like" sialic acid (SA) receptor recognition via a single amino acid change at position 226 (H3 numbering), from glutamine (Q226) to leucine (L226), within the HA's receptor-binding site (RBS). We sought to determine the plasticity of the amino acid 226 and the biological effects of alternative amino acids on variant viruses. We created a library of viruses with the potential of having any of the 20 amino acids at position 226 on a prototypic H9 HA subtype IAV. We isolated H9 variant viruses that carried natural and alternative amino acids at position 226, some of which have yet to be identified in any reported influenza subtype. Interestingly, some identified amino acids conferred a broader receptor-binding profile to resulting viruses. Furthermore, *in vivo* fitness studies revealed some variant amino acids to confer an *in vivo* replication advantage. This study shows the plasticity of position 226 of the HA of H9 influenza viruses and the resulting effect of single amino acid changes on the phenotype of variants *in vivo* and *in vitro*.

Introduction

The hemagglutinin (HA) of influenza A viruses (IAV) plays a central role in the virus' life cycle through its involvement in receptor recognition, virus attachment, and membrane fusion, and virus entry (1). The HA is also the most significant target of neutralizing antibodies during infection. Antigenic differences led to the classification of the HA into 16 subtypes (H1-H16) that are phylogenetically separated into two major groups: Group 1 contains H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16, and group 2 contains H3, H4, H7, H10, H14, and H15. It is widely accepted that wild waterfowl species are the natural hosts of IAVs where these viruses establish an infection in the intestinal tract and spread by the fecal-oral route through the water.

The HA is a single-pass type I transmembrane glycoprotein present as a homotrimer on the virus' surface that extends ~130 Å from the membrane. The HA undergoes N-linked glycosylation at asparagine residues in the classical N-X-S/T consensus sequences. N-linked glycosylation is essential for the protein's structural integrity as well as to mask antigenic sites. Between 3 to 9 potential N-linked glycosylation sites have been described depending on subtype and origin of the virus. Each HA monomer contains two subunits, HA1 and HA2, produced by the cleavage of the inactive precursor HA0. The cleavage of the immature HA0 occurs typically at a single arginine residue by trypsin-like proteases present in the lumen of the intestinal and/or respiratory tract in birds and in the respiratory tract of mammals. Disulfide bonds between the two subunits maintain the integrity of each monomer. Each monomer carries a transmembrane anchor and a small cytoplasmic tail. Three monomers bundle in coiled-coil structures to make the trimers.

The globular head of the HA1 carries a shallow grove, the receptor-binding site (RBS), which is responsible for the receptor recognition function (2-4). On the target cell, the RBS binds glycan structures terminating in N-acetylneuraminic acid, also referred to as sialic acid (SA). In nature, the SAs are linked to the penultimate galactose (Gal) in two major conformations: α2,3SA or α2,6SA. The structure of the RBS is conserved across all HA subtypes and is made up of the 130 loop (residues 135-138), 190 helix (190-198), and 220 loop (221-228) (5). It is well established that small amino acid changes in the RBS as well as differences in the type of SA linkage expressed in different host species are major determinants of the host range and tissue tropism of IAVs. Through a hydrogen-bonded network, the conserved residues Tyrosine 98 (Y98), Tryptophan 153 (W153), Histidine 183 (H183) and Tyrosine 195 (Y195) form the base of the RBS (6). Seminal work by Rogers and Paulson identified that a change at amino acid position 226 from glutamine (Q226) to leucine (L226) within the RBS of the H3 HA subtype altered the SA receptor recognition from an “avian-like” (α2-3SA) to “human-like” (α2-6SA) specificity (7). The Q226L mutation, along with the G228S mutation, played an important role in the emergence of the 1968 Hong Kong H3N2 pandemic influenza virus. In general, the HAs of avian origin IAVs have the Q226 residue allowing for the recognition and attachment α2-3SAs, which are the predominant forms in the avian digestive tract (8, 9). In contrast, the HA of H3 subtype IAVs established in the human population carry L226 at this position (which has transitioned to valine (V226) and, in most recent isolates, to isoleucine (I226)) allowing binding to α2,6SAs found primarily in the respiratory tract of humans (10, 11). The Q226L change and its influence on the SA recognition is not limited to H3 viruses. This phenomenon has been observed with other subtypes such as H2, H4, H5, H7, and H9 (12-14). While the effect of the

Q226L change has been well characterized, the plasticity at this position and the resulting effect on receptor recognition and host adaptation is less understood.

The World Animal Health Organization considers the H9N2 viruses as low pathogenic avian influenza viruses (LPAIVs). Low mortality, coughing, sneezing, and marked decrease in egg production and hatchability characterize H9N2 LPAIV outbreaks. The first description of a LPAIV of the H9N2 subtype dates back to an outbreak in turkeys in February 1966 in northern Wisconsin (U.S.A.)(15-17). H9N2 viruses are endemic in most of Asia, the Middle East and parts of Africa, where they have caused disease outbreaks in chickens, quail, and other minor poultry species (20). H9N2 viruses have been involved in the emergence of zoonotic strains by the contribution of gene segments to, most notably, the goose/Guangdong H5N1 lineage and the Asian-lineage H7N9 and H10N8 viruses, all of which have caused human fatalities (18-21). The H9N2 viruses continue to evolve through intra-subtype and inter-subtype reassortment events. While older avian H9N2 virus isolates have the Q226 residue (216 in the H9 HA sequence), which provides binding to avian-like receptors, the vast majority of H9N2 strains carry L226, which favors human-like receptor-binding (25). It is therefore not surprising that H9N2 viruses are capable of crossing the avian-mammalian host barrier and cause human infections (26-28). The expanding geographical spread and enzootic nature of H9N2 viruses as well as the presence of molecular markers favoring transmission to humans make these viruses of pandemic concern. We sought to determine the flexibility of position 226 (216 H9 numbering) in the context of a prototypic H9 virus of the G1 lineage by perturbing this position using partial PCR based reverse genetics (22). Our study reveals that position 226 is plastic and can accommodate several different amino acids. We identified natural and alternative amino acids at position 226 in the context of H9 HA as well as an amino acid that has yet to be found in any influenza virus subtype. In vivo, however, strong selection towards L226 was observed in quail, highlighting the limitations of in vitro systems to fully recapitulate virus-host interactions in natural hosts. The results provide new insights into the biology of H9N2 influenza viruses and potential avenues for development of live attenuated virus vaccines against the H9 and other influenza virus subtypes.

Materials and methods

Ethics Statement

All animal studies were conducted under Biosafety level 2 (ABSL-2) containment and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia (Protocol: A201506-026-Y3-A5)

Cells

MDCK, 293T and DF1 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum, 1% Antibiotic/Antimycotic (Ab/Am) (Sigma-Aldrich, St. Louis, MO), 1% L-Glutamine and 2.5% HEPES (Sigma-Aldrich, St Louis, MO). Cells were cultured at 37 °C in a humidified incubator under 5% CO_2.

Generation of reverse genetics-ready PCR $_{226}$HA library

The full-length cDNA copy of the HA gene segment of the strain A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10) was split into two overlapping fragments and cloned into two separate plasmids. The plasmid pHA_I contains nucleotides 1-767 of H9 HA gene segment preceded by the 5' end sequence corresponding to the mouse RNA polymerase I terminator (t1). The plasmid pHA_{II} contains nucleotides 738-1742 of the H9 HA segment, immediately upstream of the human RNA polymerase I promoter (pol I) (**Figure 4.1**a). The $_{226}$HA PCR library was generated focusing on the codon encoding amino acid 226 using two different approaches. In the first approach a PCR product was produced from pHA_I using the primer set T1FragFw (5'-

ACCGGAGTACTGGTCGACCGAAGTTGGGGGGGAGCAAAAGCAGG-3’) and WF10-HA713-757$_{nnn}$ (5’-CAATAATAATCAATTCTTCCCTG*NNN*ACCATTGACAAGGGGCC-3’). The resulting *5’-t1*$_{nnn}$*HA757-3’* PCR product included the t1 terminator sequence and the HA fragment with the NNN codon at position 226. In the second approach, an equivalent PCR product, *5’-t1*$_{equi}$*HA757-3’,* was generated from pHA$_{I}$ using the primers T1FragFwd and 50 pmol/µl of a primer mix containing 20 primers designed to introduce every possible amino acid at position 226 (**Table 4.4**). The second half of the HA was amplified from $_{p}$HA$_{II}$ using the set of primers WF10 HA 738-770 (5’-GGAAGAATTGATTATTATTGGTCGGTACTAAAA-3’) and hPol1Rev (5’-ATGCTGACAACGTCCCCGGCCCGGCGCTGCT-3’) to generate the PCR product *5’-HA738pol1-3’* that included the second half of the HA followed by the pol1 promoter sequence. The full-length reverse genetics-ready HA PCR libraries, $_{nnn226}$H9PCR and $_{equi226}$H9PCR, were obtained by overlapping PCR using either the *5’-t1*$_{nnn}$*HA757-3’* or *5’-t1*$_{equi}$*HA757-3’* PCR fragments along with the *5’-HA738pol1-3’* PCR fragment and the primer pair T1FragFwd and hPol1Rev**.** The 50 µl PCR reaction contained 10 ng of each PCR product, 25 µl of the PCR Master mix, 50 pmol/µl of each primer and 1.5µl DMSO. PCR amplification was done using Phusion high-fidelity PCR master mix with GC Buffer (New England Biolabs, Ipswich, MA) under the following cycling parameters: 98ºC 30 sec, (98ºC for 8 sec, 56ºC for 1min and 72ºC for 3 min) x 30 cycles, and 72ºC for 10 min.

Generation of $_{nnn226}$***H9N2,*** $_{nnn226}$***H9N1,*** $_{equi226}$***H9N2, and*** $_{equi226}$***H9N1 virus libraries***

Independent virus rescue experiments were performed with the $_{nnn226}$H9PCR and $_{equi226}$H9PCR amplicon libraries, as previously described (22). To generate the $_{nnn226}$H9N2 and $_{equi226}$H9N2 virus libraries, the corresponding PCR amplicons were co-transfected along 7 reverse genetics plasmids encoding the rest of the WF10 genome **(Figure 4.1b).** To generate the $_{nnn226}$H9N1 and

$_{\text{equi226}}$H9N1virus libraries, the PCR amplicons were co-transfected along 7 reverse genetics plasmids encoding the PB2, PB1, PA, NP, NA, M, and NS gene segments of the laboratory-adapted strain A/Puerto Rico/8/1934 (H1N1) (PR8) **(Figure 4.1b)**. For transfection, co-cultures of MDCK and 293T cells were seeded in each well of a 6 well plate overnight at 37ºC. The following day, 1µg of each of the 7 plasmids of either WF10 or PR8 and 1µg of PCR amplicon was mixed with 16 µl of TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI) and incubated for 45 min. After 45 min, the MDCK/293T cells were overlaid with transfection mixture and incubated at 37ºC for 24 h. At 24 h post transfection (hpt), the transfection mixture was replaced with fresh OptiMEM media containing 1 µg/ml of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemicals, Lakewood, NJ). Supernatants containing rescued viruses were collected at 96 hpt.

Isolation and identification of individual virus variants and growth of virus stocks

Single virus variants (*var*) in the virus libraries were isolated by limiting dilution assays as previously described (23). Briefly, MDCK cells (2x10^4 cells/well) in a 96 well plate were infected with 8 serial 10-fold dilution of the rescued virus library in OptiMEM media containing 1µg/ml of TPCK-trypsin (Worthington Biochemicals, Lakewood, NJ). The *var* strains were produced starting from the $_{\text{nnn226}}$H9N2 virus library (n=12), the $_{\text{nnn226}}$H9N1 virus library (n=12), the $_{\text{equi226}}$H9N2 virus library (n=24), and the $_{\text{equi226}}$H9N1 virus library (n=24). After 72 h incubation at 37ºC, *var* virus supernatants were collected from wells infected with the most diluted sample displaying the cytopathic effect (CPE). This process was repeated twice, followed by Sanger sequencing to determine the amino acid at position 226 in HA. A third round of limiting dilution was carried out for samples yet to resolve at position 226 after the second limiting dilution. The *var* viruses were further expanded in MDCK cells and stocks aliquoted and stored at -80ºC until used. The *var* virus stocks were titrated by 50% tissue culture infectious dose ($TCID_{50}$) in MDCK cells and titers determined by the Reed-Muench method (24).

Generation of Quail antisera

Three-week-old Japanese quails (*Cortunix japonica*) were infected with 10^6 $TCID_{50}$ of WF10 or the M226 *var* virus. Quails were then boosted with the respective virus containing 1:1 (v/v) of Montanide as an adjuvant at 14 dpi. Quails were bled for serum collection at 14 days post boost and antisera collected used in HI assays.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) assays

Standard HA assays were performed using either 1% horse red blood cells (RBCs), 0.5% chicken RBCs or 0.5% turkey RBCs and expressed in HA units (HAU) as previously described (25). HI assays were performed as previously described (33) using the H9 *var* viruses as antigens and quail anti-sera generated against the WF10 (H9N2) virus and the M226 *var* virus.

Preparation of viral RNA and cDNA

vRNA and cDNA from *var* viruses was prepared as previously described (22). Total RNA was extracted from *var* viruses using RNeasy Kit (Qiagen, Valencia, CA) following manufacturer's protocol. To obtain cDNA, reverse transcription was carried out using the Avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and Uni12 primer (5'-AGCAAAAGCAAGG-3').

In vitro growth kinetics assays

MDCK and DF1 cells were infected with wt and *var* H9 viruses at an MOI of 0.01 in 6 well plates for 15 min at 4ºC and 45 min at 37ºC. Two independent growth kinetics experiments were carried out and infections were carried out in triplicates for all viruses. Following 3 washes with Phosphate buffered saline (PBS) to remove any unbound virus, MDCK cells were overlaid with 2 ml OptiMEM media containing Ab/Am (Sigma-Aldrich, St. Louis, MO) and 1µg/ml TPCK-trypsin. For infected DF1 cells, TPCK-trypsin was replaced with 5% allantoic fluid. At 6, 12, 24, 48, and 72 h post-infection (hpi), supernatant was collected and stored at -80ºC until viral titration in MDCK cells. Viral titer was determined by $TCID_{50}$ using the Reed and Muench method (24).

Solid phase binding assays

The receptor-binding specificity was determined using two versions of the solid phase direct binding assay as previously described (12, 26). Briefly, ELISA 96 well plates coated with Fetuin were incubated overnight at 4ºC with 128 HAU of purified *var* virus in 50µl. Next, plates were blocked with PBS containing 0.1% neuraminidase-treated Bovine serum albumin (BSA-NA) for 2 h at room temp. For the first version of the assay, following 3 washes with ice cold PBS + 0.01% Tween 80 washing buffer (WB), wells were incubated at 4ºC for 1 h with 50 µl of 2-fold serial dilution of 3'SLN or 6' SLN high molecular weight biotinylated sialyglycopolymer (Glycotek, Gaithersburg MD) in reaction solution (RS) (PBS containing 0.02% Tween-80, 0.1% BSA-NA and 2 µM oseltamivir carboxylate). Subsequently, wells were washed 5 times with WB and incubated with a 1:1000 dilution of Streptavidin-HRP (ThermoFisher) for 1hr at 4ºC Alternatively, for the second version of the assay, wells were incubated with 50 µl of 3-Fet-HRP and 6-Fet-HRP in RS for 1hr at 4ºC. After incubation, plates were washed 5 times with WB and the wells replenished with 100 µl of freshly prepared substrate solution added (0.1% TMB in 0.05M sodium acetate and H_20_2). The reaction was stopped with 3% H_2SO_4 after 30 min unless otherwise stated. Absorbance readings obtained at 450 nm using a Victor X3 multilabel plate reader (PerkinElmer, Waltham, MA).

Glycan array analysis

Glycan array analysis was performed using an NHS ester-coated glass microarray slide containing six replicates of 128 diverse sialic acid-containing glycans, including terminal sequences and intact N-linked and O-linked glycans found on mammalian and avian glycoproteins and glycolipids. Whole H9 influenza virus samples where diluted to 256 HAU (final) in PBS containing 3% BSA and incubated on the array surface for one hour at room temperature in a humidity-controlled chamber. After one hour, slides were washed in PBS and incubated with a virus-specific anti-H9 mouse antibody (derived from mouse ascitic fluid) diluted 1:200 in PBS, 3%BSA for a further hour. Slides were washed in PBS and incubated for a final hour in Goat anti-Mouse IgG-Alexa Flour Plus 488 (A32723, Thermo Fisher Scientific; 10 ug/ml final) diluted in PBS, 3% BSA. Slides were washed twice in PBS, and in dH2O, then dried prior to detection. Slide scanning to detect bound virus was conducted using an Innoscan1100AL (Innopsys) fluorescent microarray scanner. Fluorescent signal intensity was measured using Mapix (Innopsys) and mean intensity minus mean background of 4 replicate spots was calculated.

Cell avidity assays

The cell avidity assay was performed as previously described (27, 28). Chicken RBCs were treated for 1 h at 37ºC with serial dilutions of neuraminidase from *Clostridium perfringes* (Sigma-Aldrich, St Louis, MO) to remove sialic acids. RBCs were washed twice with PBS and resuspended into a 1% solution with PBS. Then 50µl of 1% RBCs were incubated with 50 µl of virus (8 HAU) at room temperature for 45 min to determine the HA titer.

Virus Histochemistry with avian tracheal and mouse lung tissues

Tracheal tissues from influenza virus negative 4-6 week old Japanese quail, 6 week old broad breasted white turkeys (*Meleagris gallopavo*), 6 week old chickens (*Gallus gallus*) and lung tissue from 5-6 week old female DBA2J mice (The Jackson Laboratory, Bar Harbor, ME) were used for virus histochemical studies. Virus histochemistry was performed as previously described using fluorescein isothiocyanate (FITC) labeled viruses (29, 30). Briefly, tissue culture grown *var* viruses were clarified by low speed centrifugation. Following clarification, viruses were concentrated by centrifugation at 28000 rpm for 2 hr at 4°C and resuspended in PBS. Concentrated viruses were labeled with FITC by mixing equal volume of virus and 0.1mg/ml of freshly prepared FITC in 0.5 M bicarbonate buffer at pH 9.5. Formalin fixed, paraffin embedded tissues were deparaffinized using xylene rehydrated with graded alcohol and subsequently incubated with FITC-labeled viruses (100 HAU/50 µl) at 4ºC overnight in a humidified chamber. Following washing with 0.2M Tris-HCl, 0.1M NaCl, 0.5% Tween20 (TNT) buffer, FITC was detected using a peroxidase labeled rabbit anti FITC antibody (Dako). To enhance detection, signal was amplified using a Tyramide Signal Amplification system (Perkin Elmer, Akron, OH) according to manufacturers’ protocol. 3-amino-9-ethyl-carbazole (AEC) (Sigma-Aldrich, St.

Louis, MO) was used to reveal the peroxidase and counter staining was done with Mayer Hematoxylin. Viral attachment was seen as red staining on the surface.

Virus thermal stability assays

Var virus stocks were diluted to 128 HAU/50 μl and then heat-treated on a heat block at 56°C for various times (0, 15, 30, 60, 120, 180, and 240 min). Subsequently, HA assays were carried out on heat-treated viruses in quadruplicate.

Ex-vivo *growth kinetics*

Ex-vivo chicken lung and tracheal explants were used to assess variant virus replication. 6-week-old SPF chickens free of antibodies to influenza were euthanized following deep sedation with Isoflurane (Akron Inc., Lake Forest IL) and cervical dislocation. Full-length trachea and whole lungs were collected and washed in PBS containing Ab/Am (PBS+Ab/Am) to remove debris. Biopsy cores were made from the lungs using a disposable biopsy punch (Integra York PA Inc., York, PA) while trachea was cut into ~2mm rings using a scalpel. Tracheal rings were placed in tracheal explant media containing 1:1 DMEM media (Sigma-Aldrich, St. Louis, MO) and RPMI media (Life technologies, Grand Island, NY), Ab/Am (Sigma-Aldrich, St. Louis, MO), 0.1mg/ml Gentamicin (Sigma-Aldrich, St. Louis, MO) and 0.3ng/ml Glutamine (Sigma-Aldrich, St. Louis, MO) until infected. Lung cores were placed in lung explant media containing Medium 199 (Life technologies, Grand Island, NY), Ab/Am (Sigma-Aldrich, St. Louis, MO), 0.1mg/ml Gentamicin (Sigma-Aldrich, St. Louis, MO), Vitamin supplement (ATCC, Manassas, VA), ITS Liquid media supplement (Sigma-Aldrich, St. Louis, MO) and 0.5ug/ml Hydrocortisone (Sigma-Aldrich, St. Louis, MO) until infected. Infection of the tracheal and lungs were carried out by incubating explant samples in $1x10^6$ $TCID_{50}$/ml for 1hr at 37°C followed by 3 washes in PBS+Ab/Am. After washing, each explant was placed on a sterile gauze plug in a 12 well plate containing 2 ml of the appropriate explant media without submerging the explant to create an air-liquid interface. Plates of explant replicates were maintained at 37°C in a 5% CO_2 incubator for 72 hours. To assess replication kinetics, 200µl of media was collected at 0, 12, 24, 48 and 72 hpi and stored at -80°C until used in viral titrations. Media was replaced following each collection to maintain initial volume.

***In vivo* competition study**

Four groups of 4-5-week-old Japanese quail (n=6/group) were used. Quail eggs obtained from the College of Veterinary Medicine University of Georgia were hatched at the Poultry Diagnostic and Research Center, University of Georgia. A week before virus inoculation, quails were bled and confirmed seronegative for IAV exposure. Quails were inoculated with 1 ml of virus mix (0.25 ml administered via the trachea and nares and 0.5 ml via the cloaca) containing a homogenous mixture of variant viruses with or without Leucine or Glutamine virus. The first group of 6 birds served as the negative control receiving 1ml PBS through the same routes (Group 1-PBS). In group 2 (*var*ΔLQ), birds were inoculated with 10^6TCID$_{50}$ virus mix containing the following 10 *var* viruses on the WF10 backbone: I226, S226, T226, M226, H226, N226, F226, V226, C226 and G226 viruses. Group 3 (*var*+Q) birds received all 10 *var* viruses and the Q226 virus while group 4 birds (*var*+L) were infected with the 10 *var* viruses and the L226 (WF10) virus. Group 5 (*var*+LQ) birds were infected with a mix of all 10 *var* viruses along with the L226 and Q226 viruses (*var*+LQ). One day post infection (dpi), naïve quail (n=6/group) were introduced as direct contact quail to determine transmission. At 5dpi and 6dpc, 3 quails were randomly selected from each directly inoculated and contact group and sacrificed for virus titration in tissues. Tracheal and cloacal swabs were collected daily from each bird until

14 dpi. Swabs were suspended in 1 ml 3.7% Brain Heart infusion media (BHI) (Becton Dickinson, Sparks, MD) containing 10,000 U Penicillin, 10 mg Streptomycin and 25 µL Amphotericin B and stored at -80ºC until used in virus titrations.

Quantification of virus shedding in quail samples

Virus RNA was isolated from tracheal swab samples using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions. RNA was eluted in 50 µl of nuclease-free molecular grade water. One step quantitative polymerase chain reaction (qPCR) based on the avian influenza matrix gene as surrogate of virus shedding was carried out using the primers/probe set previously described (31). The qPCR was performed in a LightCycler 480 Real Time PCR instrument (Roche Diagnostics, Rotkreuz, Switzerland) using the LightCycler 480 RNA Master Hydrolysis Probes kit (Roche Life Science, Mannheim, Germany) in a final reaction volume of 20 µl. Each reaction contained 1X LightCycler 480 Probes Master mix, 0.5 µM of forward and reverse primers, 0.3 µM of probe and 5 µl of RNA. The qPCR cycling conditions ran at 61°C for 10 min, a denaturation step of 95°C for 30 sec, followed by 45 cycles of amplification at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 1 sec, with a final cooling step at 40°C for 10 s. A standard curve was generated using 10-fold serial dilutions from a virus stock of A/guinea fowl/Hong Kong/WF10/1999 (H9N2) of known titer to correlate qPCR crossing point (Cp) values with the amount of virus shedding from each bird, as previously described (32).

Sequencing

Standard Sanger sequencing was performed on the full length ${}_{nnn226}$HA and ${}_{equi226}$HA PCR product prior to transfection and on HA from all *var* virus stocks. Sequences were generated using specific primers, Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Calsbad, CA) and a 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions. RNA isolated from *var* virus stocks were used to amplify the *var* viruses whole genome by one-step reverse transcription PCR (RT-PCR) and subjected to whole genome sequencing using the Illumina Miseq platform for NGS.

Database analysis of HA sequences

For molecular analyses, all H9 subtype HA sequences, as well as all H2, H4, H6, H8, H10-H16 and a subset of H1, H3, H5, and H7 HA subtypes were obtained from the Influenza Research Database (IRD) (33) through the web site at http://www.fludb.org.

Statistical analysis

All data analyses were performed using GraphPad Prism Software Version 7 (GraphPad Software Inc., San Diego, CA). For multiple comparisons, either one-way or two-way analysis of variance (ANOVA) was performed followed by a post-hoc Tukey test. Differences in survival curves were analyzed using the log-rank test. A *p* value below 0.05 ($P<0.05$) was considered significant.

Results

Position 226 of H9 HA is flexible

To determine the plasticity of amino acids at position 226, two reverse genetics-ready PCR libraries of the H9 HA gene segment were generated: the first one containing a degenerate codon at position 226 and the second one produced with an equimolar mixture of 20 primers capable of introducing every possible amino acid at this position. The PCR libraries were subsequently used to generate four virus variant libraries in two subtype combinations: Two paired with the N2 NA gene segment in the background of the WF10 virus ($_{nnn226}$H9N2 and $_{equi226}$H9N2 virus libraries) and two paired the N1 NA gene segment in the background of the laboratory-adapted strain PR8 (${}_{\text{nnn226}}$H9N1 and ${}_{\text{equi226}}$H9N1 virus libraries). Following, limiting dilution in MDCK cells, partial sequencing of the HA gene segment revealed the presence of natural and alternative amino acids at position 226. Six variants were produced from the ${}_{\text{nnn226}}$H9N2 library after analysis of 12 individual virus clones. The most common variants identified among this group were asparagine (N226, n=4), and alanine (A226, n=3). Other variants included histidine (H226, n=2), isoleucine (I226, n=1), serine (S226, n=1) and methionine (M226, n=1) (**Figure 4.2a**). Interestingly, none of the variants generated using this approach contained the most common amino acids found in nature, either L226 or Q226. In the context of the ${}_{\text{nnn226}}$H9N1 virus library, only 4 variants were obtained by limiting dilution out of 12 independent virus clones. Notably, the naturally occurring amino acids Q226 (n=4) and L226 (n=5) were the most commonly isolated variants from the ${}_{\text{nnn226}}$H9N1 virus library, and the remaining 2 variants included valine (V226, n=2) and arginine (R226, n=1) (**Figure 4.2a**).

Given that the codon usage for amino acids differ and the probability that the presence of an amino acid in a virus variant may stem from increased codon usage of that amino acid (6 codons code for leucine compared to 1 codon for methionine), we proceeded with an alternative approach to give each amino acid the same representation in the library. Analysis of 24 individual virus clones produced from the ${}_{\text{equi226}}$H9N2 virus library revealed that N226 (n=8) was the most commonly selected amino acid as was seen with clones obtained from the ${}_{\text{nnn226}}$H9N2 virus library. Also consistent with results using the ${}_{\text{nnn226}}$H9N2 virus library, clones produced from the ${}_{\text{equi226}}$H9N2 virus library contained H226 (n=6), I226 (n=1), M226 (n=1), and S226 (n=1). In addition, clone selection from the ${}_{\text{equi226}}$H9N2 virus library resulted in clones with threonine (T226, n=2), phenylalanine (F226, n=2), cysteine (C226, n=1), and V226 (n=1). Once again, L226 and Q226 viruses were absent after clonal selection of the $_{equi226}$H9N2 virus library (**Figure 4.2a**). It must be noted that the absence of these two variants is not due to a misrepresentation of the corresponding codons in the preparation of the virus library or a defect in virus rescue. Both of these viruses were independently recovered in the H9N2 background using PCR reverse genetics and the corresponding primer set indicating that they are viable, as expected (13). On the $_{equi226}$H9N1 virus library background 9 different variants resulted from 24 clones with L226 (n=7) being the most frequent. Other variants had H226 (n=5), N226 (n=4), V226 (n=2) while Q226, T226, Y226, S226 and F226 were found in only one variant each (**Figure 4.2a).**

Limited effects in hemagglutination activity by 226 var viruses

We assessed the ability of *var* viruses to agglutinate red blood cells from different species using stocks grown in MDCK cells. Turkey and chicken red blood cells (RBCs) have both α2,3SAs and α2,6SAs while horse RBCs have predominantly α2,3SAs (34, 35). In general, HA titers of *var* viruses were similar or within 1-fold for all viruses tested (**Table 4.1**). A difference in agglutinating ability however, was observed with the horse RBCs. While most H9N2 and H9N1 *var* viruses and the WF10 (L226) virus were unable to agglutinate horse RBCs, the variants with Q226, N226, and Y226 agglutinated to varying degrees suggesting that these viruses have the ability to recognize α2,3SAs.

Position 226 does not impact the antigenicity of the H9 HA protein

Amino acid changes around the receptor-binding site are known to contribute to the antigenic drift of resulting viruses (36, 37). The L226Q mutation has previously been identified in H9N2 monoclonal antibody escape mutants (38). To evaluate if other amino acids can alter the antigenicity of variant viruses we carried out HI assays using quail antisera against the WF10 and the M226 *var* viruses (**Table 4.1**). For all *var* viruses tested, the HI titers were the same or within 1-fold of the homologous HI titer. The lowest HI titer observed was with the Q226 virus, which had a titer 2-fold less that obtained for the WF10 virus.

Receptor-binding avidity is decreased for var viruses

To investigate receptor-binding avidity, we carried out HA assays using chicken RBCs treated with varying concentrations of neuraminidase. As shown in **Figure 4.2b**, all *var* viruses had lower receptor avidity compared to the WF10. Of note, the *var* viruses with the 2 most commonly found amino acids at position 226 i.e. Q and L, had receptor-binding avidity similar to the WF10 virus. Though the avidity for receptors for most variants was lower than that of the WF10 virus, the avidity was similar to the PR8 virus with the exception of C226 (H9N2), H226 (H9N1) and T226 (H9N1) *var* viruses, which had greatly reduced receptor avidity.

Var viruses retain replicative fitness in cells of avian and mammalian origin

To investigate the contribution of the various amino acids to viral replication, we evaluated and compared the growth kinetics of *var* viruses to WF10 and PR8 viruses in both mammalian and avian cell lines. Confluent canine-origin MDCK or chicken-origin DF1 cells were infected at an MOI of 0.01 and supernatants collected at different times post-infection. *Var* viruses exhibited diverse replication profiles in MDCK and DF1 cells compared to L226 wtWF10 virus (**Figure 4.3**). At 24hpi of MDCK cells, all H9N2 *var* viruses replicated to titers similar to the L226 virus with the exception of the I226 ($P < 0.0001$), M226 ($P < 0.05$) and V226 ($P < 0.05$) that grew to significantly less titers while the Q226 virus grew to higher titers compared to the L226 virus (**Figure 4.3a**). At 72hpi, G226 and Q226 replicated to higher titers compared to L226 ($P < 0.001$

and $P < 0.0001$ respectively). The I226 replicated at consistently lower titers during the course of the experiments ($P < 0.0001$) both at 48 and 72hpi. Similar to kinetics in MDCK, I226 replicated to lower titers at every time point except at 24hpi and Q226 replicated to higher titers than other variants tested in the DF1 cells (**Figure 4.3c**). By 72hpi, Q226 and G226 replicated better than L226 as observed in MDCK ($P < 0.0001$ and $P < 0.01$ respectively). S226 on the other hand grew to titer similar to I226 at 72hpi while there was no difference between the L226 and other *var* viruses at 72hpi. In MDCK cells, all H9N1 *var* viruses replicated to less titers compared to wtPR8 at 48hpi and by 72hpi there was no significant differences between *var* viruses and wtPR8 except for L226 ($P < 0.0001$), N226 ($P < 0.001$) and F226, S226 and H226 ($P < 0.05$) that grew to lower titers (**Figure 4.3b**). In DF1 cells, wtPR8 grew to significantly lower titers compared to the H9N1 *var* viruses between 24 and 72hpi (**Figure 4.3d**).

Temperature stability profile of var viruses is dependent on amino acid at 226

We used a temperature stability assay as a surrogate to determine if the single amino acid changes at position 226 can contribute to pH stability. Interestingly, we observed differences depending on which amino acid was present at position 226. By 1 hpi at 56ºC, all H9N2 viruses had HA titers less than 2 HAU, except viruses with aliphatic amino acids, V226, L226 and I226, as well as the C226 and Q226 *var* viruses (**Figure 4.3e**). At 4 hpi only the viruses with V226 and L226 showed any HA titer and were the most stable viruses. For H9N1 variants, at 1 hpi, the Q226, V226 and L226 *var* viruses had titers ≥ 2 HAU as well at the PR8 virus. By 2 hpi, only V226, Q226 and the PR8 viruses had detectable HA titers (**Figure 4.3f**).

Sialic acid specificity modulated by amino acid 226

We performed solid phase direct binding assays using 3-Fet-HRP or 6-Fet-HRP to test the specificity of *var* viruses for α2,3SA or α2,6SA receptors. The WF10 (L226) virus bound strictly to 6-Fet-HRP indicating a preference for α2,6SA. In contrast, the Q226 *var* virus bound exclusively to 3-Fet-HRP, consistent with previous observations (**Figure 4.4a-left panel**)(13). The M226, N226 and V226 *var* viruses on the WF10 backbone displayed dual α2,3SA and α2,6SA preference while the C226 *var* virus appears to bind exclusively to α2,3SA receptors (**Figure 4.4b-c**: **left panel**). Due to low avidity for receptors, it was not possible to clearly distinguish receptor specificity for the other variants using the Fet-HRP assay (**Figure 4.4b-c**: **left panel**). To define the specificity for the low avidity viruses as well as confirm the dual specificity observed for some *var* viruses with the Fet-HRP assay, we used a modified version of the assay using synthetic high molecular weight biotinylated macromolecular probes having multiple copies of monospecific sialyoligosaccharide moieties (39). As expected, the L226 and Q226 viruses still bound mainly 6SLN-PAA-biot and 3SLN-PAA-biot respectively (**Figure 4.4a: middle panel**). C226, S226, T226 and H226 *var* viruses bound more to the 3SLN-PAA-biot moiety (**Figure 4.4c**: **middle panel)**. Variants that displayed dual receptor specificity in the Fet-HRP assay also show the same behavior in this assay. In addition to the M226, V226 and N226 *var* viruses, the variants with G226, I226 and F226 also exhibited preference for both 3SLN and 6SLN sugars (**Figure 4.4a-b**: **middle panel**). While biochemical characterization of variants was limited to variants identified using the equi226 approach to exclude bias in variants generated, we tested the receptor specificity of the A226 and R226 variants, 2 amino acid residues not identified via the equi226 approach (**Figure 4.8**). Both viruses displayed a preference for α2,3SA in the solid phase binding assay (**Figure 4.8**). Interestingly, while the binding profile of the A226 virus on the glycan microarray was predominantly α2,3, the R226 virus displayed lack of binding on the glycan microarray (**Figure 4.8**).

To further corroborate the results from the solid phase binding assay, we tested the receptor specificity of variant viruses on a custom glycan microarray with linear, O-linked, and N-linked sialosides. L226 and Q226 variants exhibited preferences same as has been previously reported and similar to our findings with the solid phase binding assay (40, 41). L226 virus bound almost all α2,6SA on the array resulting in fewer gaps and a less 'comb-like' appearance (**Figure 4.4a**: **right panel)**. C226, S226, T226 and H226 viruses bound exclusively to α2,3SA on the glycan array also corroborating the solid phase binding data (**Figure 4.4c**: **right panel)**. We did observe limited binding of these variants to some α2,6 N-linked sialosides with 4-5 LacNac repeat with the exception of H226 where binding was limited to only the α2,3SA. Also, while Q226, C226 and S226 bound the fucosylated sugars on the array, this was more limited with H226 and T226 variants (**Figure 4.4c**: **right panel)**. Preference for the M226 variant was restricted to only the N-linked subset of the α2,6SA terminating sialosides (**Figure 4.4a**: **right panel)**. This was in contrast to the solid phase data where M226 bound both 3SLN and 6SLN sugars equally well **Figure 4.4a**: **left and middle panel)**. The I226 and V226 viruses bound α2,6SA in linear as well as O-and N-linked conformations (**Figure 4.4b**: **right panel)**. A more expanded binding phenotype was seen with the N226, G226 and F226 viruses (**Figure 4.4a-b**: **right panel)**. These 3 variants bound linear and N-linked α2,3SA with variable and low binding to O-lined α2,3SA. Further inspection showed that the F226 virus bound fewer linear α2,3SA and no interaction with the L^x α2,3SA unlike the G226 and N226 viruses (**Figure 4.4b**: **right panel)**. In the α2,6SA conformation, F226 did not bind linear sugars and showed a preference for longer branched sugars. The binding phenotype for the N226 and G226 were both similar in appearance.

A subset of the *var* viruses were further tested on an expanded glycan microarray for comparison. Unlike the previous array with only 135 sugars, the CFG array v5.3 consists of 609 sugars. The binding profile of the N226 from the CFG microarray was similar to that from the Scripps microarray though the binding of virus to α2,3SA was more pronounced in the CFG array (**Figure 4.9**). We also observed that the binding profile was similar irrespective of the origin of the neuraminidase and internal genes when we compared the H9N2 and H9N1 *var* viruses with N226 (**Figure 4.9**). With the L226 virus, a preference for α2,6SA was apparent on both glycan microarrays tested. We did observe more α2,3SA binding on the CFG array for the subset of viruses tested (**Figure 4.9**). Overall, the receptor binding profile of viruses tested were comparable irrespective of the source of the microarray used in the experiments.

Intensity and pattern of virus attachment differs with amino acid at position 226 in avian and mammalian tissues:

The pattern of influenza virus attachment (PVA) in the respiratory tract is an indicator of the virus tropism, which can determine the efficiency of viral replication and transmission (42). Viral attachment pattern of *var* viruses was determined using virus histochemistry comparing tracheal tissues obtained from Japanese quail, turkeys, chickens and mice (**Table 4.3**). Several distinct patterns of attachment were observed with the quail tracheal tissue. In general, the PVA of variant viruses can be classified as intense, moderate or mild. The N226, F226, Q226 *var* viruses and the WF10 (L226) attached strongly to the trachea compared to the M226 variant which had moderate binding. The H226, I226, V226, S226, G226 and T226 variants bound with mild intensity to the quail trachea tissue. Attachment of the T226 variant was restricted to the basal structures of the tracheal epithelium (**Figure 4.5**). I226 and H226 attached mildly in punctate and discrete areas near the base of the cilia, unlike the M226 variant with intense staining restricted to the base of the ciliated tracheal epithelium (**Figure 4.5**). For the S226 variant, though binding to the cilia was moderate, the basal membrane of the cilia was intensely stained. Similar attachment patterns were observed for the N226, F226 and L226 viruses displaying abundant attachment to the cilia as well as moderate binding to the basement membrane of the trachea, which matches the pattern of α2,6SA receptor expression in quail (43). The G226 virus bound in a similar pattern but with reduced intensity. The most distinct pattern was observed for the Q226 virus, which attached abundantly to discrete areas on the ciliated epithelium along with strong staining of the goblet cells. The Q226 staining pattern matches the pattern of α2,3SAs staining in quail trachea (43).

We also tested these *var* viruses in turkey tracheal tissues based on the historical infections of turkeys with H9 viruses particularly in North America. The pattern of virus attachment in turkey tracheal tissues was somewhat different compared to quail for some variants and similar for others. It has been previously reported that the pattern of receptor expression in the respiratory tract of turkey shows noticeable α2,3SA presence at all ages but increases in α2,6SA expression as birds get older (≥4 weeks old) (44). V226, N226, and Q226 variants bound with greater intensity compared to other variants. H226, G226, S226, Y226 and L226 had moderate binding intensity while T226, I226, M226, F226 and C226 were the least with regards to binding intensity (**Figure 4.5**). As observed in the quail, the I226 virus attached very poorly with few discrete areas of binding on the ciliated cells of the tracheal epithelium of the turkey. An increased binding intensity but similar pattern to the I226 was seen with the M226 virus. Binding was mainly restricted to the epithelial surface of the trachea for all variants except the F226, G226, N226, and H226 viruses. The distinct pattern observed with the Q226 virus in the quail tissue was also seen in the turkey tracheal tissue where binding was well defined to the ciliated epithelium and the goblet cells (**Figure 4.5)**

In chicken tracheal tissues, the N226 and Q226 viruses bound with greatest intensity similar to observations in the quail and turkey tissues and consistent with the pattern of expression of α2,3SA and α2,6SA receptors in the trachea of chickens (43). The characteristic intense staining of the goblet cells with the Q226 virus was also observed in the chicken trachea tissue. Variants with H226, G226, S226, C226 and L226 bound with moderate intensity while T226, M226, V226 and Y226 had minimal binding. An apparent lack of binding was observed with the I226 and F226 *var* in chicken tracheal tissues despite intense staining of the basement membranes by the F226 virus. As seen in the turkey, staining of the basement membranes was associated with the N226, L226, S226, T226 and G226 viruses (**Figure 4.5**).

We further compared the virus binding characteristics of *var* viruses in a mammalian model of influenza virus infection. Mice are often used to study influenza virus replication and pathogenesis and are considered to have mainly 2,3SA in the lower respiratory tract thus we sought to determine how the *var* viruses would behave using mouse tissue. No binding was observed with I226, F226, V226 and G226 viruses. Binding to the alveoli and bronchiolar mucosa was mild for the S226, T226, M226, H226, C226 and L226 (wt). Binding profile of Q226 var virus was intense, similar to observations for all tissues tested in this study while N226 and Y226 bound moderately to the alveoli (**Figure 4.5**)

Poor replication of viruses in **ex-vivo** *avian cultures*

We tested the replication of the isolated variants in ex-vivo avian explants using chicken tracheal and lung tissues. The highest average virus titers of about 10^3 $TCID_{50}$ was reached at 48hpi in both lungs and tracheal tissues (**Figure 4.6a-b**). For viruses on the WF10 backbone in both tissue types, N226, F226, H226 and M226 variant viruses replicated to the highest titers. We also observed some tissue dependent differences for some variants on this backbone. I226 and G226 variants titers in both lung and tracheal tissues were below the limit of detection by $TCID_{50}$, C226 and V226 replicated better in the lungs compared to the trachea where virus titer could not be detected. The wtWF10 virus replicated poorly in the tracheal explants and had delayed replication in the lung explants with a high titer evident at 72hpi compared to other variants. For variants on the PR8 backbone, the N226 and F226 replicated to the highest titers in both trachea and lungs similar to observations with these variants on WF10 backbone (**Figure 4.6c-d**). The Q226 variant behaved similar to the N226 and F226 virus in the lungs while the L226 and T226 replicated to levels similar to the N226 variant in the trachea.

In-vivo replication advantage of H9 variants

To investigate the competitive replication and/or transmission advantage conferred by specific amino acids, we inoculated quail with mixtures of the *var* viruses on the H9N2 backbone with or without the commonly found L226 and Q226 residues. At 1 dpi, we introduced naïve quail (n=6/group) to monitor transmission. Quail are known to replicate and transmit H9 viruses and possess both α2,3SA and α2,6SA receptors in their respiratory tract. Quail were randomly assigned to 5 groups (n=6/group). Birds in group 1 (PBS) received PBS and those in group 2 received a mix of the H9N2 *var* viruses only without L226 and Q226 (*var*ΔLQ). Birds in group 3 (*var*+Q) received the H9N2 *var* virus mix along with the Q226 *var* virus, group 4 (*var*+L) birds received a similar mix with the L226 virus while a final group 5 (*var*+LQ) received a mix of all the H9N2 variants and both the L226 and Q226 viruses. Over the course of 14 days, infected birds displayed minimal signs of infection as has been previously reported. No shedding was observed in the PBS group during the duration of this study (data not shown). By 3 dpi, all inoculated birds were positive with all groups shedding similar levels of virus (10^6 TCID50/ml equivalent) (**Figure 4.7a)**. Virus titers reduced by 7dpi with quails shedding an average of 10^3 in the *var*+Q and *var*+L groups. In the *var*+LQ group, 2 birds shed between 10^4 and 10^6 $TCID_{50}$ while the last bird in the group had titers similar to birds in the *var*+Q and *var*+L groups. Only 2 birds in the *var*ΔLQ group were positive for virus by 7dpi with one bird shedding up to 10^6 $TCID_{50}$ (**Figure 4.7a**). No significant differences in virus titers were observed among the different groups of inoculated birds. Direct contact quail in the *var*ΔLQ group showed delayed replication kinetics. While direct contact quail in other groups shed an average of 10^6 $TCID_{50}$ equivalent by 2 dpc, the average virus titer in tracheal swabs of *var*ΔLQ direct contact birds was significantly less that for all other contact groups ($P < 0.001$) (**Figure 4.7b**). By 7 dpi (6 dpc), the average virus titer shed by birds in the *var*ΔLQ group was significantly higher than other groups ($P<0.05$). At 5dpi and 6dpc, 3 birds were sacrificed from each group for virus titration in lungs and trachea. Viral titers were higher in the upper respiratory tract compared to the lower respiratory tract in both inoculated and contact (**Figure 4.7c-f**). Virus titer in lung homogenates from contact birds were at the level of detection by 6dpc.

To determine what amino acids were associated with contact transmission events, tracheal samples collected at 9 dpi (n=3/group) and 8 dpc (n=3/group) were subjected to virus sanger sequencing (**Table 4.3**). In the majority of directly inoculated quail, there was a lack of amino acid resolution at position 226 (labeled as “X”) with the exception of 2 quail showing M226 in the *var*ΔLQ inoculated group and 1 quail showing L226 in the *var*+LQ inoculated group. In contrast, the vast majority of contact quail shed the L226 variant by 8 dpc, indicating a natural fitness advantage of this variant in this bird species, clearly in contact birds in the *var*+L and *var*+LQ groups. The exceptions were in the *var*ΔLQ direct contact group with 1 bird shedding an undefined (at position 226 of HA) virus population and 1 bird shedding the N226 variant, whereas the third bird in this group surprisingly selecting for a L226 variant. The L226 variant in the *var*ΔLQ contact group could have easily emerged from a single mutation in the first nt of codons encoding either F226, I226, M226, or V226. Likewise, for contact birds in the *var*+Q group showing the L226 variant, such variant could have been selected by a similar mechanism or from a single mutation in the second nt of the codon encoding for the Q226 variant. This quail study strongly suggests strong natural selection of H9 HA variants with L226 in this species, which is consistent with the prevalence of L226 variants in poultry.

Discussion

Receptor engagement is a critical step in influenza virus infection and is mediated by the HA. It is well known that changes to amino acids in the receptor-binding site of HA modulate receptor recognition, which in turn influences host adaptation. In this study, we evaluated the flexibility of amino acids in the receptor-binding site with a focus on a previously characterized position capable of altering receptor recognition. Several studies have linked the changes at position 226 and 228 (H3 numbering) to receptor preference. The combination of glutamine and glycine (Q226/G228) allows for avian-like α2,3SA recognition while leucine and serine (L226/S228) favor human α2,6SA recognition in the context of H2, H3 and H4 HA subtype viruses (7, 45-47). For H9 influenza viruses, position 226 has been implicated in changes in receptor preference (13, 41). The Q226L change has also been associated with improved direct contact transmission of H9N2 viruses in the ferret model (40). Analysis of the 4,829 H9 HA sequences available to date in the Influenza Research database revealed that 81.28% of isolates have L226, 18.24% have Q226 and the remaining 0.48% of the sequences carries M226, I226, H226, P226, S226, or F226. Given the rather limited repertoire of amino acid usage at this position in the context of H9 viruses and the increasing number of H9N2 isolates with the human L226 residue, we sought to determine the plasticity of this position and its effect on virus phenotype. Our results reveal the flexibility at this position, identifying a diverse set of amino acids that can be accommodated. Using 2 approaches, an H9 HA library was used to generate viruses with heterogeneity only at position 226 on either a wtH9N2 or wtH1N1 backbone such that any changes observed are due to the change at position 226. From isolated variants, 14 different amino acids were identified, 8 of which (A226, C226, G226, N226, R226, T226, V226 and Y226) are yet to be found in natural isolates of H9 viruses while one (C226) is yet to be found in any influenza virus HA irrespective of subtype. We did not obtain a variant with P226, which is represented by a single isolate in the database, or variants with acidic amino acids (D226, E226) or basic amino acid (K226) or with an indole side chain (W226). We proceeded to characterize these *var* viruses with a focus on those generated using the approach with less bias (equi226). It was surprising to observe that some of the most favored variants from the H9N2 and H9N1 virus libraries corresponded to amino acids that have yet to be found in nature (N226, n=17 overall) or that are uncommon (H226, n=13 overall) in the background of the H9 HA. Equally surprising was the lack of selection of the L226 variant from the $_{nnn}$H9N2 virus library considering that L226 could have been selected from 6 different codons in the $_{nnn226}$H9PCR library versus 2 codons for the N226 or the H226 variants. It is unlikely that the lack of selection for the L226 variant in the WF10 background was because of lack of representation in the

$_{nnn226}$H9PCR library. The same $_{nnn226}$H9PCR library was used to generate the $_{nnn}$H9N1 virus library, resulting in the L226 as the most prominent variant in the virus pool (n=5, and n=12 overall). Like the L226 variant, the Q226 variant was only obtained when paired with the N1 NA in the PR8 background. These results indicate that *in vitro* and *in vivo* fitness do not necessarily correlate and questions whether some of the variants observed in isolates could indeed be artifacts of the method used for virus growth, something that could be easily ascertained by sequencing the virus directly from the field sample.

Using solid phase binding assays, we determined the receptor preference of these single amino acid variants. In concert with previous studies with H9 viruses, virus with Q226 bound strongly to sugars containing α2,3SAs while the virus with L226 preferentially bound sugars containing α2,6SAs (13, 41). We isolated viruses with V226 and I226, these residues are commonly found in H3 viruses and are associated with α2,6SA binding (48). In the H9 variants, these viruses behaved similarly, binding α2,6SAs in addition to α2,3SA binding. Though the amino acids involved with receptor specificity in H1 influenza viruses do not include position 226, R226 has been found in an H1 virus following adaptation to egg and allowed for α2,3SA recognition an observation consistent with the R226 H9 *var* virus (49) (**Figure 4.9**). Furthermore, we observed a pattern of polar amino acid residues recognizing the 3SLN sugars exclusively while hydrophobic amino acid residues were able to interact with both α2,3SA and α2,6SA sugars using the solid phase binding assay. The exception to this observation was with A226 (a hydrophobic residue binding α2,3SA) (**Figure 4.9**) and N226 (a polar residue able to bind to both sugars). On the crystal structure of A/swine/Hong Kong/9/98 (H9N2) HA, the L226 makes non-polar interactions with the C6 atom of the SA (50). With the exception of N226, all the dual binding *var* viruses contain hydrophobic amino acid residues similar to leucine and are capable of making van der Waals interaction that allow recognition of SAs in the α2,6 conformation. Other amino acids in the RBS have been implicated in the receptor-binding preference of H9 viruses. In a recent study, A190V mutation was identified in an H9N2 as being able to expand SA recognition and interaction by widening the RBS pocket (51) while in H3 viruses E190D increased α2,6SA recognition (52). All variant viruses in this study possessed an E190 residue, which is fairly common in influenza viruses of avian origin. In addition to position 190, the I155T, H183N and G228S (H3 numbering) mutations have also been associated with changes in receptor preference (53). It has been suggested that the I155T mutation plays an important role in H9N2 virus binding to human-like SA receptors. An H9N2 virus engineered to carry I155 lost its ability to bind α2,6SA despite carrying the L226 profile (54). The *var* viruses described in this report have distinct patterns of α2,3SA and/or α2,6SA receptor binding depending on the amino acid residue at position 226 despite carrying the T155 mutation, suggesting that the contribution of the I155T change may be minimal for human-like receptor recognition.

Glycan microarrays provide an additional tool to investigate virus-receptor interaction. Our data from the microarray analysis are in concert with the solid phase binding data for most of the *var* viruses. Similar to previous studies with H7N9 and H3N2 viruses (55, 56), there was an overall preference for N-linked glycans by all viruses tested. N-linked glycans are known to be predominant in the human and ferret glycome (57, 58). *Var* viruses show an ability to bind sugars in different conformations to varying degrees though binding to N-linked sugars seen for both α2,3SA and α2,6SA conformations irrespective of the amino acid at position 226. We observed a few differences between the glycan microarray data and the solid phase direct binding assay for some *var* viruses. The M226, V226 and I226 *var* viruses had a preference for both α2,3SA and α2,6SA on the direct binding assay but displayed a preference for only α2,6SA on the glycan microarray while the F226, G226 and N226 showed an expanded binding profile in both the direct binding assay and glycan microarray. Two variants showed considerable difference between glycan microarray and solid phase binding assay. The R226 variant showed exclusive α2,3SA preference with the solid phase binding assay while the Y226 virus had low but detectable α2,6 binding (**Figure 4.9**), for both variants however, binding was undetectable with the glycan microarray. The comparatively lower binding intensities of both variants on the solid phase assay despite the use of the multivalent biotinylated SGPs, may account for the lack of binding on the microarray.

In addition to receptor-binding preference, recent studies have highlighted the role of pH stability in the adaptation of avian viruses to humans (59, 60). Amino acid residues in the RBS have been shown to contribute to the pH stability of influenza viruses. On the H9N2 backbone, the V226, L226, I226, C226 and Q226 *var* viruses were the more stable variants, with V226 and L226 *var* viruses still adsorbing RBCs after 4 h incubation at 56ºC. On the H1N1 backbone, V226 and Q226 were stable for 2 h at 56ºC. Our results with the H9N1 *var* viruses are similar to observations with an H7N9 virus where Q226 (217 in H7 numbering) had increased thermostability compared to a virus with L226 when incubated at 50ºC (61). The reduce stability of the H9N1 *var* viruses compared to the H9N2 *var* viruses suggest a role of NA in thermostability. As previously reported, the activities of HA and NA are functionally linked (62). It is possible that the reduced compatibility between the H9 and the N1 is responsible for the differences observed in the stability of the H9N1 variants.

The *in vitro* data suggests that all of the mutants can be viable in nature as they all replicated to similar titers in both avian and mammalian cell lines although with lower average titers in DF1 cells compared with MDCK cells. Results from the *in vivo* competition studies in quail however, indicate that while all variants are 'fit' *in vitro* (and *ex vivo*, not shown), there are bottlenecks affecting virus phenotype *in vivo* that cannot be recapitulated by *in vitro* systems. Due to limitations in the number of animal studies that could be performed, we decided to ascertain the relative fitness advantage of the 226 *var* viruses in an *in vivo* competition model. Undoubtedly, the L226 *var* virus showed an *in vivo* advantage in the quail model. L226 was the predominant *var* virus in contact quails in groups *var*+L and *var*+LQ where this variant was included in the infection mix. This may reflect what is seen in natural isolates where L226 is found in 80% of isolates. Interestingly, in the *var*+Q group where Q226 but not L226 was included in the virus mix, the L226 *var* virus was ultimately selected in both inoculated and direct contact birds. This is somewhat consistent with previous studies that have shown that position 226 is under selective pressure: a change from Q226 to I226 was observed in an H9N2 virus following only 3 passages in turkey tracheal tissues and a V226I mutation was acquired in an H3N2 virus within 5 passages in tissue culture (48, 63). In addition to L226, we isolated a M226 *var* virus from 2 directly inoculated quails in the *var*ΔLQ group at 9 dpi. The M226 variation occurs naturally in H9 isolates and confers preference for mostly α2,6SA with possible residual binding to α2,3 as seen in the direct binding in this study. Isolation of M226 from 2 of the 3 inoculated birds in the *var*ΔLQ group suggests that this amino acid conferred a replication advantage. Sanger sequencing however did not identify M226 in any of the contact birds though there was a lack of resolution in 1 DC bird. Without temporal replication data, we cannot exclude the possibility that at earlier time points, other var viruses such as N226 isolated from a DC bird, was shed by inoculated birds. It is also possible that the absence of M226 in DC birds was a direct consequence of reduced transmission fitness in quails. L226 was also identified from one DC bird in this group. It is interesting that a L226 var was found in one contact quail in this group and as mentioned above could be the result of selective pressure on this position. Furthermore, only one nucleotide change is necessary for a change from M226 (ATG) to L226 (CTG). It is interesting to note that the *var* viruses identified in contact birds all bound with the most intensity in the quail virus histochemistry study and the ability of these var to bind and initiate infection in the contact birds.

Overall, this study identified amino acids that have yet to be seen in H9 viruses and that have the ability to expand the SA preference of H9 viruses. This is important not only for surveillance efforts to identify viruses of greater pandemic concern but also can be harnessed in areas such as vaccine production, particularly live attenuated vaccines. In addition, to those approved for use in humans, live attenuated influenza virus vaccines are approved for use in horses, and recently, in pigs. These live virus vaccines carry HA gene segments that are indistinguishable from those circulating in the field. Our studies indicate that vaccine strains could be produced with 226 (or alternative RBS) mutations with optimal or improved *in vitro* growth characteristics without affecting antigenicity. Generation of vaccine candidates with these mutations could preclude the current issues accompanying growth of H3N2 vaccine viruses in eggs. RBS *var* vaccine viruses would complement the attenuation phenotype of live virus vaccines. RBS *var* vaccine viruses would be less likely to transmit and reassort which are the major concerns for mass implementation of live attenuated influenza virus vaccines in agriculture, particularly in poultry. The RBS *var* virus vaccine approach could be implemented regardless of the live attenuated platform used and would improve the safety profile of such vaccines. We believe that approaches that improve vaccine production *in vitro* but decrease HA fitness *in vivo* without sacrificing its immunogenicity is important in the context of major efforts towards universal influenza virus vaccines based on live attenuated approaches.

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Table 4.1**:** Hemagglutination and Hemagglutination inhibition assay titers of variant viruses.

***Var* virus**	**HA titers**			**HAI titers**	
	0.5% cRBC	**0.5% tRBC**	**1% hRBC**	**WF10 Antisera**	**M226 Antisera**
I226 (H9N2)	512	512	0	2560	2560
S226 (H9N2)	256	128	0	2560	2560
T226 (H9N2)	512	256	0	5120	2560
M226 (H9N2)	512	512	0	2560	2560
H226 (H9N2)	256	512	0	2560	2560
N226 (H9N2)	1024	512	4	2560	2560
F226 (H9N2)	256	256	0	2560	5120
V226 (H9N2)	256	256	0	2560	2560
C226 (H9N2)	256	128	0	2560	2560
G226 (H9N2)	512	256	0	2560	2560
L226 (H9N2)	256	256	0	2560	2560
G226 (H9N2)	512	512	128	320	640
Y226 (H9N1)	128	512	8	5120	10240
R226 (H9N1)	512	512	128	ND	ND
A226 (H9N2)	256	256	64	ND	ND
PR8 (H1N1)	1024	1024	0	<10	<10

Table 4.2: Summary of sialic acid specificity and virus histochemistry data for *var* viruses

***Var* virus**[#]	**Direct binding**[$]				**Glycan array**[*]							**Virus Histochemistry**[%]			
	3 Fet-HRP	**6-Fet-HRP**	**3SLN**	**6SLN**	**2,3 L**	**2,3 O**	**2,3 N**	**Lx**	**2,6 L**	**2,6 O**	**2,6 N**	**Quail**	**Tky**	**Chicken**	**mouse**
I226	+/-	+/-	+	+	-	-	+++	-	++	++	++	+	+	-	-
S226	+/-	-	+	-	++	++	+++	++	-	-	+	+	++	++	+
T226	+/-	-	+	-	++	+	+++	+	-	-	+	+	+	+	+
M226	+	++	+	+	-	-	+/-	-	+	+	+++	++	+	+	+
H226	+	-	+	-	++	+/-	+++	-	-	-	-	+	++	++	+
N226	++	+	+	+	++	+	+++	+++	+++	++	+++	+++	+++	+++	++
F226	-	-	+	+	+	+	+++	+	+	++	+++	+++	+	-	-
V226	++	++	+	+	+/-	+/-	+	-	+++	++	+++	+	+++	+	-
C226	++	-	+	-	+++	++	+++	+++	-	-	-	**ND**	+	++	+
G226	+	+	+	+	+++	++	+++	+++	+	++	+++	+	++	++	-
L226	-	++	+/-	+	+/-	+/-	+++	-	++++	++++	++++	+++	++	++	+
Q226	++	-	+	-	++++	++++	++++	-	-	-	-	+++	+++	+++	+++
Y226 (H9N1)	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-	+++	++	+	++

#: Viruses on H9N2 backbone except Y226 virus on H9N1 backbone.

$: Scoring for direct binding assay classified as + (binding to sialic acid substrate), - (did not bind sialic acid), +/- low binding to both 2,3 and 2,6 and binding cannot be determined.

*: Glycan microarray data classed as – (no binding), + (narrow binding), ++ (moderate binding to some sugars), +++ (strong binding at most/all sugars of the micro array)

%: Scoring for virus histochemistry- + mild, ++ moderate, +++ intense, - no binding

Table 4.3**:** Amino acid identified at position 226 in inoculated and contact quails following infection with virus mix.

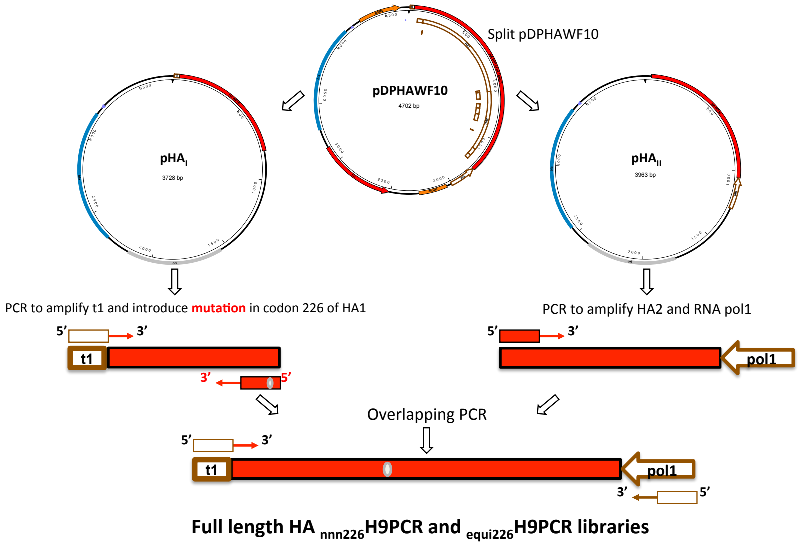
Group	**Directly inoculated quail (9dpi)**		**Direct contact quail (8dpc)**	
	Amino acid at 226	**Nucleotide**	**Amino acid at 226**	**Nucleotide**
***var*ΔLQ**	**M, M, X***	**ATG/ATG/MTR**	**X, N, L**	**WTG/AAT/CTS**
***var*+L**	**X, X, X**	**MWR, MTG, MTG**	**L, L, L**	**CTG, CTG, CTG**
***var*+Q**	**X, X, X**	**ATG/MTG/CKG/MTR**	**L, Q, L**	**CTG, CAA, CTA**
***var*+LQ**	**X, X, L**	**ATD/MTG/CTG/MTG**	**L, L, L**	**CTG, CTG, CTG**

*: Unresolved amino acid at position 226 following Sanger sequencing

Table 4.4: List of primers used in equi226 primer mix.

Primer name	Primer Sequence
WF10_HA-A226 Rev	5’CAATAATAATCAATTCTTCCCTGTGCACCATTGACAAGGGGCCTT 3’
WF10_HA-C226 Rev	5’CAATAATAATCAATTCTTCCCTGACAACCATTGACAAGGGGCCTT 3’
WF10_HA-D226 Rev	5’CAATAATAATCAATTCTTCCCTGATCACCATTGACAAGGGGCCTT 3’
WF10_HA-E226 Rev	5’CAATAATAATCAATTCTTCCCTGTTCACCATTGACAAGGGGCCTT 3’
WF10_HA-F226 Rev	5’CAATAATAATCAATTCTTCCCTGGAAACCATTGACAAGGGGCCTT 3’
WF10_HA-G226 Rev	5’CAATAATAATCAATTCTTCCCTGTCCACCATTGACAAGGGGCCTT 3’
WF10_HA-H226 Rev	5’CAATAATAATCAATTCTTCCCTGATGACCATTGACAAGGGGCCTT 3’
WF10_HA-I226 Rev	5’CAATAATAATCAATTCTTCCCTGTATACCATTGACAAGGGGCCTT 3’
WF10_HA-K226 Rev	5’CAATAATAATCAATTCTTCCCTGTTTACCATTGACAAGGGGCCTT 3’
WF10_HA-L226 Rev	5’CAATAATAATCAATTCTTCCCTGTAGACCATTGACAAGGGGCCTT 3’
WF10_HA-M226 Rev	5’CAATAATAATCAATTCTTCCCTGCATACCATTGACAAGGGGCCTT 3’
WF10_HA-N226 Rev	5’CAATAATAATCAATTCTTCCCTGATTACCATTGACAAGGGGCCTT 3’
WF10_HA-P226 Rev	5’CAATAATAATCAATTCTTCCCTGTGGACCATTGACAAGGGGCCTT 3’
WF10_HA-Q226 Rev	5’CAATAATAATCAATTCTTCCCTGTTGACCATTGACAAGGGGCCTT 3’
WF10_HA-R226 Rev	5’CAATAATAATCAATTCTTCCCTGCCTACCATTGACAAGGGGCCTT 3’
WF10_HA-S226 Rev	5’CAATAATAATCAATTCTTCCCTGTGAACCATTGACAAGGGGCCTT 3’
WF10_HA-T226 Rev	5’CAATAATAATCAATTCTTCCCTGTGTACCATTGACAAGGGGCCTT 3’
WF10_HA-V226 Rev	5’CAATAATAATCAATTCTTCCCTGTACACCATTGACAAGGGGCCTT 3’
WF10_HA-W226 Rev	5’CAATAATAATCAATTCTTCCCTGCCAACCATTGACAAGGGGCCTT 3’
WF10_HA-Y226 Rev	5’CAATAATAATCAATTCTTCCCTGATAACCATTGACAAGGGGCCTT 3’

Generation of reverse genetics-ready PCR $_{226}$HA library



Generation of $_{nnn226}$H9N2, $_{nnn226}$H9N1, $_{equi226}$H9N2, and $_{equi226}$H9N1 virus libraries

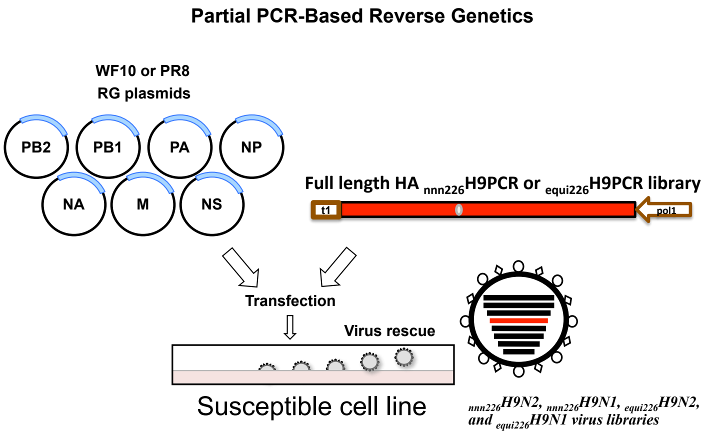


Figure 4.1: Schematic overview of the steps to generate degenerate H9 HA PCR product and rescue of H9 HA virus library. (A) WT WF10 HA plasmid was split in 2 plasmids pHA1 and pHA2 with designed primers. HA PCR product (1-767) carrying the mouse RNA polymerase terminator sequence and the degenerate NNN codon at position 226 was generated

from the pHA1 plasmid either by a specific primer with the NNN codon (nnn226) or with a mix of primers able to introduce all 20 amino acids (equi226). Another PCR product with the remaining HA (738-1742) and human polymerase 1 promoter generated from pHA2. Using overlapping PCR, a full-length HA was obtained with the degenerate codon at 226 flanked by the mouse RNA polymerase terminator sequence and the human pol 1 promoter. (B) Generation of virus library by PCR based reverse genetics using $_{226}$HA PCR product and 7 plasmids from WF10 (H9N2) or PR8 (H1N1)

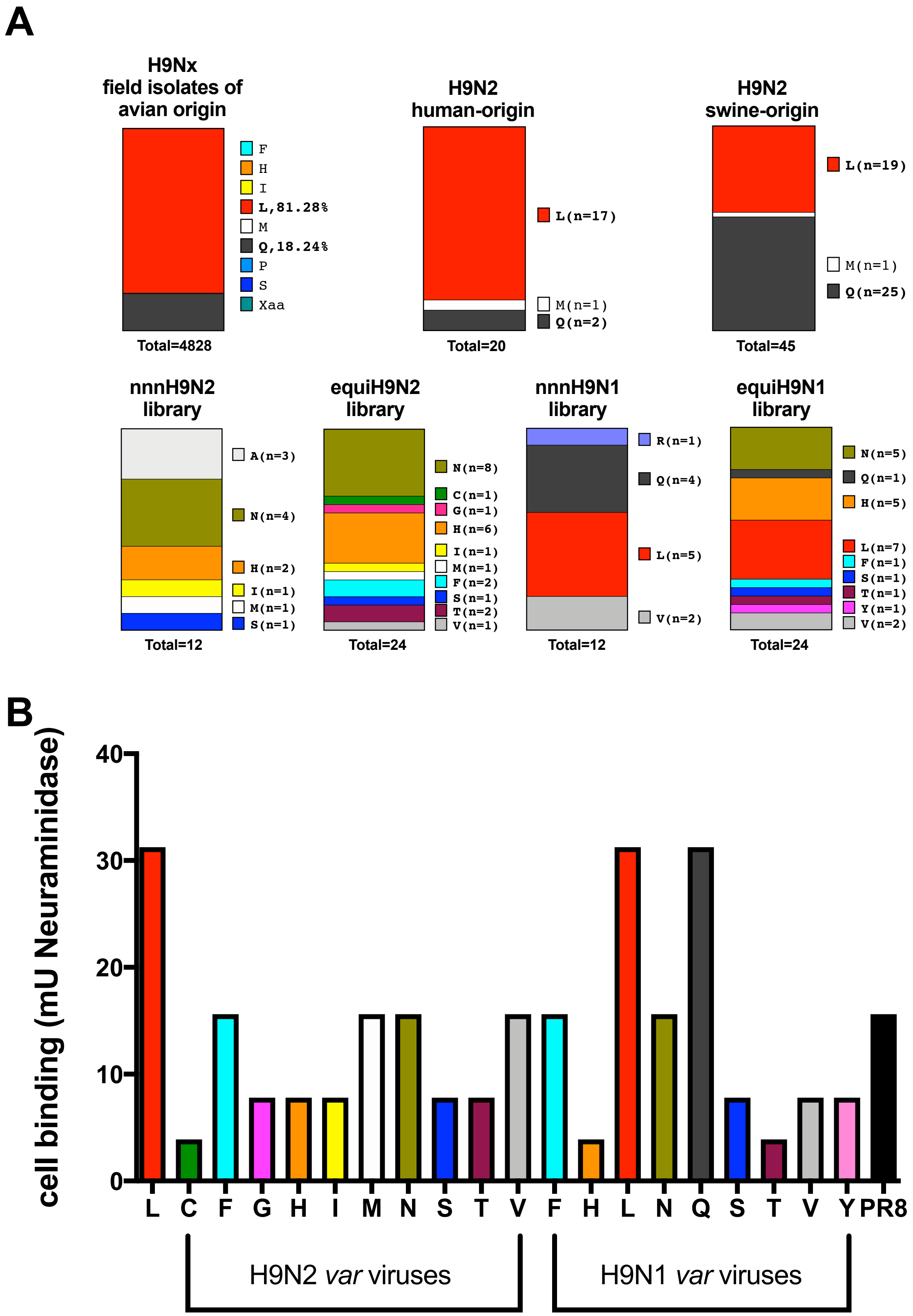

A
H9Nx field isolates of avian origin
F
H
I
L,81.28%
M
Q,18.24%
P
S
Xaa
Total=4828
H9N2 human-origin
L(n=17)
M(n=1)
Q(n=2)
Total=20
H9N2 swine-origin
L(n=19)
M(n=1)
Q(n=25)
Total=45
nnnH9N2 library
A(n=3)
N(n=4)
H(n=2)
I(n=1)
M(n=1)
S(n=1)
Total=12
equiH9N2 library
N(n=8)
C(n=1)
G(n=1)
H(n=6)
I(n=1)
M(n=1)
F(n=2)
S(n=1)
T(n=2)
V(n=1)
Total=24
nnnH9N1 library
R(n=1)
Q(n=4)
L(n=5)
V(n=2)
Total=12
equiH9N1 library
N(n=5)
Q(n=1)
H(n=5)
L(n=7)
F(n=1)
S(n=1)
T(n=1)
Y(n=1)
V(n=2)
Total=24
B
cell binding (mU Neuraminidase)
0
10
20
30
40
L
C
F
G
H
I
M
N
S
T
V
F
H
L
N
Q
S
T
V
Y
PR8
H9N2 var viruses
H9N1 var viruses

Figure 4.2: Amino acid diversity at position 226 following limiting dilution and receptor avidity of isolated variants. (A) Amino acid present at position 226 in natural isolates of H9Nx viruses by species of origin compared to amino acid identified experimentally using either the nnn226 approach or the equi226 approach on either the H9N1 or H9N2 backbone. (B) Receptor binding avidity of *var* viruses using cells treated with increasing concentration of neuraminidase *Clostridum perfringes* and compared to wt WF10 (L) virus and wt PR8 virus.

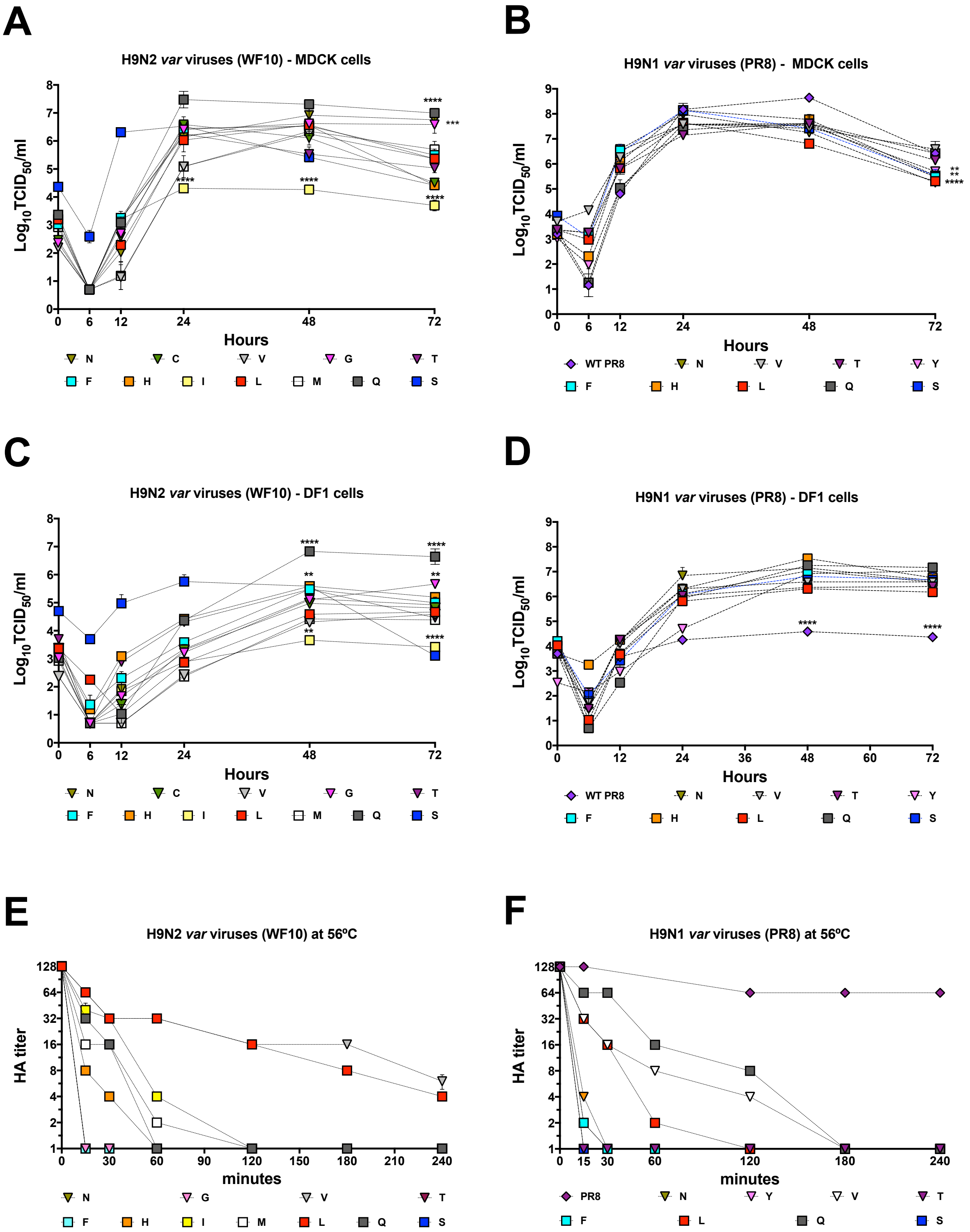
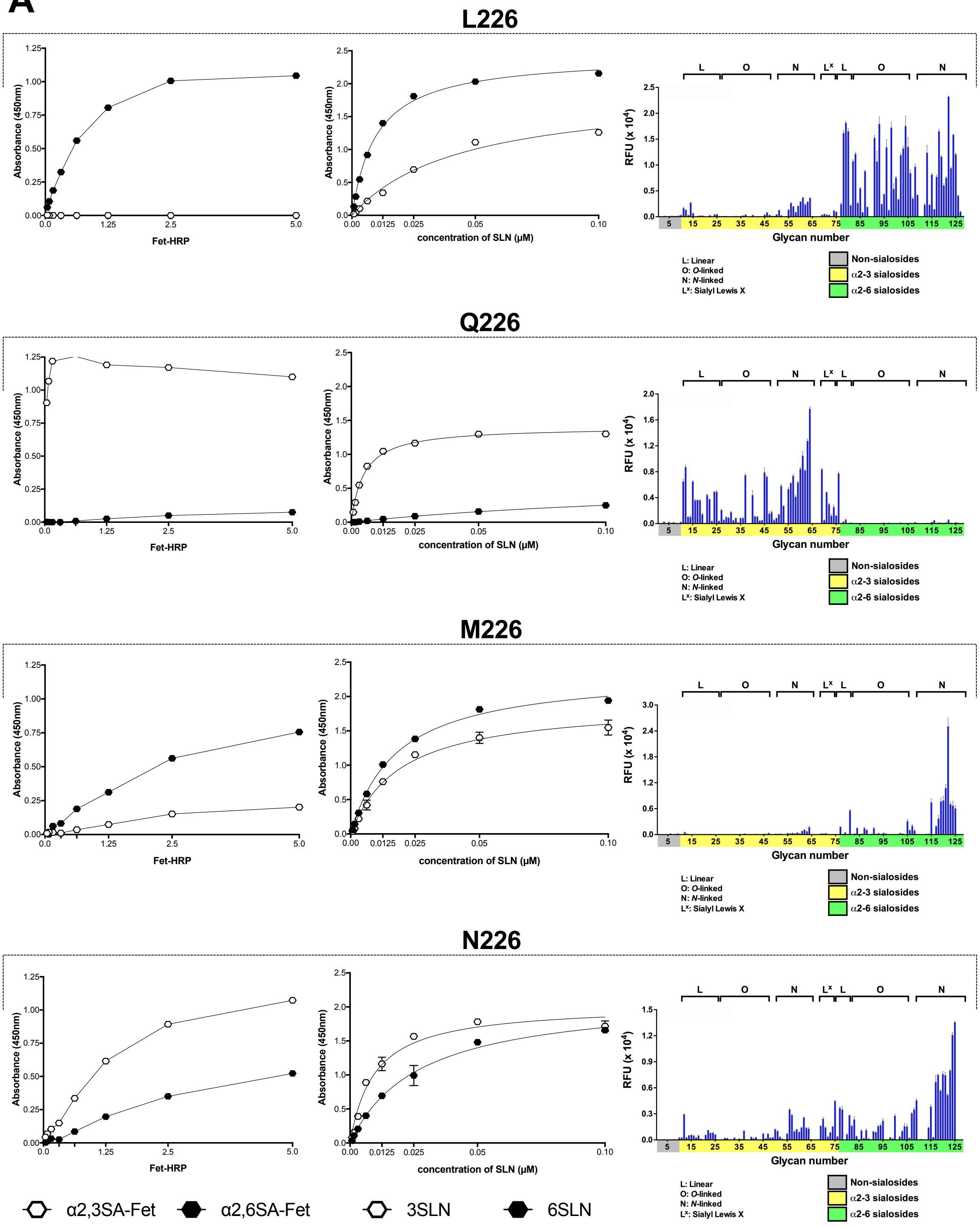
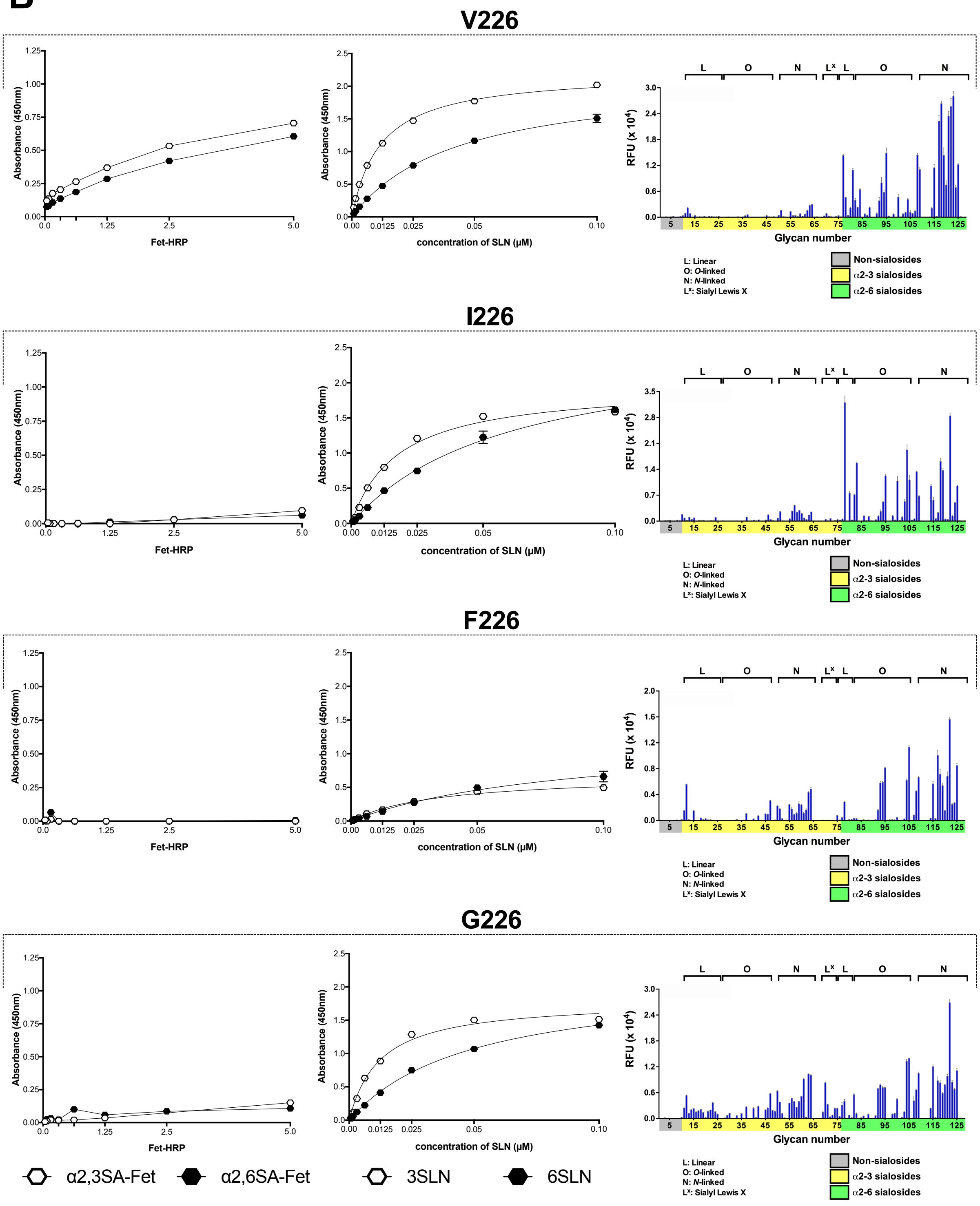

A
H9N2 var viruses (WF10) - MDCK cells
Log10TCID50/ml
Hours
0
6
12
24
48
72
****
***
****
****
****
N
C
V
G
T
F
H
I
L
M
Q
S
B
H9N1 var viruses (PR8) - MDCK cells
Log10TCID50/ml
Hours
**
**
****
WT PR8
N
V
T
Y
F
H
L
Q
S
C
H9N2 var viruses (WF10) - DF1 cells
Log10TCID50/ml
Hours
****
**
**
****
**
****
N
C
V
G
T
F
H
I
L
M
Q
S
D
H9N1 var viruses (PR8) - DF1 cells
Log10TCID50/ml
Hours
0
12
24
36
48
60
72
****
****
WT PR8
N
V
T
Y
F
H
L
Q
S
E
H9N2 var viruses (WF10) at 56ºC
HA titer
minutes
128
64
32
16
8
4
2
1
0
30
60
90
120
150
180
210
240
N
G
V
T
F
H
I
M
L
Q
S
F
H9N1 var viruses (PR8) at 56ºC
HA titer
minutes
0
15
30
60
120
180
240
PR8
N
Y
V
T
F
L
Q
S

Figure 4.3: ***In-vitro* replication and temperature stability of isolated variants.** Replication of H9N2 and H9N1 *var* viruses in mammalian MDCK (A and B) and avian origin DF1 cells (C and D) at 37°C. Confluent monolayers of MDCK or DFI cells were infected with var viruses at an MOI of 0.01 and supernatant collected at 0, 6, 12, 24, 48 and 72hpi. Collected supernatant was quantified in MDCK cells by $TCID_{50}$ using the Reed and Muench method. Plotted data represents means ± standard error. Temperature stability of H9N2 (E) and H9N1 (F) *var* viruses at 56°C. *Var* viruses diluted to 128 HAU/50µl were incubated at 56°C. Samples collected at 0, 15, 30, 60, 120, 180 and 240 min post incubation were used for HA assays. Treatment was carried out in quadruplicate.

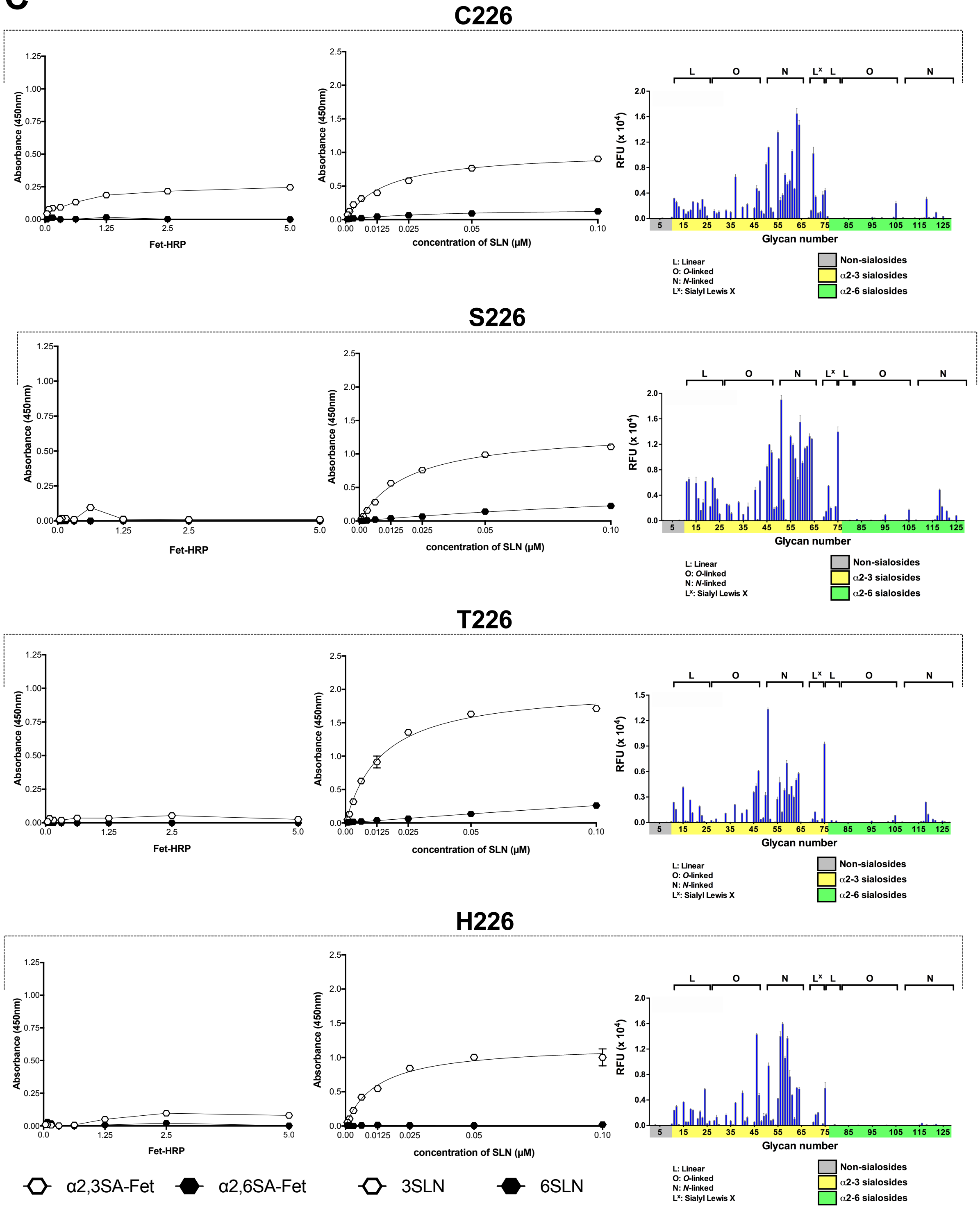
A


L226
Q226
M226
N226
Absorbance (450nm)
Fet-HRP
0.0
1.25
2.5
5.0
0.00
0.25
0.50
0.75
1.00
1.25
concentration of SLN (μM)
0.00
0.0125
0.025
0.05
0.10
0.0
0.5
1.0
1.5
2.0
2.5
RFU (x 10⁴)
Glycan number
5
15
25
35
45
55
65
75
85
95
105
115
125
L
O
N
Lˣ
L
O
N
L: Linear
O: O-linked
N: N-linked
Lˣ: Sialyl Lewis X
Non-sialosides
α2-3 sialosides
α2-6 sialosides
α2,3SA-Fet
α2,6SA-Fet
3SLN
6SLN

B


V226
I226
F226
G226
Absorbance (450nm)
Fet-HRP
concentration of SLN (μM)
RFU (x 10⁴)
Glycan number
L
O
N
Lˣ
L: Linear
O: O-linked
N: N-linked
Lˣ: Sialyl Lewis X
Non-sialosides
α2-3 sialosides
α2-6 sialosides
5
15
25
35
45
55
65
75
85
95
105
115
125
0.0
1.25
2.5
5.0
0.00
0.0125
0.025
0.05
0.10
α2,3SA-Fet
α2,6SA-Fet
3SLN
6SLN

C



C226
S226
T226
H226
Absorbance (450nm)
Fet-HRP
concentration of SLN (μM)
RFU (x 10⁴)
Glycan number
L
O
N
Lˣ
L: Linear
O: O-linked
N: N-linked
Lˣ: Sialyl Lewis X
Non-sialosides
α2-3 sialosides
α2-6 sialosides
α2,3SA-Fet
α2,6SA-Fet
3SLN
6SLN

Figure 4.4: Sialic acid specificity of *var* viruses is dependent on amino acid at position 226. Receptor specificity of *var* viruses on the H9N2 backbone comparing direct binding solid phase assays with Fet-HRP (4.4a-c left panel), direct binding solid phase assay with biotinylated sialylglycopolymers (4.4a-c middle panel) and glycan microarray (Scripps) (4a-c right panel). Two independent assays were conducted in duplicate for the solid phase binding assays.

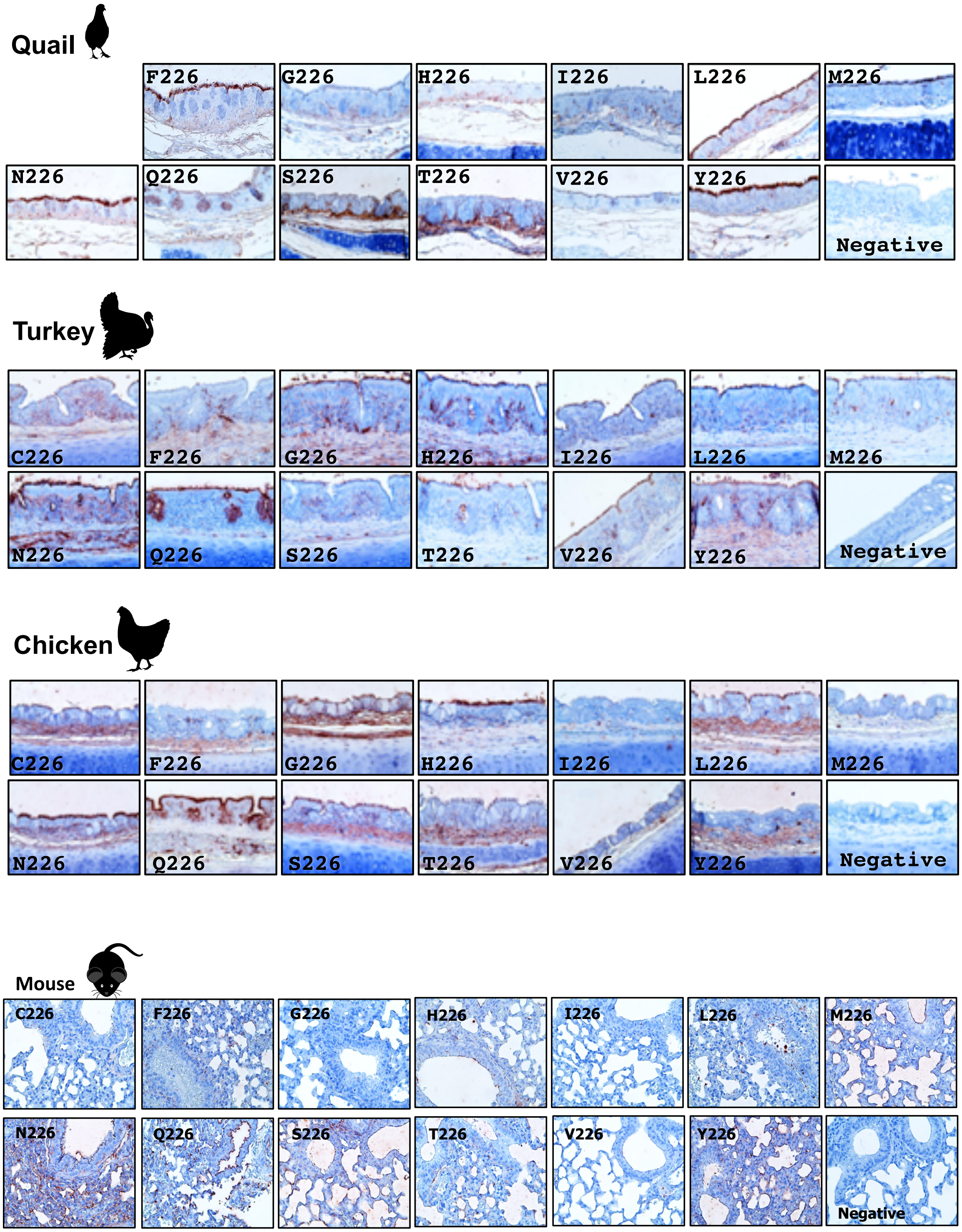

Quail
F226
G226
H226
I226
L226
M226
N226
Q226
S226
T226
V226
Y226
Negative
Turkey
C226
F226
G226
H226
I226
L226
M226
N226
Q226
S226
T226
V226
Y226
Negative
Chicken
C226
F226
G226
H226
I226
L226
M226
N226
Q226
S226
T226
V226
Y226
Negative
Mouse
C226
F226
G226
H226
I226
L226
M226
N226
Q226
S226
T226
V226
Y226
Negative

Figure 4.5: Pattern of virus attachment to avian and mammalian tissues. Bright field images comparing the pattern of viral attachment of different *var* viruses in quail, chicken and turkey tracheal tissues and in mouse lung by virus histochemistry**.**

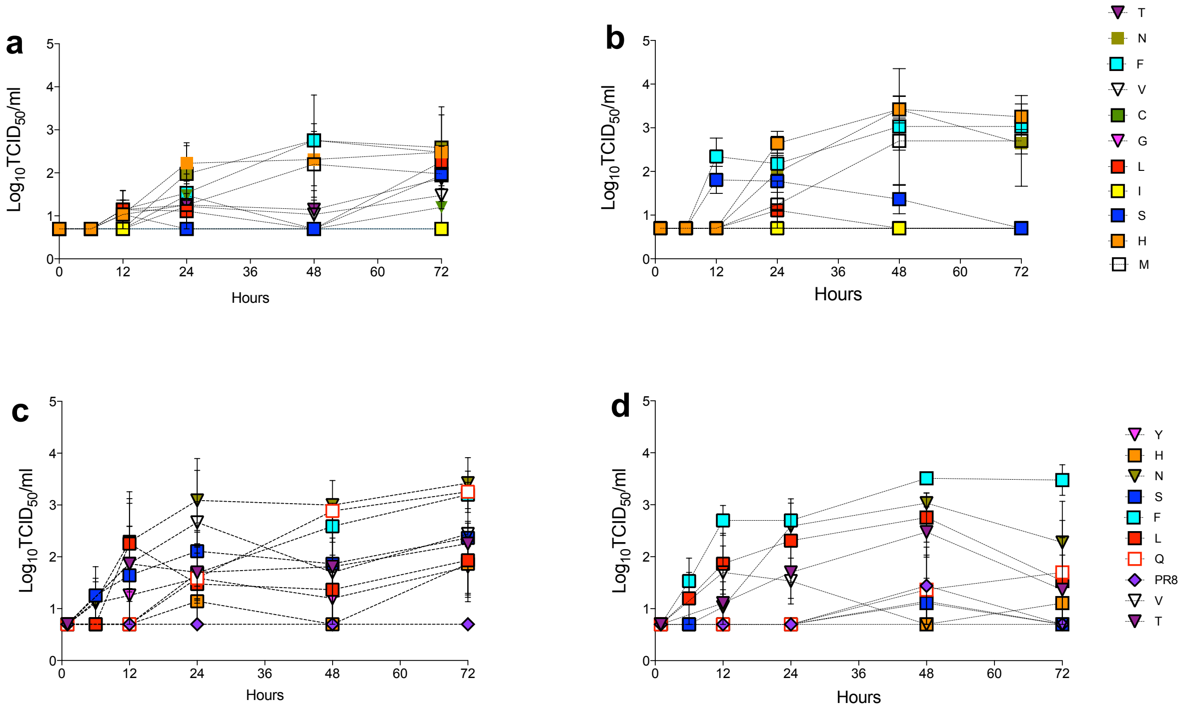


Figure 4.6: *Ex-vivo* replication of *var* viruses. *Ex-vivo* chicken lung (**a, c**) and tracheal (**b, d**) cultures from 6-week-old birds were infected with 10^6 $TCID_{50}$ of H9N2 *var* viruses (**a, b**) or H9N1 var viruses (**c, d**) in culture specific media at 37°C. Supernatant was collected at 0, 6, 12, 24, 48 and 72hpi. Quantification of virus shedding in explants was determined by titration of supernatant in MDCK cells. Experiment was carried out twice in triplicate.

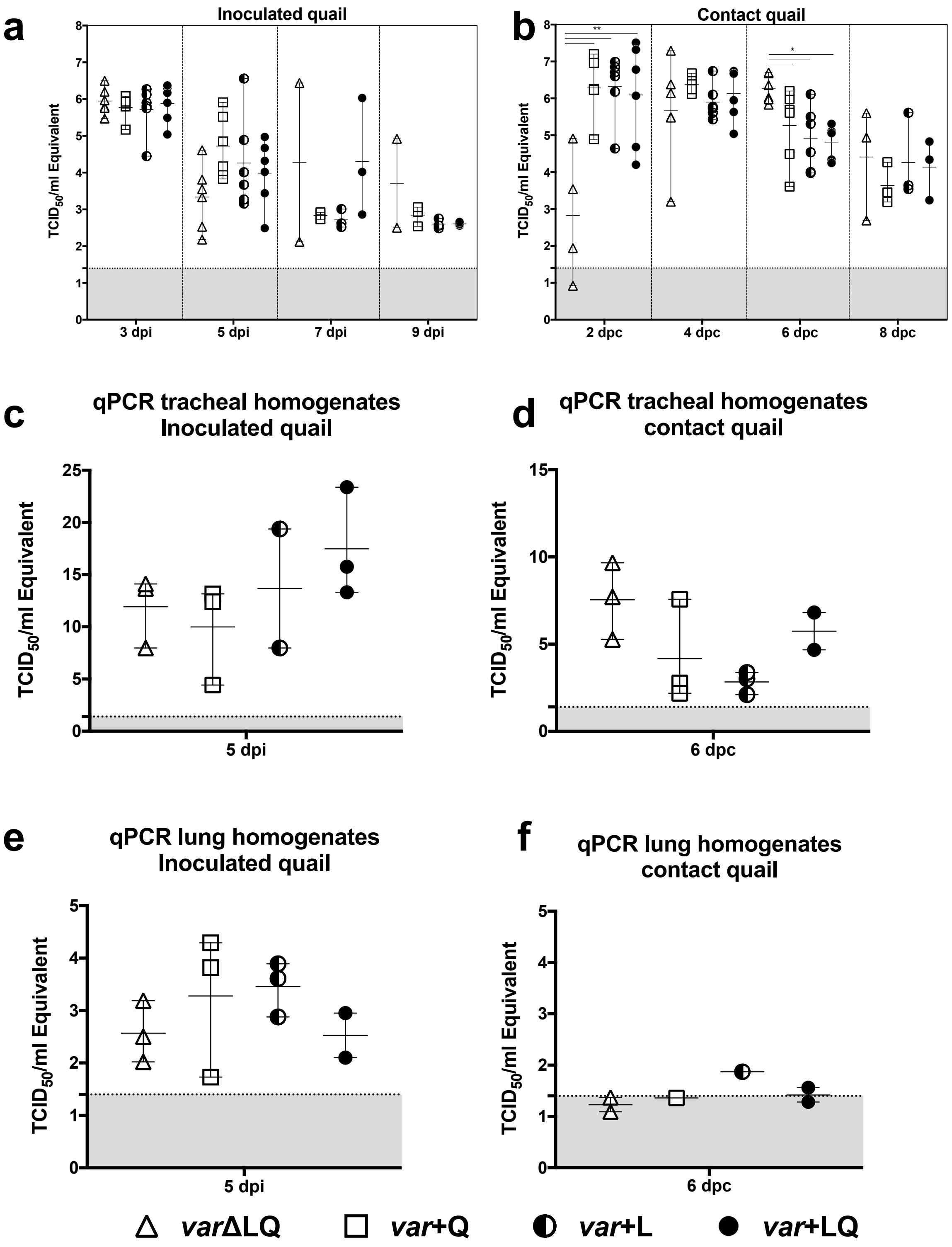

a
Inoculated quail
TCID50/ml Equivalent
0
1
2
3
4
5
6
7
8
3 dpi
5 dpi
7 dpi
9 dpi
b
Contact quail
**
*
2 dpc
4 dpc
6 dpc
8 dpc
c
qPCR tracheal homogenates
Inoculated quail
0
5
10
15
20
25
5 dpi
d
qPCR tracheal homogenates
contact quail
15
10
5
0
6 dpc
e
qPCR lung homogenates
Inoculated quail
5 dpi
f
qPCR lung homogenates
contact quail
6 dpc
△ varΔLQ
□ var+Q
◐ var+L
● var+LQ

Figure 4.7. In-vivo replication and transmission phenotype of var viruses. Quails were randomly divided in 5 groups, one group served as a control. At 4-6-week of age, 6 birds were inoculated intranasally, intratracheally and via the cloaca with a mixture of variant viruses with or without Glutamine and Leucine at 10^6 $TCID_{50}$ per ml per bird. Group 2 received only H9N2 *var* viruses (*var* ΔLQ), group 3 received *var* viruses and glutamine virus (*var* +Q), group 4 received *var* viruses and leucine virus (*var*+L) and the final group received all *var* viruses and the leucine and glutamine viruses (*var* +LQ). On day 1 post infection, 6 naïve quails were introduced as direct contact animals. (A, C, E) Virus shedding quantified by qRT-PCR in tracheal swabs at 3, 5, 7 and 9dpi and in tracheal and lung homogenates at 5dpi for inoculated birds. (B, D, F) Virus shedding quantified by qRT-PCR in tracheal swabs at 2, 4, 6 and 8dpc and in tracheal and lung homogenates at 6dpc for direct contact birds.

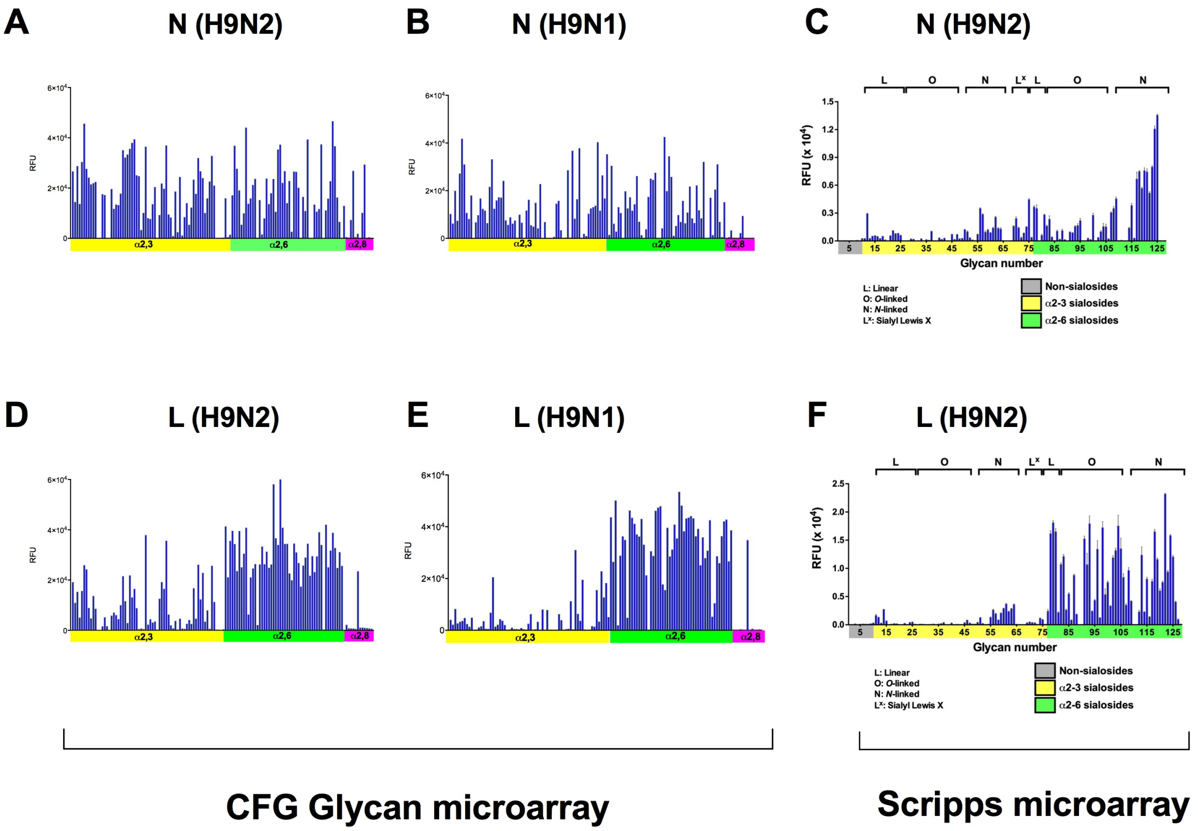
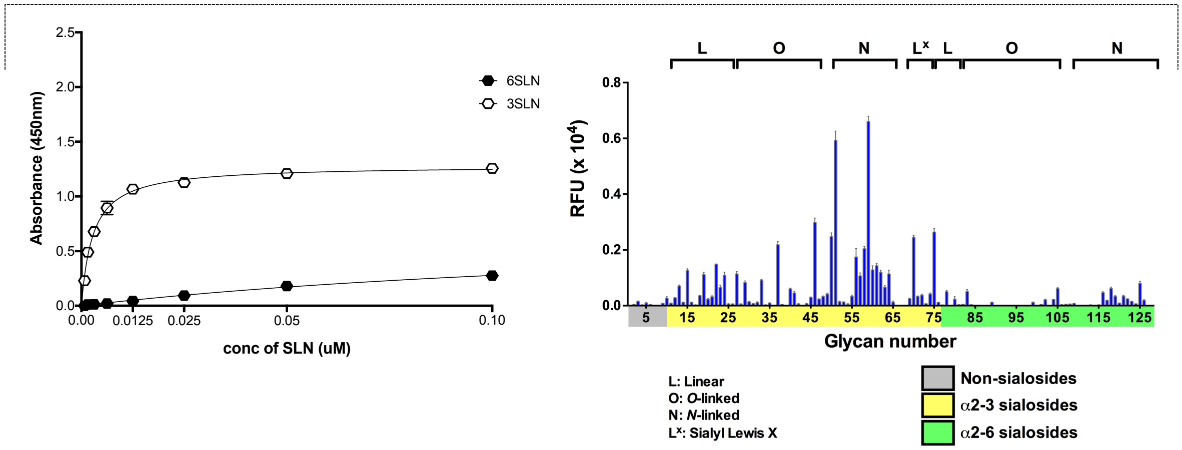
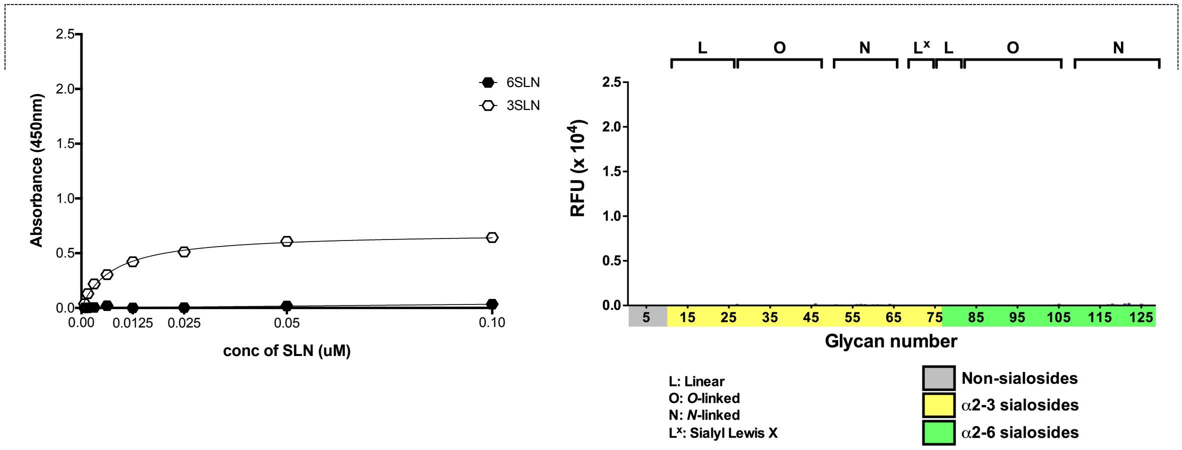


Figure 4.8 Glycan microarray analysis of *var* subset tested on different glycan microarrays. Comparison of glycan microarray analysis data obtained using the CFG v5.3 glycan microarray (A, B, D and E) and data obtained from the Scripps microarray (C and F). Similar binding patterns were observed using the 2 different arrays. Comparison of binding phenotype of H9N2 and H9N1 *var* viruses with same amino acid at position 226 show that subtype origin of the internal gene and neuraminidase genes did not significantly alter binding phenotype.

A226 (H9N2)



R226 (H9N1)



Y226 (H9N1)

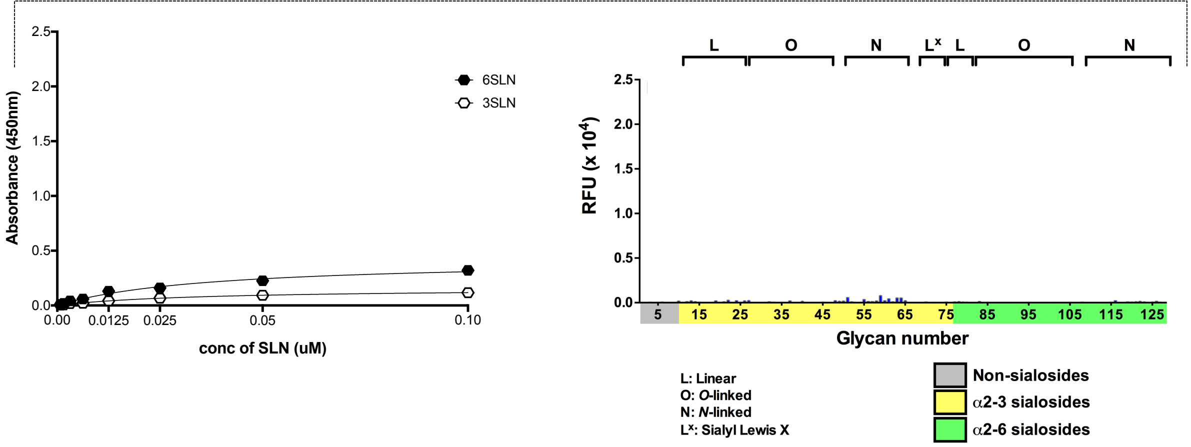
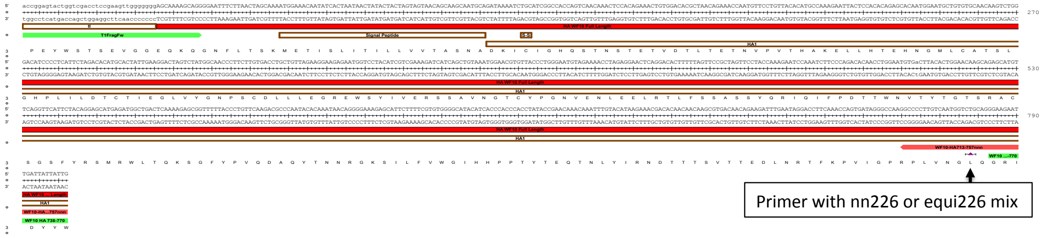
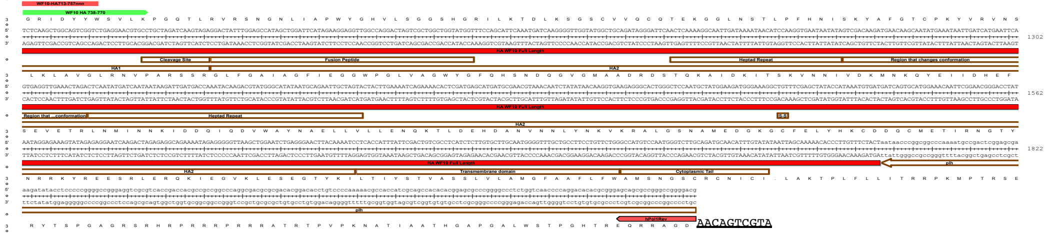


Figure 4.9 Comparison of the receptor binding specificity of 3 *var* viruses. Solid phase binding assays and glycan microarray analysis were used to determine the specificity of the A226 (top panel), R226 (middle panel) and Y226 (bottom panel) variants.

PCR to amplify t1 and introduce mutation in codon 226 of HA1



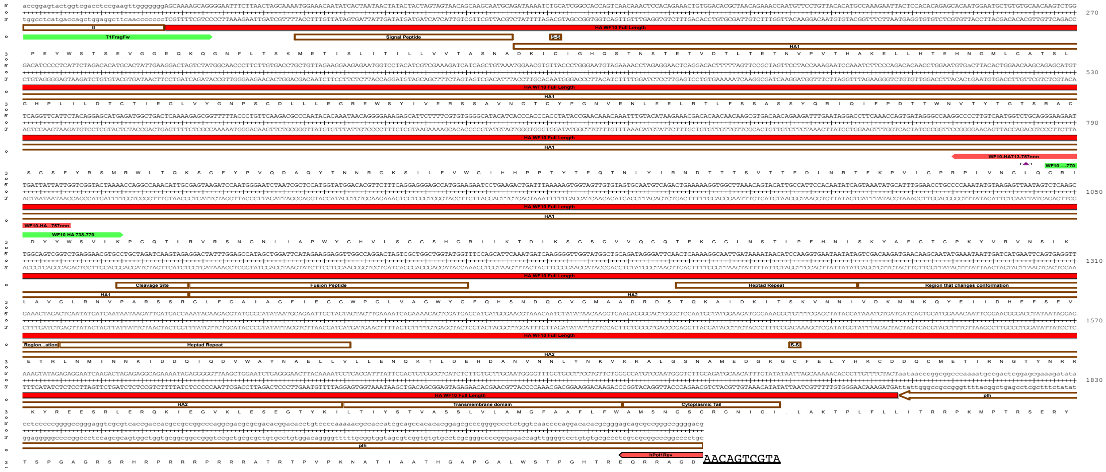
PCR to amplify HA2 and RNA pol1



Primer tail, irrelevant sequence

Overlapping PCR

Full length HA $_{nnn226}$H9PCR and $_{equi226}$H9PCR libraries



Primer tail, irrelevant sequence

Figure 4.10 Schematic representation of PCR products used to generate H9PCR libraries. Top panel schematic showing the sequence of the PCR products and primers used to generate the HA fragment from pHA1 and pHA2. Bottom panel showing the sequence of the full length HA PCR product obtained from the overlapping PCR reaction.

CHAPTER 5

CONCLUSIONS AND FUTURE PROSPECTS

Every year, influenza A viruses (IAVs) cause seasonal epidemics and are a threat to public health worldwide. This threat is not limited to currently circulating human H1 and H3 subtype viruses that continually change antigenically to evade existing population immunity but also IAVs from other species can cross the species barrier and establish infections in humans. The H5N1 outbreak in the late 1990s caused a shift in the influenza transmission paradigm that had previously portrayed that IAVs of poultry origin do not cause human infections. Following this event, research interest into the mechanisms that allow for interspecies transmission has increased, expanding our understanding of the virus-host relationship. In addition to the H5 subtype, H7, H9, H10 and H6 subtype viruses have caused human infections. While the molecular mechanisms and viral factors that enable cross species transmission of avian-origin IAVs are not completely understood, it is evident that changes to the surface genes, particularly the HA, and the exchange of genetic material via reassortment are key factors in this process.

In this dissertation, I examined the effects of molecular changes in the surface genes and reassortment with a focus on an H9 subtype IAV. I tested the contribution of these changes using an avian model of influenza virus transmission, quail (*Coturnix japonica*). Quail are land-based poultry that have the potential to act as intermediate hosts for influenza virus. They are capable of being infected with and replicating diverse avian-origin IAVs with minimal or no clinical signs, making these birds ideal for disseminating IAVs. Quail also possess receptors for both mammalian and avian IAVs similar to pigs. Pigs have historically been considered the intermediate hosts for the emergence of novel IAVs due to their ability to be infected with both human- and avian-origin IAVs creating an opportunity for reassortment to occur. Reassortment between from humans and avian species played a major role in the emergence of pandemic influenza in 1957 (H2N2), 1968 (H3N2) and 2009 (pH1N1). I sought to investigate if avian-human H9N1 reassortant virus would it still be viable for replication and transmission in an avian natural host such as the quail after acquiring adaptive mutations in the mammalian host, or if the mammalian adaptive mutations would be a hindrance to the fitness of such virus in the avian host. I hypothesized that in the quail such reassortant viruses would be capable of replication and transmission to naïve animals without any need for further adaptation. In Chapter 3, reassortant viruses carrying adaptive mutations in the surface genes (arising from passage in ferrets) were tested for replication and transmission fitness in quail. From the results described in Chapter 3, I can conclude that the 3 mutations that arose following mammalian adaptation did not prevent replication and transmission in the quail thus these land-based poultry could serve as intermediate host for reassortant viruses with the ability to efficiently cross to mammals. Furthermore, I identified a role for the internal gene constellation in the efficiency of reassortant virus transmission in the quail with the avian-origin internal gene cassette conferring a greater transmission efficiency compared to mammalian-adapted internal gene segments. I also observed the effect of the functional balance between the HA and NA and their co-evolution on virus replication in a natural host. A reassortant virus with the surface genes from different host origins (H9 avian, N1 mammalian) was unable to replicate and transmit in quail. My conclusions confirm the importance of quail as an intermediate host of IAVs. This is of particular importance in regions where multiple species are kept in confined spaces such as is commonly found in backyard farms and live animal markets. This can provide an optimal ecosystem where the risk of bidirectional transmission of IAVs is greatly enhanced.

In the second half of the thesis, my focus was on molecular changes in the receptor binding site of the HA. This shallow groove at the tip of the HA engages the viral receptor, the first step in virus-host interaction. Receptor specificity is a critical determinant of host range and is important for interspecies transmission of IAVs. Changes at position 226 can alter sialic acid receptor recognition and seminal studies identified Q226L mutation as responsible for a shift from avian to human receptor preference of H3 IAVs. In the context of an H9 virus, the same amino acid change confers a shift to a human receptor preference. An increasing number of recent H9 viruses have acquired the mammalian L226 residue at this position, increasing the public health risk posed by H9 viruses. I sought to determine the flexibility of position 226 in H9 viruses, to understand which amino acids other than leucine and glutamine are tolerated at this position. As discussed, in Chapter 4, my studies showed that this position is flexible. I identified variants with 14 different amino acids, 7 of which have yet to be identified in an H9 influenza virus. Of these 7 variants, one variant with cysteine at position 226 is not present in any naturally occurring influenza virus. To my knowledge, this is the first time that the plasticity of the RBS has been studied in this manner. All *var* viruses isolated were viable, and replicated in avian-origin and mammalian-origin cells. Using biochemical assays, I determined the effect of the changes at position 226 on receptor recognition and found that changes at this position do influence the specificity of the variant virus for cellular receptors. Some single amino acid changes restricted binding of variants to avian receptors or human receptors while some others expanded the receptor recognition to both human and avian receptors.

Future prospects

Based on these results, future prospects and studies should include:

1. ***Effects of mammalian adaptation and reassortment on replication and transmission.***
    a. I tested the replication and transmission of the mammalian adapted reassortant viruses in quail. Future studies can include other avian species including aquatic and land based poultry.
    b. I observed a lack of replication and transmission 1P10 reassortant virus in quail due to the lack of balance between the HA and NA genes. Serial passage of this reassortant virus in quails to yield a transmissible virus can be carried out to identify the molecular changes needed to shift the HA-NA balance such that this reassortant virus is able to transmit.
    c. The transmission studies done focused on direct contact transmission. Future studies into the effect of the mammalian-adapted mutation on indirect (airborne) transmission should be considered particularly for the viruses that showed contact transmission.
2. ***Effects of amino acid changes at the RBS of an H9 virus***
    a. In the studies described in Chapter 4, I focused on only one position in the 220 loop of the receptor binding site – position 226. The RBS is composed of other structures such as the 130 loop and the 190 helix. Changes to amino acids in these structures could alter the virus-receptor engagement. Future studies should include mutagenesis of other single positions in any of these 3 main structures or combination of amino acids to determine and identify receptor binding motifs and its effect on the virus phenotype.

b. The in-vivo fitness studies carried out in quail identified amino acids that possess dual receptor specificity, which could be a function of the quail host that carry both α2,3 and α2,6 sialic acid receptors. To identify if there are any species related preference to amino acid usage at position 226, a similar fitness study can be carried out in other land-based poultry such as turkeys, which have historically been infected with H9N2 viruses, or in aquatic birds.

c. In this study I identified variants with asparagine (N226) and valine (V226) both of which were capable of binding both 2,3 and 2,6 sialic acid in the context of an H9 virus. These variant viruses can be tested in mammalian models to determine if the dual binding phenotype can allow for transmission by direct or airborne routes.

d. This study further identified amino acids that are exclusively bind α2,3 receptors in particular cysteine (C226) and histidine (H226). Recent H3N2 exhibit poor growth in established substrates (embryonated eggs), which has been linked to binding of only long branched receptors. Introduction of either of these amino acids in the context can be done to determine if mutating position 226 can rescue the replication and agglutination phenotype of these H3 viruses.