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Experimental Evolution of Human Influenza Virus Hemagglutinin (H3) in the Mouse Lung

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**Experimental Evolution of Human Influenza Virus Hemagglutinin (H3)
in the Mouse Lung**

By

Liya Keleta

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Faculty of Graduate and Postdoctoral Studies
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for the degree of Doctor of Philosophy

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Abstract

Molecular basis of Influenza A virulence is not fully understood. This study focuses on the Hemagglutinin (HA) protein since it is known to be a critical determinant of virulence. The experimental approach used was mouse adaptation. The prototype clinical isolate A/HK/1/68 (H3N2) was subjected to serial mouse to mouse passages. Following longitudinal and parallel studies 11 mouse adapted populations were generated. Sequence analysis of all 11 populations identified a total of 24 mutations within the HA gene. These mutations clustered in two areas within the 3 dimensional structure. One adaptive region resides within the HA1 while the other is located in the HA2 domain. Four of the mouse adapted HA mutations exhibited evidence of convergent evolution. Three of these mutations (P162L/S, Q210R and G218W/E) reside in HA1 while one mutation (N154S/K) is located in HA2. Recombinant viruses possessing convergent HA mutations exhibited altered receptor binding and pH of fusion. These mutations increased infection and replication within the mouse lung (in vivo) and/or mTECs (in vitro). However, different infection patterns were observed indicating that distinct α 2,3 SA receptors might be present in the tracheal, bronchial and alveolar cells. In addition, adaptive mutations in HA1 as well as HA2 were associated with enhanced virulence. Certain mouse adapted mutations parallel changes observed in other virulent variants. This identifies them as putative virulence determinants. Hence, these mutations can serve as predictors of virulence.

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I dedicate this thesis to my parents.

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Introduction

1.1. Pathogenesis of Influenza A

In humans, influenza A virus causes acute infection of the respiratory epithelium. Non-fatal infections primarily affect the upper airways, while fatal cases are associated with infection of the lower airways (110). Seasonal influenza occurs annually in Winter months where complications from the virus cause 4000 to 8000 deaths in Canada (Public Health Agency of Canada). The virus is transmitted from person to person through aerosolized droplets or from touching contaminated surfaces. Adults shed virus starting 24 hours prior to and approximately five days following the onset of symptoms. On the other hand, children are able to shed virus for over 10 days while individuals with weakened immune systems are even more contagious, shedding virus for weeks or even longer (36).

The outcome of infection can range from asymptomatic to severe illness causing death. Symptoms consist of fever, headache, cough, muscle ache, malaise, anorexia and diarrhea, which is usually associated with children (12, 123). Certain groups such as the elderly, children, pregnant woman and people with underlying diseases are at a greater risk from infection. In children, infection can lead to complications such as croup, reye's syndrome and otitis media (6, 110, 125). Infection of individuals with diabetes, pre-existing respiratory or cardiovascular illnesses can lead to complications such as hemorrhagic bronchitis and

pneumonia (110). Influenza infection can result in primary viral pneumonia, secondary bacterial pneumonia or combined viral-bacterial pneumonia. Secondary bacterial pneumonia caused by species such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae* tends to be the most common (15). Interestingly, proteases secreted by *Staphylococcus aureus* are known to be involved in the cleavage activation of HA0. This indicates that the co-infecting bacteria can play a role in enhancing the pathogenicity of the virus (108). Other complications associated with influenza include toxic shock syndrome, renal failure, encephalopathy, myocarditis and pericarditis (62).

For the last 50 years avian viruses such as H5N1, H7N7, H7N3 and H9N2 have jumped the species barrier and infected humans (88). Most of these viruses cause mild symptoms with some fatal cases except for H5N1 that has emerged as a possible candidate for a pandemic with a mortality rate of 61% (WHO). Since 1997 the virus has spread from China to various other Asian countries, Europe and Africa. The main source of transmission has been infected poultry but limited human to human transmission has been documented (88). The incubation period for this virus is 2 to 8 days, which is long compared to seasonal influenza (36). Disease ranges from mild cases to severe pneumonia that progresses to death (88). Enhanced replication and an intense cytokine response are thought to be important aspects of H5N1 pathogenesis (26). The majority of the patients displayed influenza-like symptoms, which

include fever, cough, myalgia and pneumonia. In addition, many of the patients exhibited gastrointestinal symptoms like diarrhea and vomiting. In fatal cases, complications consisted of acute respiratory distress syndrome (ARDS) and multi-organ failure (25).

H5N1 has the ability to infect the upper as well as lower respiratory tract. However, studies suggest that the virus might replicate more efficiently within the lower respiratory tract. Isolation of the virus from blood and cerebrospinal fluid of critical cases suggests disseminated infection. In some patients, diarrhea preceded other symptoms and virus was isolated from feces pointing to the gastrointestinal tract as a possible replication site for H5N1 (1, 25). In addition, viral RNA has also been isolated from the placenta and fetus identifying a new route of transmission (1).

1.2. Virus classification

Orthomyxoviridae comes from the Greek words *orthos* and *myxa* which translate to standard and mucus respectively. This family consists of RNA viruses containing segmented, single-stranded and linear negative-sense genomes. The classification of negative-stranded viruses comes from the observation that the genomes of these viruses are complementary in sequence to the messenger RNA (mRNA), which by convention is considered positive stranded (62).

The family *orthomyxoviridae* consists of five genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, *Thogotovirus* and *Isavirus* (42). The Influenzaviruses are differentiated based on the antigenic properties of the nucleoprotein (NP) and matrix (M) proteins (117, 25). Host range is another distinguishing factor with influenza A viruses infecting a wide range of species such as birds and mammals including humans, while Influenza B and C infections are generally restricted to humans. The genomes of Influenza A and B consist of eight linear segments compared to Influenza C that have seven. Influenza C viruses have one envelope glycoprotein, the hemagglutinin-esterase-fusion protein (HEF) that is equivalent to the two glycoproteins present in Influenza A and B, the hemagglutinin (HA) and neuraminidase (NA) (62). Influenza A viruses are also divided into subtypes according to the antigenic properties of the surface glycoproteins. Currently 16 HA (H1-16) and 9 NA (N1-9) subtypes of influenza A have been isolated from aquatic birds. However only H1N1, H2N2 and H3N2 have established lineages in humans (9, 25).

Thogoto virus (THOV) and Dhori virus (DHOV) are classified under the genus *Thogotovirus*. The genome of these viruses contains six strands that code for seven proteins. These viruses are distinguished from the Influenzaviruses based on their ability to replicate in ticks and mammalian cells (43). The newest discovery in the family *orthomyxoviridae* is the genus *Isavirus*. Infectious salmon anemia virus

(ISAV) is the sole member of this genus and targets salmonid fish species. The genome of the ISAV contains eight strands of RNA that code for 10 proteins (20).

1.3. Nomenclature of Influenza A viruses

Influenza viruses are identified according to the following nomenclature: the genus of influenza virus, host (except humans), location of origin, strain number, year of isolation and virus subtype (e.g. A/HongKong/1/68 (H3N2)) (25).

1.4. Virus structure

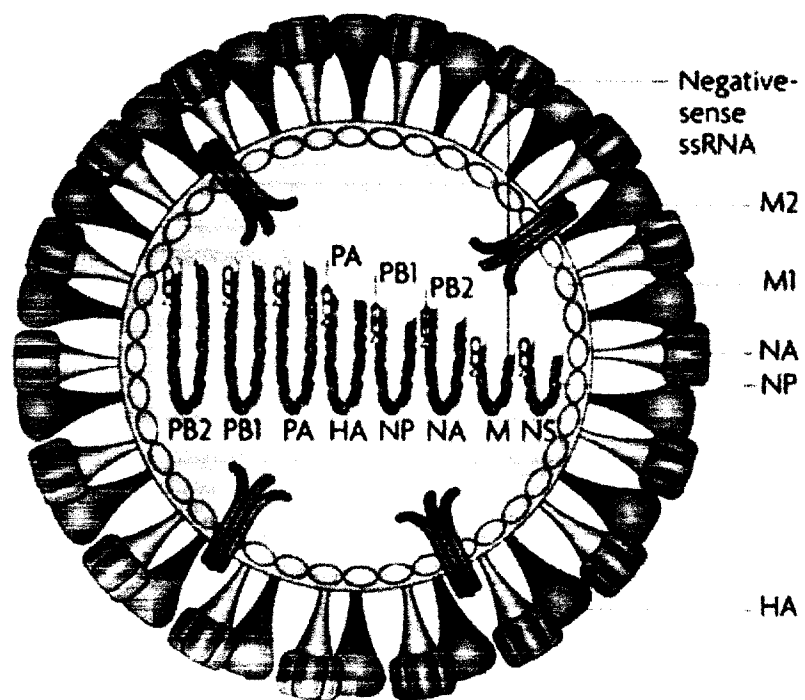
Influenza A virus particles consist of 70% protein, 20% lipid, 5 to 8% carbohydrate and 1% RNA. Influenza viruses have a lipid membrane that is acquired by budding from the host cell at the end of the replication cycle (62). The particles are pleomorphic with the spherical forms being roughly 100 nm in diameter while the filamentous forms are longer and can reach micrometers in length. Early clinical human isolates are mainly filamentous but viruses shift to a more spherical morphology following continuous propagation in eggs or tissue culture (8, 105). Since viruses are obligate parasites, they must replicate in host cells. While several interacting host factors have been identified, most of the current knowledge on viral replication has focused on the structures and functions of the viral components (97).

1.5. Genome Organization

The genome of influenza A viruses consist of 8 segments of viral RNA that code for 11 proteins (18) (Fig 1). The three largest segments encode the polymerase basic 1 subunit (PB1), polymerase basic 2 subunit (PB2) and polymerase acidic subunit (PA) proteins, which makeup the RNA-dependent RNA polymerase complex. These proteins were named according to their characteristics on isoelectric gels, PB2 and PB1 (basic) and PA (acidic) (105). The PB2 protein encoded by segment 1 interacts with 5'cap of the host. These cap structures are subsequently excised by PA to be used as primers in viral mRNA synthesis (12, 27, 117). The cap binding ability of PB2 is activated when the PB1 protein interacts with 5' end of vRNA. Different studies have identified three possible cap binding regions within PB2. These sites consist of residues 242-252, 533-577 as well as a central domain. This domain contains aromatic amino acids Phe363 and Phe404. It is proposed that the aromatic residues sandwich the N- methyl guanine present within the cap structures. The PB2 protein bound to the cap has recently been crystallized. According to this study, minimal cap binding site consists of residues 318-483 (31, 41).

The PB1 protein encoded by segment 2 can be considered the backbone of the RNA polymerase since it contains binding sites for both PB2 and PA located within the C-terminus and N-terminus

Fig 1. Structure of Influenza A virus. The genome consists of 8 segments of RNA that code for 11 proteins
(www.scq.ubc.ca)



respectively (28, 57). The PB1 protein contains conserved motifs I through IV that are present in other RNA-dependent RNA polymerases. These motifs contain one invariant amino acid each which are essential for the activity of the protein, mutating any of these residues results in a loss of function (7, 57). PB1 also serves as the elongation protein by consecutively adding nucleotides during synthesis of the vRNA, mRNA and cRNA (complementary RNA). The SDD sequence located within motif III of PB1 is thought to be important during chain elongation (68, 77, 117). Recently an alternative open reading frame was identified within the PB1 gene. This generated a novel protein called PB1-F2 which is present in some but not all influenza A viruses. This protein localizes within the mitochondrial membrane where it interacts with host proteins such as adenine nucleotide translocator 3 (ANT3) and voltage-dependent anion channel 1 (VDAC1) resulting in the release of cytochrome c which leads to apoptosis (22, 18,122).

PA encoded by segment 3 is a phosphorylated protein (89). It is implicated in the synthesis of vRNA, cap-binding and viral assembly (12, 45, 68). Recent studies indicate that endonuclease activity required for cap snatching is provided by PA and not PB1. This protein excises cap structures 10-13 nucleotides downstream of the 5' end (27). Cleavage of the host 5' cap generates 3'-OH ends which allows these structures to act as primers for transcription (64). The N-terminal end (residues 1-247) of this protein has been shown to possess proteolytic

activity even though the consequence of this function is not understood (44, 65, 80). However, this activity has been implicated in affecting the replication potential of the polymerase (84).

The Hemagglutinin (HA) protein encoded by segment 4 is a glycoprotein that is post-translationally cleaved into two subunits: HA1 and HA2. The HA1 subunit is primarily involved in receptor binding, while HA2 is responsible for fusion between the viral and endosomal membranes. As a result, cleavage of HA is necessary for the virus to be infectious. HA also serves as the major surface antigen that is targeted by the host immune system (12, 117, 119).

The Nucleoprotein (NP) encoded by segment 5 is one of the most abundant structural proteins. A major role is to encapsidate the viral RNA within the nucleus. The encapsidated vRNA along with the polymerase proteins make up the ribonucleoprotein (RNP) complex. Even though all the proteins within the RNP contain a nuclear localizing signal (NLS), NP is sufficient for the import of RNPs into the nucleus. During the life cycle of the virus this protein is essential in the switch from transcription to replication (12, 117, 120). NP is also phosphorylated in a host dependent manner. As a result, phosphorylation might play a role in host range restriction (117).

The Neuraminidase (NA) protein encoded by segment 6 is the second glycoprotein found in influenza A viruses. It facilitates release of newly synthesized progeny by cleaving terminal sialic acid (SA) from

oligosaccharides found on the surface of the cell (12, 40, 117). In addition, NA cleaves SA found on the surface of the newly synthesized viral glycoproteins thereby preventing self-aggregation. It is also involved in removing SA from mucins which aids in facilitating viral spread (12, 40). Furthermore, it is known to play a role in the activation of latent transforming growth factor β (TGF- β) with enhancement of these proteins resulting in the induction of apoptosis (92). It also serves as the second major antigen that is targeted by neutralizing antibodies (25).

The Matrix (M1) and M2 proteins are encoded by segment 7 through alternative splicing (12). The M1 protein is the most abundant structural protein found within the virion. It forms a shell beneath the host derived lipid envelope separating it from the core of the virus (63, 117). M1 is essential in the export of newly synthesized RNPs from the nucleus of infected cells. This involves M1 binding RNPs as well as the nuclear export protein (NEP) through its C-terminus and NLS respectively (79). Hiding the NLS on the M1 protein through NEP binding is important in preventing the newly exported RNPs from re-entering the nucleus (2). M1 is also important in viral assembly, the M1/RNP complex arrives at the budding site where M1 interacts with the cytoplasmic tails of HA and NA (12, 37).

The M2 protein is an integral membrane protein that functions as an ion channel. One role of M2 is to pump protons into the core of the

virus resulting in the dissociation of protein-protein interactions. This acidification results in the disruption of the M1/RNP interaction and furthermore causes a conformational change in HA resulting in fusion and release of RNPs into the cytoplasm (12, 50, 117). M2 is also involved in modulating the pH within the Golgi apparatus during maturation of the HA protein. This is important in preventing pre-mature acid-induced conformational change that could occur in the HA before it reaches the plasma membrane (50, 117). M2 has also been shown to bind cholesterol suggesting raft association and a possible role in the assembly and budding process (91). Both the M1 and M2 proteins have also been implicated in determining the morphology of influenza A virions (85).

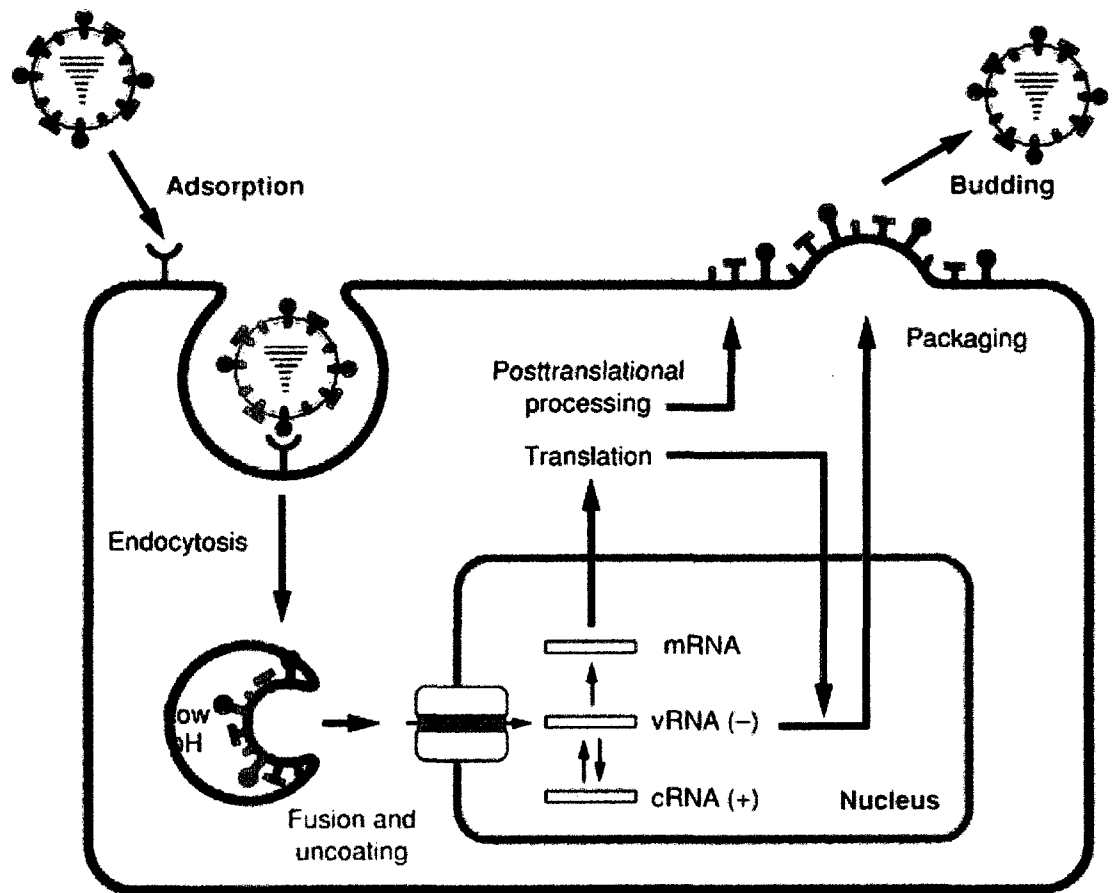
Non-structural proteins NS1 and NEP (previously referred to as NS2) are encoded by segment 8 through alternative splicing (117). NS1 is a multifunctional protein that has a variety of functions in enhancing replication. It inhibits induction of interferon by preventing activation of interferon regulatory factors (IRF3 and IRF7) and NF κ B; reduces the anti-viral state by inhibiting activation of protein kinase R (PKR) and 2'-5' oligoadenylate synthetase; inhibits cellular mRNA maturation and export from nucleus by interacting with cleavage and polyadenylation specificity factor (CPSF) and poly (A)-binding protein II (PABPII); enhances viral protein translation by recruiting poly (A)-binding protein I (PABPI) and eukaryotic translation initiation factor 4GI

(eIF4GI) to the 5' untranslated region of viral mRNA; it increases viral replication by activating phosphatidylinositol 3-kinase (PI3K) and inhibits apoptosis (44, 60). On the other hand, NEP is involved in the export of the newly synthesized vRNP's by using the host nuclear export machinery as stated above (79).

1.6. Influenza A replication

Attachment of the virus to target cells is initiated when the activated HA binds to surface receptors (Fig 2). Sialic acid (SA) found at the terminal end of oligosaccharides serve as receptors for influenza A viruses. Once bound the virion enters the cell mainly through clathrin-mediated endocytosis even though a nonclathrin, noncaveolae mediated entry has also been observed (96, 117) . After internalization, the drop in pH within the endosome (pH 5 to 6) activates the M2 ion channel causing transfer of protons into the virus. This acidification causes disruption of the M1/RNP interaction which frees the RNPs for release to the cytoplasm (50, 98). The drop in pH within the endosome also induces a structural change in HA. This in turn exposes the amino terminal fusion peptide. Once exposed the peptide gets embedded into the target membrane and initiates fusion of the two opposing membranes (viral and endosomal). This allows the free RNPs to be released into the cytoplasm and eventually migrate towards the nucleus of the infected cell (12, 117).

Fig 2. Influenza A replication cycle
(192.207.64.1/field_research/flu.htm)



Once in the nucleus the vRNPs are transcribed into mRNA by a process that is primer-dependent. This involves 'cap-snatching' whereby host cap structures are excised and used as a primer for the synthesis of viral mRNAs (12, 76). Synthesis proceeds until the polymerase is blocked by hindrance when it reaches a polyuridine (5 to 7) stretch at the 5' end of template vRNA. The polymerase then stutters resulting in the addition of a poly (A) tail to the newly synthesized mRNA (76). The mRNA's are then exported into the cytoplasm where viral proteins are synthesized at the expense of the host. Influenza A gene expression is tightly regulated where NP and NS1 are synthesized early while HA, NA and M1 are synthesized late in infection (117).

In contrast to transcription, genome replication of influenza A virus is primer independent. The switch from mRNA to cRNA synthesis requires high levels of free NP which is needed to encapsidate the cRNA. The newly generated cRNA (+ stranded) can then serve as a template for the synthesis of vRNA (- stranded). In turn, the vRNAs which are also encapsidated serve as a template for secondary transcription or for generation of vRNP complexes (12, 95, 117). Newly synthesized NP and polymerase proteins interact with the vRNA to generate vRNPs. The M1 protein then interacts with the newly assembled vRNP as well as NEP facilitating the nuclear export of the vRNP. The M1-vRNP complexes are then transported to the plasma membrane where M1 interacts with the cytoplasmic tails of the surface

proteins (HA, NA and M2). These envelope proteins are transported to the assembly site (lipid microdomains) via the endoplasmic reticulum and golgi apparatus. After assembly, release of the viral progeny is facilitated by the NA protein (95).

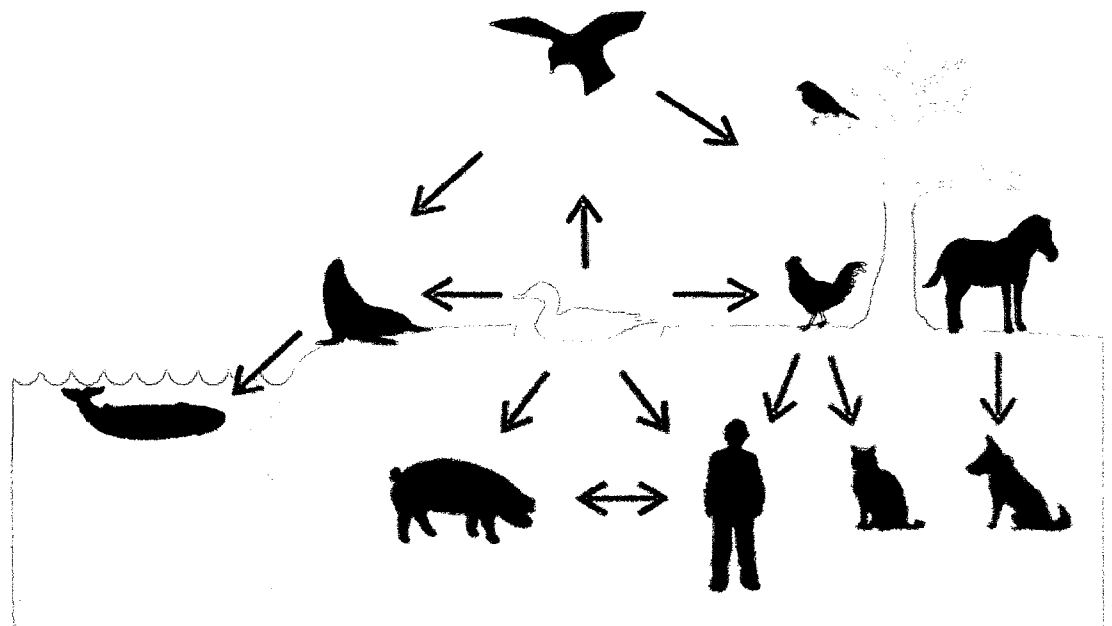
1.7. Host range of Influenza A viruses

All subtypes of influenza A can be isolated from aquatic birds. As a result, these birds serve as the natural reservoir (Fig 3) (117). Influenza A viruses are host specific. However, these viruses can become adapted to new hosts (94). For example, equine viruses (H3N8) have been shown to infect dogs and adapt to this new host (83). In addition, human strains such as A/HK/1/68 (H3N2) have been adapted to mice (13).

1.8. Influenza A Pandemics

A Pandemic is a worldwide epidemic that occurs when a novel influenza A virus enters a naïve population and causes high mortality (101). There are certain criteria that have to be met for a virus to cause a pandemic. The virus should acquire a novel HA gene, the population should have no immunity to the virus due to previous exposures, it should cause serious human illness and exhibit efficient human-to-human transmission (93). There have been 3 influenza A pandemics in the 20th century.

Fig 3. Influenza A reservoirs
(Fields virology 5th edition)



The first pandemic known as the Spanish flu occurred in 1918 (H1N1) and exhibited high mortality killing close to 40 million people worldwide. The causative agent is thought to be an avian virus that was able to infect and adapt to humans. The next pandemic, Asian flu occurred in 1957 when the H1N1's were replaced with H2N2 viruses. These viruses emerged when the existing human H1N1 gained novel HA (H2), NA (N2) and PB1 genes from an avian H2N2 virus. The last pandemic, Hong Kong flu occurred in 1968 with the emergence of the H3N2 viruses. This occurred when the human H2N2 gained novel HA (H3) and PB1 genes from an avian H3Nx virus. Both these pandemics caused close to 2 and 1 million deaths worldwide, respectively (93, 101). During the 1918 pandemic mortality was high in young people, in comparison the 1957 and 1968 pandemics targeted children and the elderly. Currently, H3N2 along with an H1N1 virus that re-emerged in 1977 co-circulate in the human population (101). The avian H5N1 viruses that have jumped the species barrier and infected humans since 1997 are thought to be the likely candidates for causing the next pandemic. These viruses have already met three of the four criteria with the exception of human-to-human transmission (93). However, limited human-to-human spread has been observed within clusters among family members (56). These viruses can acquire efficient transmissibility through two possible routes: reassortment with a human virus or adaptation (25). Studies indicate that certain viruses have already

acquired human adaptive mutations, however these viruses still exhibit inefficient transmission indicating that further adaptive changes are required (4).

1.9. Hemagglutinin

1.9.1 HA Synthesis

Hemagglutinin (HA) is a type I glycoprotein that is synthesized as a precursor (106, 119). The primary structure includes an N-terminal ectodomain, a transmembrane region and a cytoplasmic domain (Fig 4). The protein is synthesized using host translational machinery and translocated into the endoplasmic reticulum (ER). Once in the ER it is modified by removal of the signal peptide, generation of disulfide-bonds and core glycosylation. N-linked glycosylation is essential for proper folding of the protein however the number and location of the sites vary depending on the viral strain (23, 113, 117). The protein is also assembled into trimers within the ER before being transported to the golgi apparatus en route to the plasma membrane. Oligosaccharide trimming, terminal glycosylation and palmitoylation all occur within the golgi apparatus (113, 23). Palmitoylation involves the addition of palmitic acid to conserved cysteine residues (positions 555, 562 and 565) located near the carboxy terminus of the protein. The palmitate moieties appear to be important in targeting the proteins to lipid microdomains

Fig 4. Primary Structure of Hemagglutinin
(Chen et al 2005)



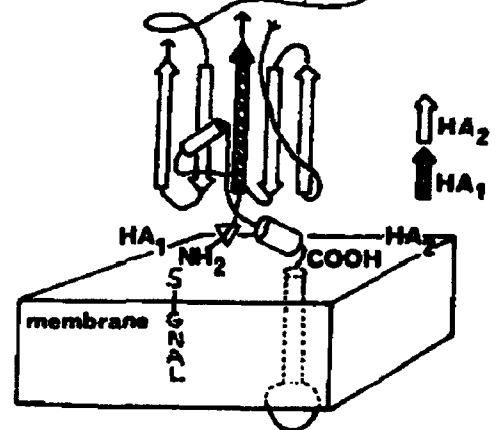
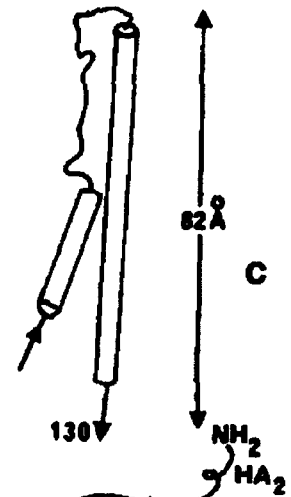
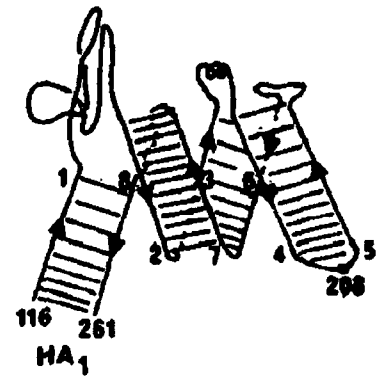
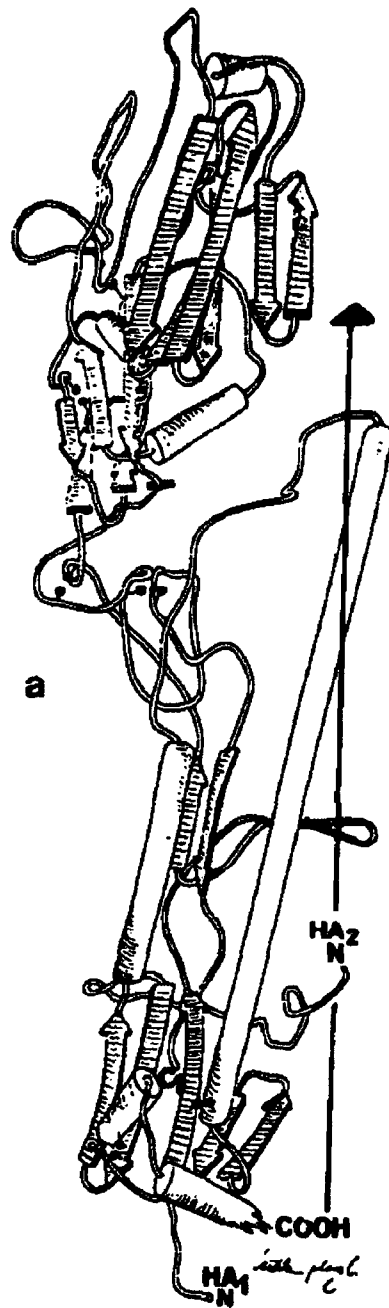
which is necessary for assembly and budding (23). The final processing step involves cleavage activation of the precursor protein (117). The precursor protein (HA0) needs to be cleaved into HA1 (324 residues) and HA2 (222 residues) in order for the newly synthesized virus to be infectious. This can occur once the protein reaches the cell surface or after the viral progeny is released (98, 109). The proteases responsible for HA0 cleavage differ based on the HA subtype. The HA's of the human pandemic viruses (H1, H2 and H3) are cleaved by serine proteases that recognize the motif Q/E-X-R. These enzymes are secreted by cells of the bronchial epithelium therefore replication of the virus is restricted to the respiratory tract. On the other hand, HA's of certain avian viruses (H5 and H7) are cleaved by ubiquitous intracellular furin-like proteases causing systemic infection in domestic poultry. These proteases recognize polybasic residues (R/L-X-R/L-R) that are inserted at the cleavage site of these proteins. One factor that can affect activation is the existence of glycosylation sites near the cleavage loop with removal of these sites causing greater accessibility of the loop (98, 105, 109). The cleavage step is important since it liberates the fusion peptide and results in a structure that is primed to undergo conformational changes necessary for fusion upon exposure to acidic pH. The uncleaved and cleaved HA structures are mostly superimposable except for minor differences.

In HA0, there is an empty cavity within the interior of the trimer that is lined by ionizable residues but upon cleavage the resulting hydrophobic fusion peptide (N-terminus of HA2) occupies this cavity and buries the ionizable residues (104).

1.9.2 HA monomer

Each monomer of HA consists of a globular head and a stem region measuring 135Å from the viral envelope (Fig 5). The globular domain is composed entirely of HA1 residues and its dominant feature is an eight-stranded antiparallel beta sheet. The receptor-binding pocket and the antibody-binding sites (A to E) are all located within this domain. On the other hand, the stem region consists of HA1 as well as HA2 residues. The most notable structure in this region is an α -helical hairpin which consists of amino acids (aa) 36-130 of HA2. The second helix of each monomer measures 82 Å long and forms a trimeric coiled coil that functions to stabilize the molecule. The hydrophobic fusion peptide is hidden within these alpha helices in the native HA. The peptide measures 100Å from the top of the molecule and 35Å from the C-terminal end. The last feature within the monomer is a five stranded beta sheet located near the membrane, the middle strand consists of the amino terminal of HA1 while the adjoining strands are composed of the carboxy terminal of HA2 (30, 119).

Fig 5. Structure of the Hemagglutinin Monomer
(Wiley and Skehel 1987)



1.9.3 Receptors binding site

The primary receptor-binding site (RBS) located in the globular domain of HA1 is composed of distinct secondary structures (Fig 6); 130 loop (aa 135-138), 190 helix (190-198) and 220 loop (aa 221-228) (23, 34). As mentioned above, Sialic acid (SA) terminating glycoproteins and glycolipids serve as receptors for influenza A viruses. Avian viruses recognize SA linked to galactose by an α 2,3 (SA α 2,3Gal) linkage while human viruses prefer SA α 2,6Gal (Fig 7). This is consistent with the observation that the enteric tract of birds which serves as the replication site of avian viruses contains abundance of α 2,3 linked receptors, while human respiratory tract which is the major site of replication for human viruses contains α 2,6 linked receptors. However, SA distribution is complex with α 2,6 linkages found predominantly on non-ciliated cells of the human respiratory tract while ciliated cells contain adequate amounts of α 2,3 linkages to support infection by avian viruses (23, 34, 70, 106). Interestingly, pig trachea which is the replication site of swine viruses contains α 2,6 as well as α 2,3 linked receptors. As a result, pigs can support replication of human and avian viruses and serve as mixing vessels for the production of novel reassortants (23, 34, 106). However, the receptor binding specificity of influenza A viruses goes beyond the identity of the terminal SA linkage. Even though avian viruses prefer SA α 2,3Gal receptors, affinity studies indicate that binding is affected by the identity of the inner saccharides of polysaccharide chains (32).

Fig 6. Receptor Binding Site
(www.ncbi.nlm.nih.gov/bookshelf/picrender)

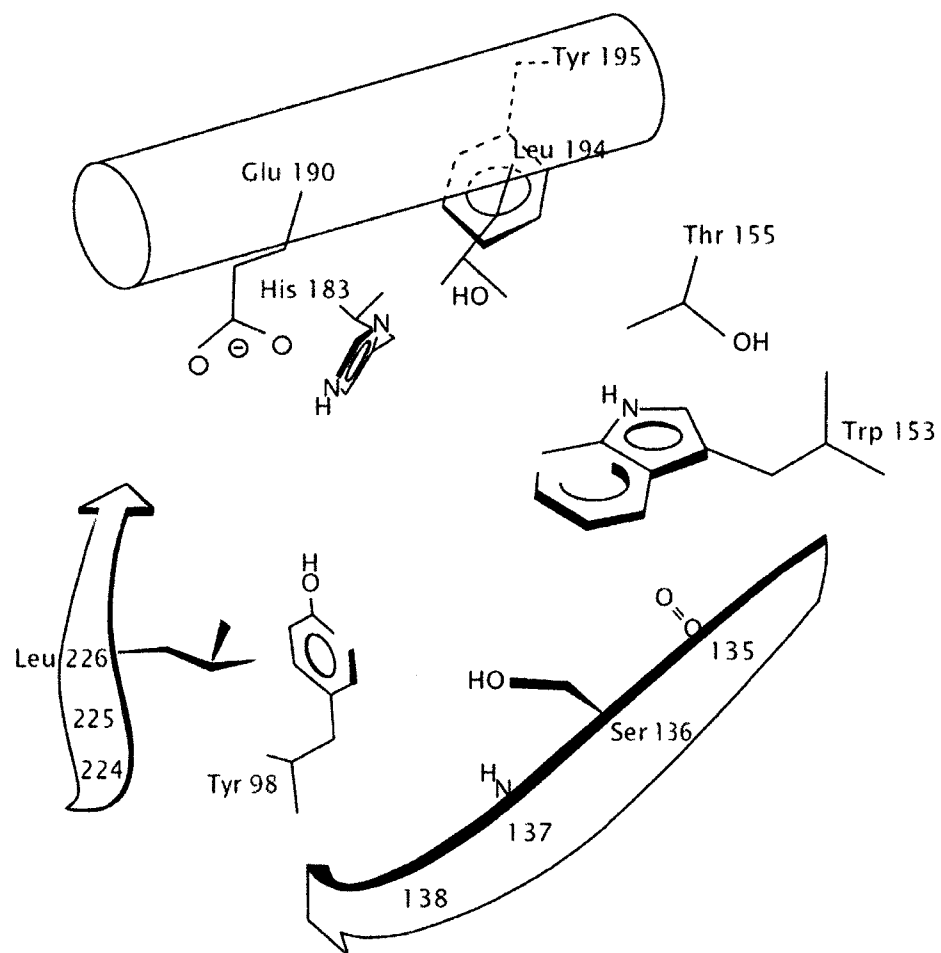
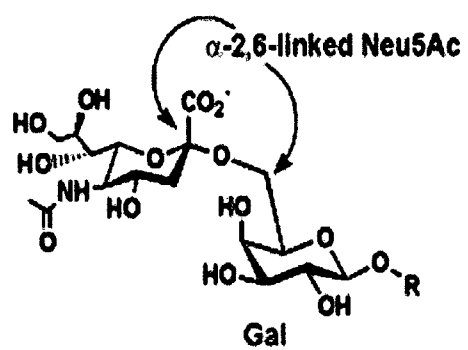
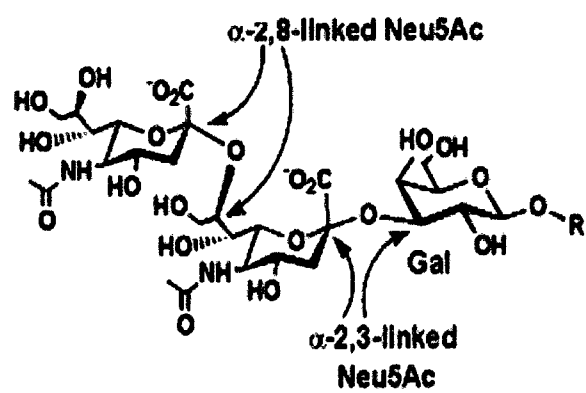


Fig 7. Different Sialic Acid Linkages
(effectmeasure/2006/10/influenza)

A



B



Another study indicated that the structural topology of the receptors plays an essential role in addition to the SA linkages. Taken together these findings indicate that receptor binding specificity depends on the SA structure as well as adjacent carbohydrate components (17).

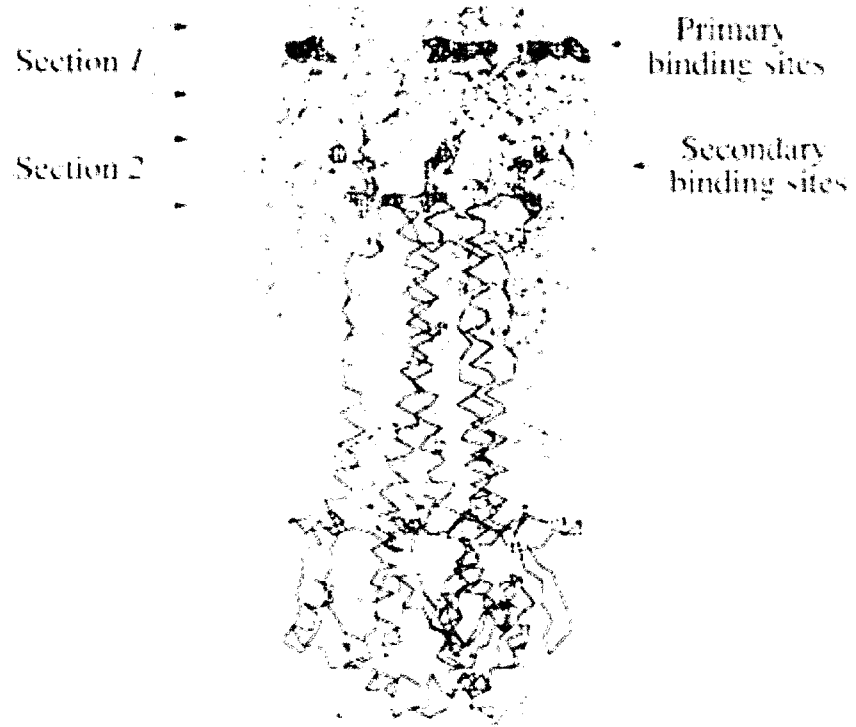
1.9.4 Receptor binding specificity

A change in receptor binding specificity appears to be one of the requirements for an avian virus to adapt to a human host. The amino acid changes that result in the shift from α 2,3 to α 2,6 binding are different based on the HA subtype. The Q226L + G228S (H2 and H3) and the E190D + G225E (H1) mutations located within the RBS are the minimum changes that are required to enhance the affinity of these viruses for human receptors (34, 69). The H5 human viruses currently circulating have the ability to bind both 2,3 and 2,6 linked receptors. Studies indicated that changes such as N186K, Q196R and S227N (H3 numberings) found in the H5 viruses were correlated with their ability to bind human receptors. However, these viruses did not show efficient human-to-human transmissibility (33, 121).

1.9.5 Secondary binding site

In addition to the primary RBS, a second sialoside binding site exists within the HA (Fig 8). While the primary site is located within the globular domain composed entirely of HA1, the second site is located at

Fig 8. Influenza A Secondary Binding site
(Sauter et al 1992)



the interface between HA1 and HA2 subunits. Studies indicated that while $\alpha 2,3$ linked receptors bound to both sites their affinity for the second site appears to be 4x weaker (90, 98). However, there is no proof that this site is physiologically significant. In addition, its inability to bind $\alpha 2,6$ containing receptors casts doubt on its relevance (98).

1.9.6 Antibody binding site

Comparison of sequences between naturally occurring viruses and antigenic mutants identified five domains (A to E) as antibody binding sites. These regions are located at the globular domain of HA1 where they surround the receptor binding pocket (Fig 9). Since these sites are in close proximity, some mutations can affect both receptor binding specificity and antibody binding (119). In general, novel antigenic variants that can re-infect the population and cause an epidemic contain a minimum of four mutations in two or more antibody binding sites (124). One factor that affects resistance is the generation of novel glycosylation sites within the antibody binding domains. The resulting carbohydrates mask the antigenic sites protecting the virus from neutralizing antibodies (98, 119).

1.9.7 Membrane fusion

The HA2 domain is involved in fusion between the viral and endosomal membranes (Fig 10). Infection starts when virus binds sialic

Fig 9. Antibody binding sites within the Hemagglutinin. The five antibody binding sites are labeled A-E
(www.ncbi.nlm.nih.gov/bookshelf/picrender)

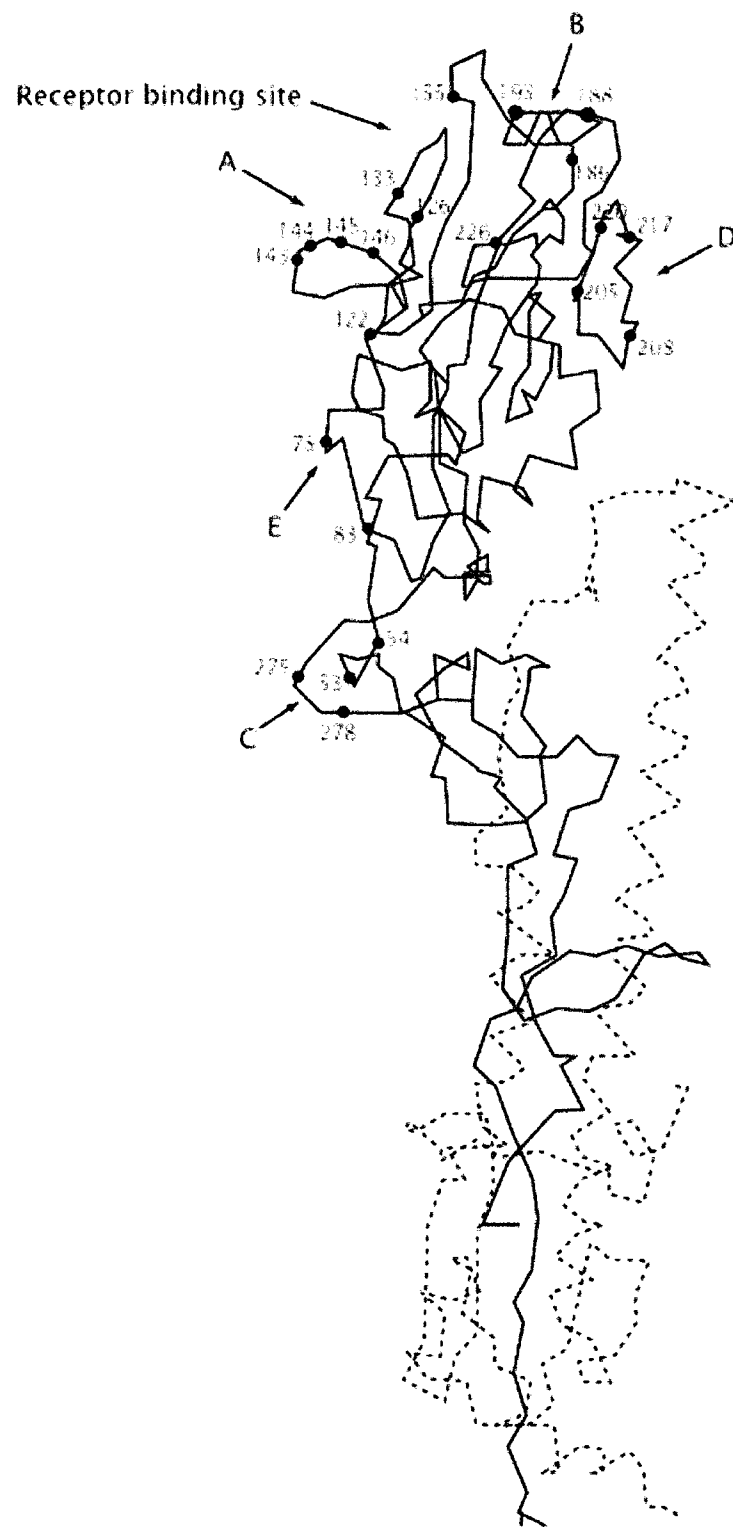
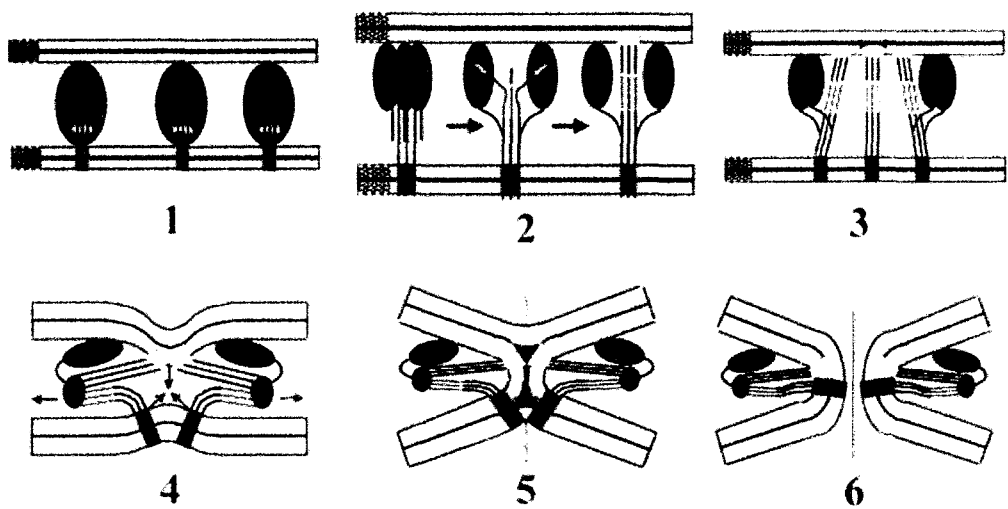


Fig 10. Mechanisms of membrane fusion
(www.healthsystem.virginia.edu/internet/resear.)

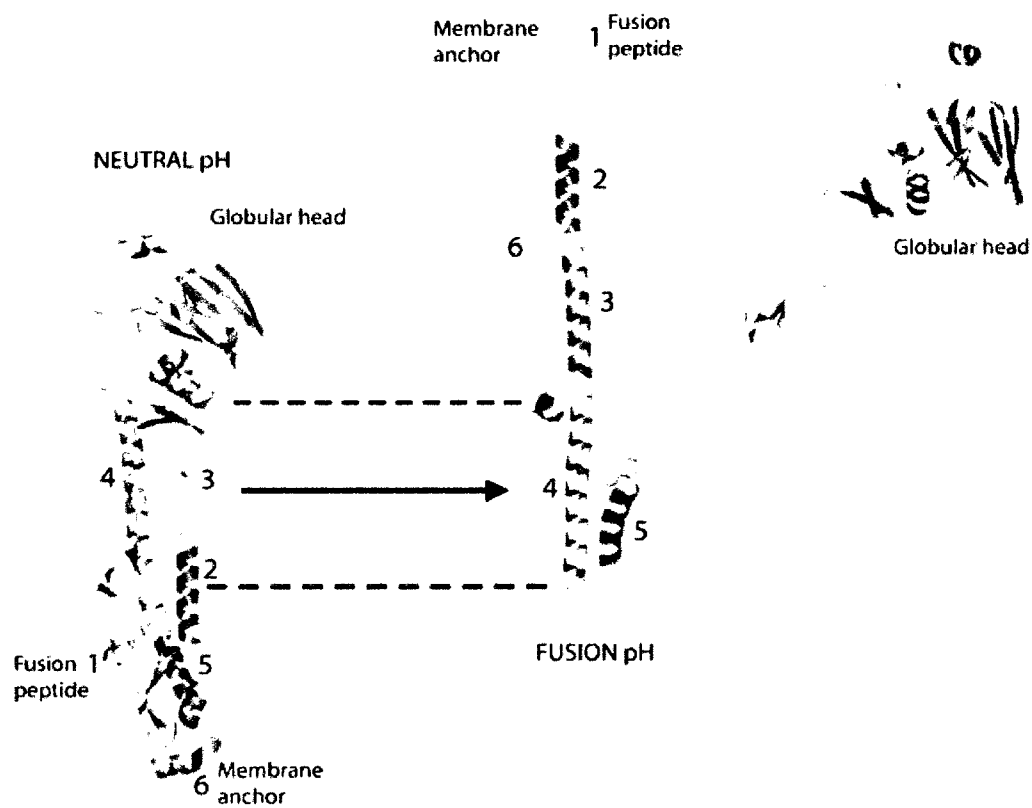


Proposed sequence of events in pH sensitive hemagglutinin membrane fusion

acid and enters the cell through receptor mediated endocytosis. Once in the endosome there is a drop in pH (between 5 and 6) which results in the activation of the M2 ion channels. This allows protons to enter the virion which cause conformational changes within the HA (50, 98). Exposure of the virus to low pH disrupts HA1 and HA2 interactions causing several structural changes.

One consequence of low pH is the dissociation of the HA1-HA1 interactions at the distal globular domain of the trimer. One of the major structural changes is referred to as the spring-loaded model and involves a loop to helix change in residues 55 to 75 of HA2 (30, 116) (Fig 11). The consequence of this change is the extension of the coiled coil which causes the translocation of the fusion peptide 100Å from the initial hidden position towards the target membrane. The exposed peptide is then close enough to be embedded into the target membrane. Another modification deals with a helix to loop switch in residues 106-112 of HA2 which are located in the proximal end of the triple stranded coiled coil. This causes amino acids at the C-terminus of the helix to reverse direction and end up anti-parallel to the coiled coil. As a consequence, the N and C-terminal regions end up on the same side of the structure (23, 30). One consequence of the helix to loop transition is the partial bending of the trimer. When several trimers bend concurrently the action brings the viral and target membranes close together. Studies indicate that a successful fusion requires 3 to 4 trimers at the site. Once the

Fig 11. Low pH form
(Fields virology, 5th edition)



membranes are close together, a short lived hemifusion intermediate is generated. In this intermediate, the outer bilayers of the opposing membranes fuse while the inner bilayers are pulled together generating a dimple. This diaphragm expands until the structure is destabilized and breaks forming a pore. The initial pore flickers before it dilates allowing release of the vRNPs into the cytoplasm of the infected cell (23, 118).

1.10. Genetic determinants of enhanced virulence

Virulence refers to the measure of the ability of a microorganism to infect and cause disease in the host. Currently, the molecular basis for increased virulence of influenza A viruses is unclear (13). However, several studies indicate that virulence is multigenic (10, 54, 104). The HA protein is considered to be a critical determinant of virulence. The identity of the HA0 cleavage site plays a role in determining pathogenicity of avian influenza viruses. As mentioned above, the cleavage site of avian viruses (H5 and H7) contain a series of basic residues which makes them susceptible to ubiquitous proteases. This allows the viruses to replicate in a wide range of tissues causing systemic infection within the host (104, 123). All human H5N1 viruses have polybasic residues in their cleavage site. However, not all infections by highly cleavable HA's are pantropic which suggests that other factors affect the pathogenicity of these viruses in humans (61).

Interestingly, mouse adapted A/WSN/33 (H1N1) causes systemic infection in mice even though it's HA cleavage site lacks the poly-basic residues present in the highly virulent avian viruses. This was attributed to the ability of the NA to sequester plasminogen that can be activated to plasmin which cleaves HA. The wide distribution of plasminogen facilitates cleavage of HA in a variety of organs and identifies WSN NA as a virulence determinant in mice (38, 104). Studies performed using the 1918 (H1N1) pandemic virus also showed that the NA affected HA cleavability. However, this virus lacks the polybasic residues in its HA cleavage site and it's NA is unable to bind and sequester plasminogen. The mechanism by which 1918 NA affects HA cleavability has yet to be determined (112).

The PB2 protein has also been implicated as a virulence determinant in mice. Human H5N1 isolates containing the E627K mutation in the PB2 gene were shown to be highly pathogenic in mice. This substitution allows the viruses to replicate efficiently within the upper respiratory tract which exhibits lower temperatures. However, only some viruses from fatal cases have this mutation which indicates that it is not a requirement for enhanced virulence in humans (61). Interestingly, most human H5N1 isolates that lack this mutation contain an alternative change at residue 701 (D701N). This mutation compensates for the lack of E627K and supports replication of these viruses in mammalian cells (26, 102). It functions to improve the

interaction with mammalian importin 1 α thereby increasing nuclear transport of PB2 (102). Even though these mutations within the PB2 appear to be essential for pathogenicity in a mammalian host, studies suggest possible roles for all the polymerase proteins (87).

The recently discovered protein PB1-F2 plays a role in pathogenicity by causing apoptosis of macrophages. As a result, these cells are unable to induce an immune response which in turn delays viral clearance. The destruction of these immune cells can also enhance the possibility of secondary bacterial infections (21). Mice infected with A/Puerto Rico/8/34 (PR8) and challenged with *Streptococcus pneumoniae* showed an enhanced induction of pneumonia and a higher mortality rate. This study also showed that the secondary bacterial pneumonia is exacerbated when the PR8 PB1-F2 was replaced with 1918-like PB1-F2. This might explain the high prevalence of pneumonia seen throughout the 1918 pandemic and the incomparable human virulence of this virus (71).

NS1 is also essential in virulence since it shields the virus from the host interferon (IFN) response, through its interactions with dsRNA and retinoic acid inducible gene I (RIG-I) (72). Recently, the C-terminus of NS1 has been recognized as a new virulence determinant. Studies indicated that four amino acids within the C-terminus region of these proteins serve as a PDZ domain ligand (PL). Proteins containing this motif have been shown to affect several signaling pathways. Studies

indicate that PL of human viruses show weak binding to human PDZ domains, in contrast PL of avian viruses show strong binding which can result in the disruption of many pathways (54, 81).

1.11. Evolution of Influenza A viruses

Influenza A viruses evolve rapidly since the RNA polymerase lacks proofreading capacity. During replication 10^{-4} errors/site are observed for RNA versus 10^{-9} for DNA polymerases (103, 124). As a result, RNA viruses exist as quasispecies which are genetically distinct but closely related isolates that comprise mutant swarms (103). Evolutionary rates are different between avian and mammalian influenza A viruses indicating differences in environmental pressure. Avian viruses appear to be in an evolutionary stasis where they acquire nucleotide changes that do not result in amino acid substitutions. This indicates that the viruses are adapted to their environment and amino acid changes may no longer be advantageous in the natural environment. In contrast, mammalian viruses are constantly evolving in response to environmental changes (117). Evolution of these viruses can be driven by point mutations, reassortment or recombination. During replication viruses accumulate point mutations throughout their genome. When these changes occur in the envelope glycoproteins (HA and NA) the process is referred to as antigenic drift. Eventually, these changes are sufficient enough that the virus escapes neutralization by pre-existing

antibodies. These antigenic variants can re-infect and cause an epidemic in a population that was previously protected (103, 124).

When a cell is infected with two different viruses, the resulting progeny contain genes from both parents. This reassortment event is attributed to the segmented nature of the viral genome and is referred to as antigenic shift when it involves exchange of surface glycoproteins. This event allows the virus to make a large evolutionary jump since the gene segments can evolve independently before coming together. In conclusion, this mechanism can generate antigenically novel strains that could potentially cause a pandemic, similar to the viruses that caused the 1957 and 1968 pandemics (103, 124). Even though it is rare, recombination plays a role in the evolution of influenza A viruses. Certain viruses have obtained pathogenic traits through insertion of nucleotides within the HA cleavage site. These include viruses such as A/turkey/Oregon/71 and A/seal/Massachusetts/1/80 that have acquired insertions derived from ribosomal RNA and viral NP respectively. Studies also speculate that the HA of the 1918 pandemic virus was a recombinant where its HA1 and HA2 subunits were derived from swine and human viruses respectively (105). A new study also points at homologous recombination as a driving force in the evolution of avian viruses. The results indicated that recombination can occur within or between subtypes and it was observed in most of the gene segments.

However, its effect on virulence and tropism of these viruses has yet to be determined (48).

1.12. Experimental Evolution

As stated above, RNA viruses such as Influenza evolve rapidly. Evolution is affected by several factors including population size (82). Studies conducted on RNA viruses indicate that plaque-to-plaque passages (bottlenecks) prevent competitive optimisation thereby decreasing fitness. This reduction in fitness is associated with large proportions of deleterious mutations and the process is referred to as Muller's ratchet. These genetic bottlenecks are commonly observed in natural infections. On the other hand, large population transfers increase fitness. Passaging population of this size allows competitive selection between the variants. The consequence is gradual optimisation of the population (29).

1.13. Treatment and Prevention

1.13.1 Anti-viral Drugs

Two types of drugs are used for prophylaxis and treatment of influenza viruses. One type referred to as M2 inhibitors (amantadine and rimantadine) are effective exclusively against influenza A viruses. These drugs target the M2 ion channel inhibiting the flow of protons into the virus which interferes with uncoating. Both these drugs are effective

when administered as prophylaxis and aid in reducing the duration of illness when used in treatment (75). However, the major problem with these inhibitors has been the generation of drug-resistant strains. Studies indicate that the resistant strains appear to be virulent and easily transmissible. Currently, the majority of seasonal H3N2 viruses show resistance to these drugs. As a result, their use is limited to H1N1 viruses since these drugs are still effective against most of the circulating strains. With respect to the M2 inhibitors, resistance to one confers resistance to the whole group. Luckily, studies indicate that these resistant variants are vulnerable to neuraminidase inhibitors. In terms of H5N1 viruses resistance to M2 inhibitors tends to be clade specific. The clade 1 and 2.1 viruses show resistance while clade 2.2 and 2.3 viruses appear to be sensitive to these inhibitors (47, 75).

The second class of drugs referred to as NA inhibitors (zanamivir and oseltamivir) are effective against influenza A and B viruses (75). The NA inhibitors (NAI) prevent the release of newly assembled viruses from the surface of infected cells causing aggregation of these viruses at the cell membrane (55). These inhibitors are administered differently, zanamivir (inhaled) vs oseltamivir (oral), however both are effective for prophylaxis and reduce complications when used in treatment. Even though these drugs are not widely used against seasonal influenza, oseltamivir-resistant variants have been identified. However, these occur at a lower percentage than the M2 inhibitor resistant variants. Some NAI

resistant variants exhibit low infectivity while others are fit and easily transmissible. Fortunately, studies indicate that zanamivir is effective against these oseltamivir-resistant variants (47, 75). Studies indicate that the NAIs are also effective against human H5N1 infections if the drug is administered early in infection. However, resistant variants have been identified in patients treated with these inhibitors. In addition, studies with ferrets indicate that a higher dose might be required to combat H5N1 infections in comparison to the amount administered against seasonal influenza. Since H5N1 is thought to cause systemic infection in humans, oseltamivir would be the drug of choice due to its oral bioavailability (75).

In order to deal with the limitations of current drugs several new antivirals have been generated. Currently, intravenously administered NAI, attachment as well as polymerase inhibitors are in clinical development. Even though all these drugs show promise, the ideal weapon against influenza infections might be combinational therapy (47).

1.13.2 Vaccines

Even though antiviral drugs are very useful, vaccines are still the most effective way to prevent influenza infections. The most commonly used is a trivalent inactivated influenza vaccine containing two influenza A (H1N1 and H3N2) and one influenza B virus. This vaccine contains

purified envelope antigens that are administered intramuscularly (111). The second is a live-attenuated influenza vaccine which accounts for <1% of global vaccine production (58). This vaccine is supplied as an intranasal spray and consists of a cold-adapted strain that grows at 25°C. As a result, replication of the vaccine strain is restricted to the nasal passages which are colder than core body temperature. Vaccines are generally produced in embryonated chicken eggs, however vaccines generated in a cell based system are currently under development. Ideally this would save time, minimize contamination and avoid the use of special facilities that are required when dealing with eggs (111).

Vaccines have to be reviewed annually in order to ensure their effectiveness against circulating strains. This is essential in dealing with any antigenic variants that arise. The Global Influenza Network monitors circulating strains to make vaccine recommendations approximately nine months prior to the flu season. Since viruses keep evolving during this time there can be a mismatch between the vaccine and the strains that circulate during the flu season reducing its efficacy. However, it has to be noted that some variants are susceptible to vaccine induced immunity. Lately, the focus has been to generate improved vaccines that can deal with potential antigenic variants. The proposed approaches include adjuvants that could illicit a broader response and the generation of a universal vaccine directed against proteins which exhibit low mutation rates such as M2 and NP (16).

There have been several challenges in developing vaccines against the highly pathogenic H5N1 viruses. One limitation is the requirement of high level containment facilities to work with these viruses. Another issue is the inability to get high yields from chicken eggs since these viruses are highly pathogenic. In addition, the genetic diversity seen within the circulating viruses makes it hard to predict the strain with pandemic potential. However, a split H5 vaccine has been approved by the FDA for use in humans. This vaccine contains HA and NA genes from A/Vietnam/1203/04 while the remaining genes are from A/Puerto Rico/8/34 (65).

Background to this project

Adaptation of influenza to the mouse generates isolates that can cause pneumonia. The lung pathology of the disease observed in mice is identical to the patterns of pathology seen in human infections. Hence, this model has been instrumental in understanding influenza pathogenesis (89). Previous studies in our laboratory involved mouse adaptation of A/FM/1/47 (H1N1). The parental virus was not virulent in mice exhibiting an LD₅₀ of 10^{6.6} plaque forming units (pfu). After 12 passages, the adapted isolate (FM-MA) was virulent with an LD₅₀ of 10² pfu. Upon sequencing, it became evident that the mouse adapted variant had acquired mutations within five (PB1, PB2, HA, NA and M1) of the segments. Further studies using reassortants indicated that each mutation was important in enhancing virulence (11).

While the above study was valuable, A/FM/1/47 has a history of mouse passage. Therefore, A/HK/1/68 (H3N2) that has no previous history in the mouse was utilized for subsequent experiments. When virulence was assessed, prototype A/HK/1/68 turned out to be avirulent with LD₅₀ >10^{7.7} pfu in CD-1 mice. Following 20 mouse lung passages virulent clonal isolates were identified. The passage 12 population exhibited an LD₅₀ of 10^{3.7} pfu. On the other hand, the passage 20 population showed an LD₅₀ of 10^{2.7} pfu. Sequence analysis of clonal isolates identified mutations that were also present in other virulent variants. This identified the mouse adapted mutations as putative

virulence determinants (13). An independent experiment was then performed to further elaborate the complexity as well as extend on these results. Ten sister clones were first isolated from A/HK/1/68. Each of these clones were then subjected to 21 serial lung passages. As a result, eleven mouse adapted populations were available for analysis at the start of this project.

Hypothesis

Identification of mutations acquired during adaptation can give insight into the changes that are required to make a viral strain virulent. This project is going to focus on the biology and fusion properties of the HA gene that are known to affect virulence in human and animal strains.

Objectives

- 1) To sequence the Hemagglutinin (HA) genes of the mouse adapted viruses
- 2) To perform receptor binding and hemolysis assays on the parental A/HK/1/68 and the mouse adapted variants
- 3) To investigate the ability of the parental and mouse adapted variants to infect and replicate in the mouse lung
- 4) To generate recombinant viruses containing convergent HA mutations

- 5) To perform receptor binding and hemolysis assays on the recombinant viruses
- 6) To investigate the ability of the recombinant viruses to infect and replicate in the mouse lung and mouse tracheal epithelial cells (mTEC)

2. Methodology

2.1. Cells

Madin-Darby canine kidney (MDCK) and Human embryonic kidney (293T) cells were grown in minimum essential medium (MEM) containing Penicillin (100 U/ML), Streptomycin (100 µg/ml), L-glutamine (200 mM) and 10% Fetal bovine serum (FBS). These cells were cultured in a 37°C incubator with 4% CO₂.

2.2. Cell Storage

Confluent cells grown in a T75 flask were trypsinized and suspended in 10 ml complete MEM. The cells were spun at 1500 rpm for 5 min (IEC DPR-6000 centrifuge) and resuspended in freezing medium containing: MEM with 40% FBS and 10% Dimethyl sulfoxide (DMSO). The cells were aliquoted into 1 ml cryo tubes (Nunc) and stored at -80°C for a minimum of 6 hrs before transferring them to liquid nitrogen.

2.3. Viruses

Prototype clinical isolate A/HK/1/68 (H3N2) was kindly provided by the Laboratory Centre for Disease Control, Health Canada, Ottawa, ON. The mouse adapted viruses were previously derived in our laboratory as described by Brown et al (13). Briefly, viruses were plaque purified twice in MDCK cells. Stocks were grown in 10 day old specific pathogen free chicken embryo's (Canadian Food Inspection Agency, Ottawa, ON). The virus was then subjected to 20 sequential lung to lung passages. Initially, groups of 3 CD-1 mice (Charles River Laboratories, Quebec) were infected intranasally with 10^6 pfu of the parental virus for 3 days under halothane anesthesia. Groups of 3 lungs were suspended in 3 ml (1 ml/lung) PBS (phosphate buffered saline) and disrupted by sonication. Then 50 μ l of 1/10 diluted lung homogenate was used for subsequent passages. Six clones were then isolated following passage 12 (HKMA12 to HKMA12E) and passage 20 (HKMA20 to HKMA20E).

An independent mouse adaptation experiment was also performed. Ten sister clones were first isolated from A/HK/1/68 (same stock as above). Stocks were then prepared in 10 day old chicken embryos. Upon sequence analysis of segment 4, all the clones exhibited wild type sequence. Each clone was then subjected to sequential lung to lung passages. The experiment was performed in CD-1 mice using 10^6 pfu of virus for passage 1 followed by 50 μ l of filtered (0.22 μ m) lung homogenates for later passages. At the end of the experiment, each clone

has been passaged 21 times under the following conditions: for the first 10 passages 1 mouse was infected for 1 day, for the next 5 passages 2 mice were infected for 2 days and for the last 6 passages 2 mice were infected for 3 days. After passage 21, three clones were derived from each of the 10 mouse adapted populations and purified (via 2 plaque isolations). These clones were designated as HK (sister clone #) MA (mouse passage #)-(clone #).

2.4. Virus amplification

2.4.1. Amplification of virus in MDCK cells

Confluent MDCK cells in 35mm dishes were washed (2X) with 2 ml of warm PBS prior to inoculation. Virus was applied in volumes between 100 and 250 μ l and cells were incubated at 37°C for 30 min to allow adsorption. After adsorption, cells were supplemented with 1X MEM containing 1% Penicillin (100 U/ml)/Streptomycin (100 μ g/ml), 1% L-glutamine (200 mM) and 1 μ g/ml trypsin (Crystallized trypsin, Sigma). The cells were incubated at 37°C until good cytopathic effect was observed. The supernatant was collected and spun down at 1500 rpm for 5 min (IEC DPR-6000 centrifuge) to remove cell debris. The samples were aliquoted and stored at -80°C.

2.4.2. Amplification of virus in Chicken embryos

Fertilized chicken eggs were incubated in a 37°C egg incubator with automatic rocking (Twice/hr cycle) for 10 days. The eggs were candled and the location of the embryo head was marked. Following sterilization with 1% iodine in 70% ETOH, the allantoic cavity of embryonated eggs was infected at this site with 100 µl of the desired dilution before sealing with melted wax. The eggs were incubated at 34°C for 3 days and chilled at 4°C overnight to euthanize the embryo and thus prevent bleeding. The allantoic fluid was collected following surface sterilization and spun at 1500 rpm for 15 min (IEC DPR-6000 centrifuge). The samples were aliquoted and stored at -80°C.

2.5. Washing Chicken Red Blood cells (RBC)

RBC must be washed before use in hemagglutination assay. In a 15 ml conical tube, 10 ml of solution (RBCs collected in an equal volume of Alsever's media) was centrifuged at 1500 rpm for 5 min (IEC DPR-6000 centrifuge). The supernatant and white blood cells were removed with a pasteur pipette and the RBCs were washed 3X in 5 volumes of PBS. After the last wash, the packed cells were resuspended in 9 ml PBS per ml to get a 10% solution. Then 0.5% RBC was prepared by using 0.5 ml of 10% and 9.5 ml PBS. The solution was mixed and stored at 4°C.

2.6. Hemagglutination (HA) assay

The assay was performed using round bottom 96-well plates. 100 µl/well of virus at the desired dilution was added to the first row in duplicates. 50 µl/well of PBS was added to the remaining wells. Two-fold serial dilutions were performed using a semi-automatic minidiluter (Cooke Engineering Co). Once the dilutions were performed 50 µl/well of 0.5% chicken RBCs were added to each well. The samples were mixed and allowed to settle at room temperature for at least 45 min. The HA titres were determined based on the titration end point (represents 1 HA unit).

2.7. Plaque assay

MDCK cell monolayers were grown to confluency in 6-well plates. Virus was serially diluted in cold phosphate buffered saline (PBS). The monolayers were washed twice with warm PBS and inoculated with 100 µl of the diluted virus in duplicate. After a 0.5 hr adsorption of virus at 37°C (rocking the plates after 15 min), 3 ml of overlay was added to the wells. The overlay consisted of 20 ml of 1.3% agarose (100°C), 20 ml of 2XMEM (4°C) [containing 2% of P/S (100X) and 2% L-glutamine (200 mM)], 1.2 ml of 7.5% sodium bicarbonate and 8 µl of Trypsin (5 mg/ml) (Sigma). The Trypsin was added last after mixing the other ingredients to prevent heat inactivation. Once the overlay was added the plates were left at room temperature (RT) to

allow solidification before the plates were incubated at 37°C for 3 days. At this time the plaques were fixed with carnoy's fixative (3 parts methanol with 1 part acetic acid) for 45 min and the overlay was removed under running tap water. The plates were dried and plaques were counted (represented as plaque forming units per ml).

2.8. Hemolysis assay

The assay was conducted using 96 well U bottom plates as previously described by Smeenk et al (100). Briefly, 50 µl of virus ($\geq 10^6$ pfu/ml) was mixed with equal amounts of 2% human RBCs (< 48 hrs old). The plates were incubated at 4°C for 15 min prior to centrifugation at 1500 rpm for 5 min (IEC DPR-6000 centrifuge). The pellet was resuspended in 150 µl of 100 mM citrate buffers (pH values ranging from 5 to 6). The samples were mixed thoroughly and incubated at room temperature (21°C) or 37°C for 60 min. The plates were then spun at 1500 rpm for 5 min (IEC DPR-6000 centrifuge). Supernatant was transferred by multichannel micropipetter to ELISA plates followed by spectrophotometric measurement of optical density at 540 nm (OD₅₄₀).

2.9. Concentration of Influenza virus by Polyethylene glycol (PEG) precipitation

Allantoic fluid (1.5 ml) containing virus was mixed with PEG 8000 to yield an 8% w/v solution. The samples were mixed and incubated in a shaker at 4°C for exactly 1 hr. The precipitate was harvested by centrifugation (Beckman Avanti) at 7000 rpm for 20 min at 4°C. The pellet was resuspended in 0.1 ml STE and stored at -80°C.

2.10. RNA extraction

Once the samples were PEG precipitated, RNA was extracted as previously described Chomczynski, 1987. Briefly, 0.5 ml of denaturing solution (4M guanidinium with lysozyme) was added to the samples stored in STE. This was followed by addition of 100 µl of 2M sodium acetate (NaOAc) to get a final concentration of 0.2M. After adding 0.5 ml of 1:1 phenol:chloroform, the tubes were shaken vigorously and incubated on ice for 15 min. The samples were centrifuged at 8500 rpm for 25 min at 4°C (Beckman Avanti). The aqueous phase was transferred to 5 ml polypropylene tubes and equal volume of isopropanol was added. The mixture was shaken vigorously and precipitated at -20°C for a minimum of 1hr to overnight (O/N). Following centrifugation at 9500 rpm for 25 min at 4°C (Beckman Avanti), the pellet was resuspended in 300 µl denaturing solution. The solution was transferred to 1.5 ml eppendorf tubes and 300 µl of isopropanol was added. The samples

were mixed vigorously and precipitated for 1 hr at -20°C. This was followed by centrifugation at 12000 rpm for 15 min at 4°C (Beckman Avanti). The supernatant was removed and the pellet was washed with 70% ethanol and dried (Speed Vac SC110). The dried pellet was resuspended in 100 µl Diethylpyrocarbonate (DEPC) treated water, mixed with 300 µl of 100% ethanol and stored at -80°C.

2.11. Reverse transcription and Polymerase chain reaction

Half of the RNA stored in ethanol was centrifuged at 13000 rpm for 15 min at 4°C (Sorvall MC12V). The pellet was dried and dissolved in 21.4 µl of DEPC treated water. The RNA was reverse transcribed using 1 µl of primer 10231 (0.1 µg/µl), 1 µl of primer 10232 (0.1 µg/µl), 8 µl of 5X first strand buffer, 4 µl of 0.1M Dithiothreitol (DTT), 1.6 µl of 25mM dNTP, 1 µl of RNase inhibitor (40 units/µl) (Invitrogen) and 2 µl of Moloney Murine Leukemia Virus Reverse Transcriptase (200 units/µl) (Invitrogen) for a total volume of 40 µl. The components were mixed together and complementary DNA (cDNA) was synthesized using a thermal cycler (Progene Techne) under the following conditions: 21°C for 10 min, 33°C for 10 min, 37°C for 10 min and 42°C for 1 hr. The cDNA was stored at -20°C.

The Hemagglutinin (HA) gene was amplified by polymerase chain reaction (PCR). The 100 µl reaction mixture contained 2 µl of cDNA, 1 µl of plus-sense primer 10232 (0.1 µg/µl), 1 µl of anti-sense

primer 10238 (0.1 µg/µl), 1 µl of 25 mM dNTP, 10 µl of 10X PCR buffer, 6 µl of 25 mM MgCl₂, 78 µl of water and 1 µl of Taq DNA Polymerase, recombinant (5 units/µl) (MBI Fermentas). The samples were mixed and the reaction was performed using a thermal cycler (Progene Techne) under the following conditions; 1 cycle (94°C for 4 min), 30 cycles (94°C for 1 min, 47°C for 30 sec, 72°C for 2 min) and 1 cycle (72°C for 10 min). The samples were analyzed on an agarose gel and stored at -20°C.

2.12. PCR Purification

After PCR amplification, a 100 µl aliquot of phenol was added to each sample. The tubes were vortexed and spun in a table top centrifuge at high speed for 4 min (Spectrafuge 16M) before the top layer was collected and put through a Sephacryl S400 (Amersham, Pharmacia) spin column to remove primers. The spin columns were prepared by putting 1 ml of Sephacryl S400 resin into the column and spinning it at 1500 rpm for 4 min (IEC DPR-6000). The columns containing the samples were also spun at 1500 rpm for 4 min. The cleaned samples were analyzed on an agarose gel prior to storage at -20°C.

2.13. Cycle sequencing

This was performed using the ABI Prism BigDye Terminator Cycle Sequencing kit (PerkinElmer). The reaction mixture included

clean PCR product (30-90 ng), 2 μ l of Big Dye mix, 2 μ l of Extender, 1 μ l of sequencing primer (3 μ M) and x μ l of water to get a total volume of 20 μ l. The sequencing reaction was performed using a thermal cycler (Progene Techne) under the following conditions: 25 cycles (96°C for 30 sec, 50°C for 15 sec, 60°C for 4 min). 1 ml of Sephadex G50 (Amersham Pharmacia) was added into a column and spun at 4000 rpm for 2 min (Spectrafuge 16M). After the above reaction was completed the samples were added to the Sephadex G50 spin column and centrifuged at 4000 rpm for 2 min to remove dNTP's. These samples were then evaporated and sent for sequencing at a commercial service.

2.14. Transformation

A 100 μ l aliquot of Library efficiency DH5 α competent cells (Invitrogen) were mixed with 1 μ l of the appropriate plasmid (>0.1 μ g) and incubated for 30 min on ice. The samples were heat shocked for 45 sec at 42°C and chilled for 2 min on ice. The samples were diluted with prewarmed 900 μ l S.O.C medium and shaken at 37°C for 1 hr. Following this, 100 μ l of each sample was plated on Luria Bertani (LB) agar plates containing ampicillin (100 μ g/ml). The plates were incubated at 37°C O/N.

2.15. Plasmid purification

2.15.1. Mini prep (small scale)

Single colonies of transformed bacteria were picked and grown overnight in 5 ml LB broth containing ampicillin (200 µg/ml). Then 1.5 ml of the culture was centrifuged at 14000 rpm for 2 min at 4°C (Sorvall MC12V). The pellet was dissolved in 200 µl of Lysozyme buffer and incubated for 5 min at 21°C. After incubation 400 µl of solution 2 (Alkaline SDS containing 1% SDS and 0.2N NaOH) was added and the samples were vortexed and incubated for 5 min at 21°C. Then 300 µl of solution 3 (8M ammonium acetate) was added and incubated for 10 min on ice. The samples were then centrifuged at 10000 rpm for 3 min at 4°C (Sorvall MC12V). The supernatant was transferred to a clean tube, mixed with 500 µl isopropanol and incubated for 10 min at 21°C. The samples were centrifuged at 10000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol, dried (Speed vac SC110) and dissolved in 100 µl TE buffer (pH 8). The samples were analyzed on an agarose gel and stored at -20°C.

2.15.2. Midi prep (large scale)

One ml of overnight culture was added to 50 ml of LB media containing ampicillin (200 µg/ml). The samples were shaken at 37°C for 4 to 5 hrs. The cells were spun down at 8000 rpm for 10 min (Beckman Avanti). Midi prep was performed using the Wizard plus DNA purification kit (Promega) in accordance with the manufactures

instructions. The samples were analyzed on an agarose gel and stored at -20°C .

2.16. Restriction digestion

2.16.1. Single digestion

The DNA (1 µg) was mixed with sterilized water to get a volume of 17 µl. Then 2 µl of the appropriate buffer and 1 unit of the restriction enzymes (New England Biolabs) were added for a total reaction mixture of 20 µl. The samples were mixed and incubated at the recommended temperature for 1 hr. Then the samples were analyzed on an agarose gel. If digestion is incomplete, the samples were re-incubated for a longer period of time.

2.16.2. Double digestion

1 µg of DNA was mixed with sterile water to get a volume of 17 µl. To this 1 µl of restriction enzyme A, 1 µl of restriction enzyme B and 2 µl of buffer (compatible with both enzymes) was added for a final volume of 21 µl. The samples were mixed and incubated at the recommended temperature for 1 hr. After incubation, samples were analyzed on an agarose gel.

2.17. Gel extraction

The samples were run on an agarose gel (0.8%) and the fragment of interest was excised with the aid of a hand held UV light (BLAK-

RAY long wave ultraviolet lamp 21). Gel extraction was performed using QIAquick Gel Extraction kit (QIAGEN) in accordance with the manufacturer's instructions.

2.18. Glycogen Precipitation of Gel purified DNA

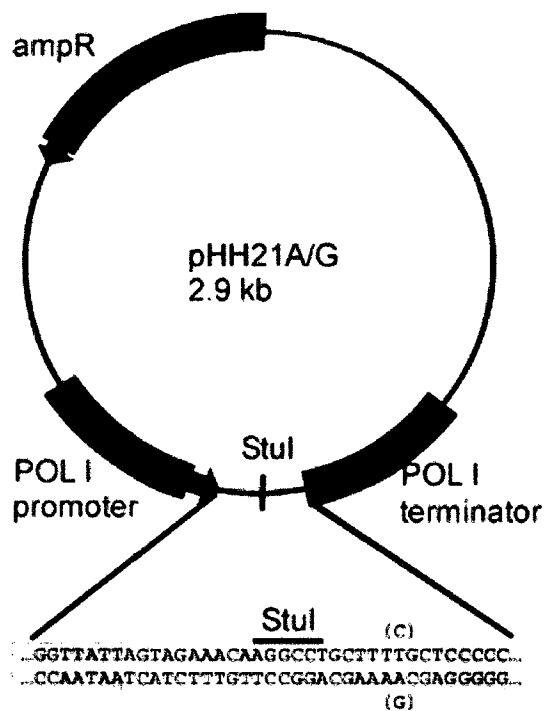
The extracted samples were mixed with 1/10V of 3M sodium acetate, 1/10V of glycogen (1 µg/µl) and 2.5V of 100% ethanol followed by a 5 min incubation on ice. The samples were centrifuged at full speed for 15 min at 4°C (Sorvall MC12V). The supernatant was removed and the pellet was rinsed with 70% ethanol, dried (Speed vac SC110) and resuspended in 10 µl of distilled water.

2.19. Cloning

2.19.1. Ligation-independent Cloning

The PHH21 vector containing the human RNA polymerase I promoter and mouse RNA polymerase I terminator was previously modified by PCR mutagenesis (115). The modified vectors designated as PHH21A and PHH21G contain sequences that are complementary to 5' and 3' ends of influenza gene segments located between the promoter and terminator (Fig 12). These two vectors are identical except for the A

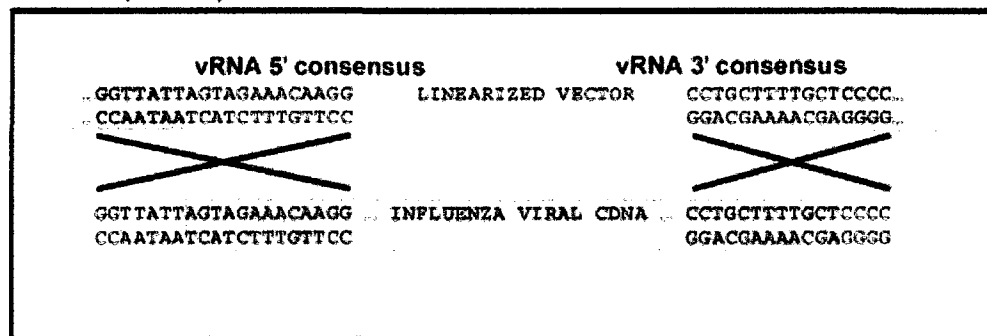
Fig 12. Ligation-Independent cloning
(Wang et al 2008)



Influenza virus recombination cassette



E. coli (RecA)



in situ recombination between linear vector and
viral cDNA in *E. coli*

or G at position 4 (plus strand). This accounts for the difference between the influenza gene segments, therefore PHH21-A is complementary to HA, NP, NA, M and NS genes while PHH21-G is complementary to PB2, PB1 and PA genes (115). The cloning was performed by using 100 ng of linear PHH21-A (digested with *Stu*I) and 500 ng of PCR sample (mutant HA gene). The mixture was incubated with 100 μ l of Library efficiency DH5 α competent cells (Invitrogen) and transformation was performed as described above. Transformation would result in homologous recombination inserting the gene of interest into the vector. Single colonies were then picked and grown on LB media containing ampicillin (200 μ g/ml) and miniprep was performed. The samples were analyzed on an agarose gel prior to sequencing (115).

2.19.2. Ligation-dependent Cloning

The PHH21 vector (containing wt HA gene) and insert (PCR sample containing mutation of interest) were digested with the same restriction enzymes as stated above. The goal was to take the fragment with the mutation of interest and ligate it into the vector. The digested samples were gel extracted and ligated using vector to insert ratio of 1:3. The following reaction mixture was used; x ng of insert, 100 ng of vector, 1 μ l of T4 DNA ligase, 10X buffer and x μ l of water for a total volume of 10 μ l. The samples were mixed and incubated at 15°C O/N. This was followed by transformation, miniprep and sequencing to

confirm existence of desired mutation. The amount of insert used was calculated based on the following equation: $\text{vector (ng)} \times \text{insert (kb)} / \text{vector (kb)} \times \text{ratio of insert/vector} = \text{insert (ng)}$.

2.20. Generation of recombinant viruses

Recombinant viruses were generated using the 12 plasmid system previously described by Neumann et al (Fig 13) (80). This system uses 8 PHH21 plasmids that possess cDNA specific for each gene segment. These genes reside between human RNA Pol I promoter and mouse RNA Pol I terminator as stated above. The remaining 4 are the protein expressing plasmids PCAGGS NP, PCDNA PB1, PCDNA PB2 and PCDNA PA.

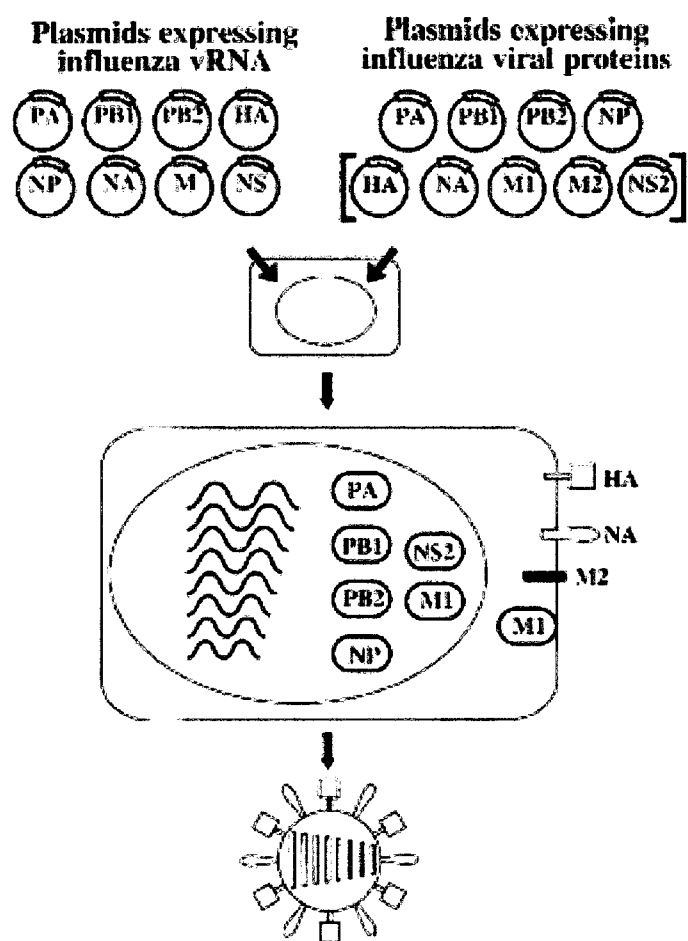
2.20.1. Plasmid mix

1 µg of each of the 11 plasmids and 0.1 µg of PCDNA PA were mixed with 1/10 vol of 2.5M sodium acetate and 2vol of 95% ethanol. The samples were incubated for 10 min on ice and spun down at 4°C for 15 min (Sorvall MC12V). The pellet was rinsed with 70% ethanol and dried (Speed Vac SC110). The samples were dissolved in 11.1 µl of sterile distilled water.

2.20.2. Virus rescue

Six well plates containing 293T plus 10% MDCK cells (80% confluency) were used for this experiment. The precipitated

Fig 13. Twelve plasmid Rescue System
(Neumann et al 1999)



plasmid mix (11.1 μ l) was diluted in 555 μ l of Opti-MEM I (solution 1). Then 22.2 μ l of Lipofectamine was diluted in 111 μ l of Opti-MEM I (solution 2). The two solutions were combined together, mixed and incubated for 30 min at RT. After the incubation, additional 600 μ l of Opti-MEM I was added to each tube and the diluted complex was added to the cells (washed 2X with warm PBS). The cells were incubated for 5 hrs at 37°C then the complex was removed and replaced with fresh Opti-MEM I containing Trypsin (1 μ g/ml) (Sigma). The cells were then incubated for 48 hrs at 37°C. The supernatant was collected spun down at 1000 rpm for 5 min (IEC DPR-6000) and stored at -80°C.

2.21. Virus concentration

The virus samples (10 ml) were centrifuged using SW41 rotor (Beckman) at 39000 rpm for 1 hr. The pellets were re-suspended in 1 ml PBS and stored at -80°C. The concentrated samples were used to perform an HA assay.

2.22. Solid-phase Sialic Acid Binding assay

The synthetic polymers used were 6' Sialyl-(N-acetyllactosamine) (6' SLN), 3' Sialyl-(N-acetyllactosamine) (3' SLN), 6' Sialyllactose (6'SL) and 3' Sialyllactose (3' SL). These ligands contain biotin attached to a polyacrylamide linker. This assay was previously described by Matrosovich et al (69). Briefly, 96 well vinyl plates (Costar) were

incubated with 40 μ l of concentrated virus (50 HAU) at 4°C O/N. The supernatant was removed and 200 μ l of blocking solution (1% BSA in PBS) was added to each well and the plates were incubated for 1 hr at RT. The blocking solution was discarded and plates were washed (3x) using buffer A (PBS with 0.01% Tween 80). Two-fold dilutions of the polymers were then prepared in buffer B (PBS with 1 μ M Zanamivir, 0.02% BSA and 0.02% Tween 80). After dilution, 25 μ l/well was added and the microplates were stored at 4°C for 2 hrs. Following incubation, plates were washed (3x) and 25 μ l/well of streptavidin-peroxidase, diluted 1/3000 in buffer B was added. This was followed by incubation for 1 hr at 4°C. Plates were washed (3x) and 50 μ l/well of 3,3',5,5' – tetramethylbenzidine (TMB) substrate (KPL, Mandel) was added for 45 min at room temperature. The reaction was stopped by adding 50 μ l/well of 1N HCL and the plates were read at 450 nm.

2.23. Resialylation of Chicken erythrocytes

2.23.1. Titration of Receptor Destroying Enzyme (RDE)

A reference antigen (A/HK/1/68) was first titrated in 0.85% sodium chloride to obtain the working dilution. The end point dilution of the HA assay was divided by 4 to get a solution that contains 4 HA units per 0.05 ml. This was used as the working dilution of the antigen for the RDE titration.

In a 96-well U-bottom plate serial two-fold dilutions of the RDE stock were prepared in 25 μ l volumes of calcium saline. Then 25 μ l of 1% RBC (in saline) was added to all the wells of the RDE dilution series as well as control (calcium saline) wells. The plate was covered, mixed and incubated for 1 hr at 37°C. The cells were re-suspended every 15 min throughout the incubation period. After this, 50 μ l of A/HK/1/68 (4 HAU) was added to all the wells in the RDE dilution series while 50 μ l of calcium saline was added to the control wells. The samples were mixed and the plate was incubated for 45 min at RT. The end point dilution of RDE is the last well of the dilution series in which hemagglutination is completely absent. The number of units of RDE contained in 1 ml of undiluted stock was determined by multiplying the end point dilution factor by 4.

2.23.2. Sialidase treatment of Chicken erythrocytes

1 ml of packed RBC's were mixed with Neuraminidase vibrio cholera (150 milliunits/1 ml of cells). To the mixture, 4mM of calcium chloride was added per 1ml of enzyme. This was followed by a 3 hr incubation at 37°C. Cells were then washed 2x in PBS (1500 rpm for 4 min) (IEC DPR-6000 centrifuge). The treated cells were used to prepare 0.5% RBC in PBS. The remaining cells were spun down for storage in equal volume of alsever's medium (AMAB). An HA assay was performed using 0.5% treated RBCs and A/HK/1/68. If the treatment was successful no agglutination was visible.

2.23.3. Re-sialylation of Chicken erythrocytes using α 2,6 or α 2,3

Sialyltransferases

The sialidase treated RBC's were first washed 2x in PBS (1000 rpm for 4 min) (IEC DPR-6000). Then 200 μ l of packed cells were mixed with 200 μ l of solution A (50 mM sodium chloride, 100 mM glucose, 11 mu sialyltransferase and 1.1 mM of the substrate CMP-Sialic acid with 50 mM MOPS used to bring the volume to 200 μ l). The mixture was vortexed and incubated for 5 hrs at 37°C. The cells were washed 3x in PBS (1000 rpm for 4 min). After the last wash, cells were resuspended in PBS and 0.8% RBC was prepared for HA assay. In this case, the assay was performed at 4°C to inhibit neuraminidase activity.

2.24. Antibody adsorption

Uninfected mouse lungs were collected and sonicated in 1 ml PBS. The sonicated lungs were mixed with 5V acetone and fixed for 5 min on ice. The samples were pelleted and the acetone was evaporated in the warm room (37°C). The lung extract was mixed with 5 ml serum and sonicated. The mixture was rotated O/N at 4°C and spun down to collect the antibodies. These were aliquoted and stored at -20°C.

2.25. Immunostaining of infected lungs

Balb/c mice were inoculated using 5×10^3 pfu (A/HK/1/68 and the MA variants) or 10^5 pfu (recombinant viruses). Lungs were then

harvested 2 days post-infection (d.p.i). After fixing the lungs O/N with 10% buffered formalin the solution was replaced with 20% sucrose (2x). Frozen lung sections were then prepared for staining. The sections were first fixed in acetone for 20 min at RT and air dried for 45 min. The slides were incubated in 10 mM PBS for 5 min at RT followed by treatment with 50 μ l of Trypsin (0.25X) for 5 min at RT. The slides were washed (3x) in 10 mM PBS and incubated with 100 μ l of anti-HK primary rabbit antibody (1/1000 in antibody buffer) at 4°C O/N. The slides were washed with 10 mM PBS followed by incubation with 100 μ l of Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch laboratories Inc) for 1 hr at RT (Cy3 was diluted 1/800 in antibody buffer). Slides were washed and incubated with 100 μ l Hoechst (0.2 μ g/ml) for 5 min in the dark at RT. The slides were washed and cover slip was applied. The pictures were taken with an Olympus BX50 Microscope (20X magnification), and figures were prepared with Adobe Photoshop 7.0.

2.26. Growth of virus in the mouse lung

Groups of mice (CD-1 or Balb/c) were intranasally inoculated with the desired amount of virus. Lungs were then harvested and 4vol (wt/wt) PBS was added. This was followed by sonication and titration to determine the yield.

2.27. Median Lethal Dose (LD₅₀)

Groups of five Balb/c mice were inoculated using 10^6 pfu of the recombinant viruses. Mortality was monitored daily for 12 days. The LD₅₀ values were calculated using Karber-Spearman method as previously described (74).

2.28. Location of mutations in the HA trimer

The figures were created using PyMOL software and A/HK/1/68 H3 hemagglutinin structure (Protein Data Bank ID: 1 HGD) as well as the low pH conformation of HA2 (Protein Data bank ID: 1 QU1).

3. Results

3.1. Mutations in the HA genes for clones of mouse adapted A/HK/1/68 following passages 12 and 20

The prototype A/HK/1/68 was previously mouse adapted by serial passage in CD-1 mouse lung. Mouse adaptation involved infection of groups of 3 mice with 10^6 pfu/mouse for 3 days before lungs were harvested. For subsequent passages, the virus extracted from a pool of 3 mice was diluted 10 fold and used to infect another set of 3 mice. This procedure involved infection with large doses of virus that will possess all possible single nucleotide substitutions to result in the selection of the most adaptive mutations because they confer the greatest increase in fitness. Assessment of virulence in the mouse lung indicated that A/HK/1/68 was avirulent with an $LD_{50} > 10^{7.7}$. In contrast, subsequent to 12 and 20 serial lung passages the median lethal dose for the mouse adapted populations of virus was reduced $>10^4$ and $>10^5$ fold respectively. These results indicated that the mouse adapted viruses have gained mutations that cause an increase in virulence (13).

Genomic sequencing showed that clones from passages 12 and 20 have mutations in most of their gene segments, however this study will focus on the changes that occur within the HA gene. This was investigated since we were interested in mapping evolution to increased virulence. Following passage 12, six viruses (HKMA-12 to -12E) were clonally isolated for sequence analysis (Table 1). We found that all of the clones

isolated from the passage 12 population were mutants that presumably contributed to the increased virulence of the mouse adapted viruses. Most of the clones were double mutants except for HKMA-12E which was a single mutant. The results indicated that the G218¹W mutation was positively selected because it was present in 4 of the 6 clones (HKMA-12, -12C, -12D, -12E). This mutation occurred alone or in combination with T156²N (superscripts 1 and 2 indicate HA1 and HA2 respectively). Other mutations identified after passage 12 include D2¹Y+N246¹D and P162¹L+D158²N found in HKMA-12A and HKMA-12B respectively.

After passage 20, six variants (HKMA-20 to -20E) were isolated and the HA genes were sequenced (Table 1). All the clones were double mutants containing the G218¹W mutation with N154²S or T156²N. These results indicate that even though several mutations were acquired following passages 12 and 20, G218¹W turned out to be the most adapted. In addition, convergent evolution was apparent with respect to the 154 and 156 mutations. These changes within HA2 cause loss of a glycosylation site bearing the sequence N₁₅₄-G₁₅₅-T₁₅₆.

Table1. Mutations in the HA genes of clones of Mouse-adapted A/HK/1/68 acquired following passage 12 (HKMA-12 to HKMA-12E) and 20 (HKMA-20 to HKMA-20E)

	HA1				HA2			LD ₅₀ PFU
	2	162	218	246	154	156	158	
HKWT	D	P	G	N	N	T	D	$>10^{7.7}$
HKMA-12	-	-	W	-	-	N	-	10^4
HKMA-12A	Y	-	-	D	-	-	-	$10^{3.9}$
HKMA-12B	-	L	-	-	-	-	N	$10^{3.6}$
HKMA-12C	-	-	W	-	-	N	-	$10^{4.6}$
HKMA-12D	-	-	W	-	-	N	-	$>10^{4.7}$
HKMA-12E	-	-	W	-	-	-	-	$>10^{4.9}$
HKMA-20	-	-	W	-	-	N	-	$10^{4.2}$
HKMA-20A	-	-	W	-	-	N	-	10^4
HKMA-20B	-	-	W	-	-	N	-	10^3
HKMA-20C	-	-	W	-	-	N	-	$10^{2.5}$
HKMA-20D	-	-	W	-	S	-	-	$10^{3.5}$
HKMA-20E	-	-	W	-	-	N	-	$10^{3.6}$

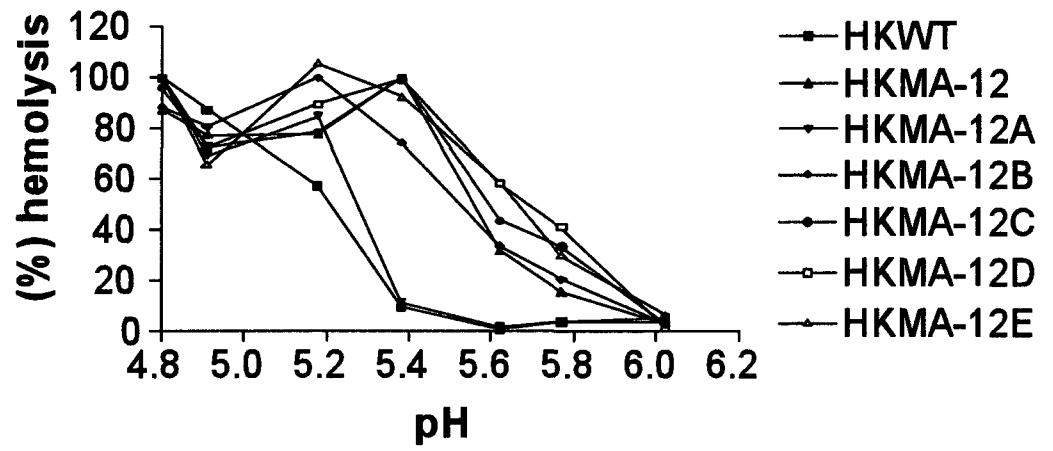
- Indicates identity with parental sequence

3.2. The pH dependence of Hemolysis by A/HK/1/68 and the mouse adapted variants

Once influenza A virus interacts with its receptor (sialic acid), it enters cells via endocytosis. After internalization, there is a drop in pH within the endosome (pH 5 to 6). This causes structural changes within HA resulting in membrane fusion (98). Previous studies indicate that mouse adapted variants fuse at an elevated pH (46). To corroborate these results hemolysis assays were conducted on the prototype A/HK/1/68 and the mouse adapted variants as described by Smeenk et al (100). When the assay was performed at 37°C, the parental virus fused at pH 5.2. In contrast, the majority of the mouse adapted variants exhibited an elevated pH, 5.6 versus 5.2 (0.4 units more than the parent) (Fig 14A and B). In contrast, HKMA-12A only showed a slight increase at pH 5.25, 0.05 units more (Fig 14A). When the assay was conducted at room temperature (21°C), a similar pattern was observed (Fig 15A and B). However, the pH of fusion was lower at 21°C when compared to 37°C for the viruses tested including the prototype. This study suggests that an enhanced pH of fusion is a constant feature of adaptation to increased virulence. In addition, we have previously shown that fusion of mouse adapted A/FM/1/47 occurs at pH 5.6. These results suggest that viruses

Fig 14. The pH dependence of hemolysis by A/HK/1/68 and the mouse adapted viruses. Hemolysis was evaluated at 0.2 pH increments (ranging from 4.8 to 6.0). The assay was performed using 2% human blood at 37°C. **(A)** Passage 12 variants (HKMA-12 to HKMA-12E). **(B)** Passage 20 variants (HKMA-20 to HKMA-20E). Most of the mouse adapted variants, with the exception of HKMA-12A, exhibited an elevated pH.

A



B

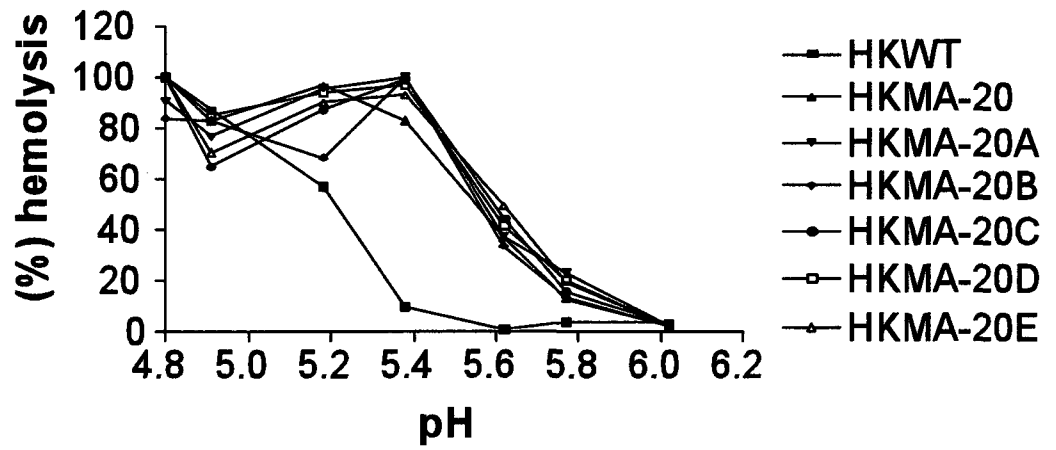
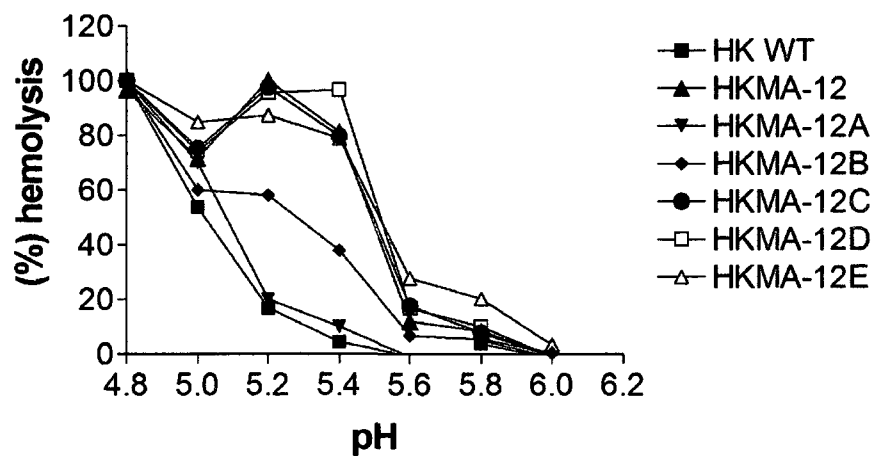
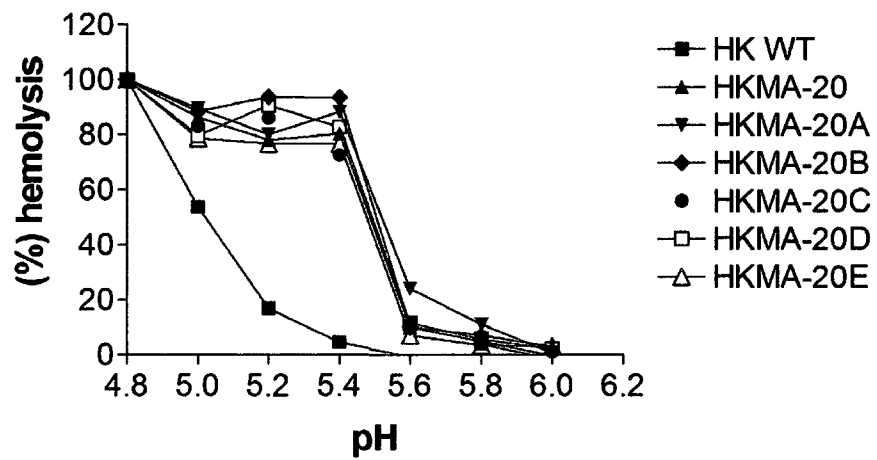


Fig 15. The pH dependence of hemolysis by A/HK/1/68 and the mouse adapted viruses. Hemolysis was evaluated at 0.2 pH increments (ranging from 4.8 to 6.0). The assay was performed using 2% human blood at room temperature (21°C). **(A)** Passage 12 variants (HKMA-12 to HKMA-12E). **(B)** Passage 20 variants (HKMA-20 to HKMA-20E).

A



B



which exhibit this elevated pH may have a replicative advantage. In these viruses, uncoating could happen earlier following internalization (46).

3.3. Changes in Receptor Specificity

3.3.1. Hemagglutination of Resialylated chicken erythrocytes by the parental and mouse adapted variants

Receptor binding specificity of A/HK/1/68 and the mouse adapted variants was investigated using resialylated chicken red blood cells (RBC). Previous studies indicate that chicken RBCs display SA α 2,6Gal as well as SA α 2,3Gal linkages. Chicken RBCs have the N-acetyl sialic acid instead of the N-glycolyl form (53). Native RBCs were first incubated with neuraminidase to eliminate sialic acid (SA) moieties found on the surface of the cell. After treatment, Hemagglutination (HA) assay was performed to verify successful removal of the linkages. Loss of agglutination observed with the asialo (treated) cells indicated that the linkages were indeed removed (Table 2). These cells were then resialylated with SA α 2,6Gal (human form) or SA α 2,3Gal (avian and mouse) moieties. As expected, the prototype showed a preference for SA α 2,6Gal resialylated cells over the SA α 2,3Gal resialylated cells (Table 2). In contrast, most of the mutants showed a change in receptor binding specificity preferentially recognizing SA α 2,3Gal resialylated

Table 2. Hemagglutination of resialylated chicken erythrocytes by A/HK/1/68 and the mouse adapted variants.

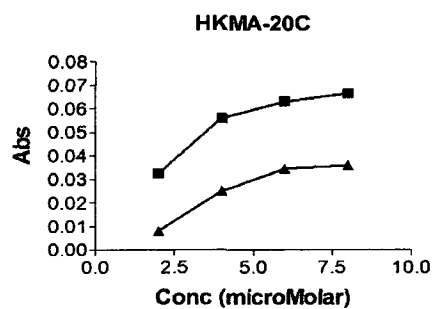
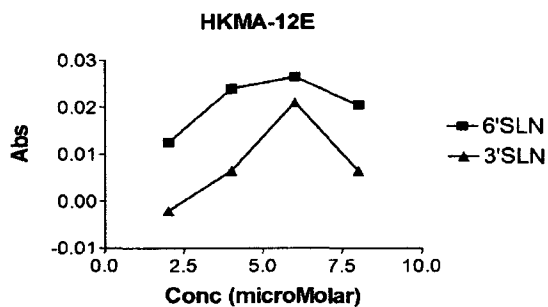
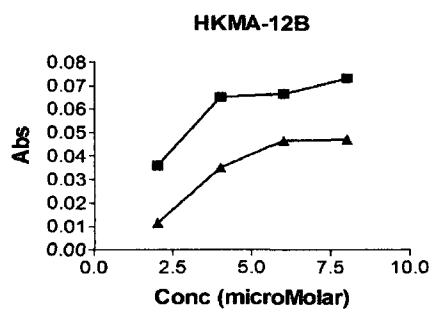
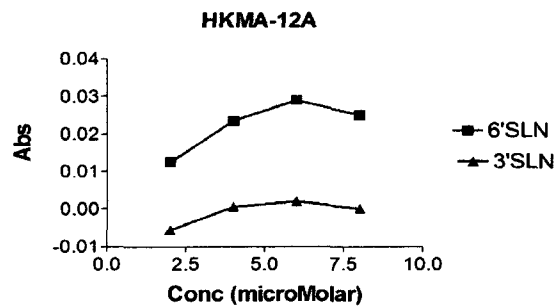
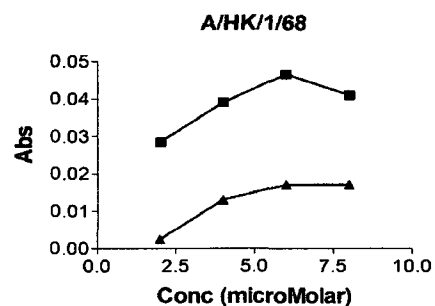
	HA titre for selected mouse adapted viruses					
Viruses	A/HK/1/68	HKMA-12A	HKMA-12B	HKMA-20C	HKMA-20D	HKMA-12E
HA mutations	wt	D2 ¹ Y N246 ¹ D	P162 ¹ L D158 ² N	G218 ¹ W T156 ² N	G218 ¹ W N154 ² S	G218 ¹ W
Native RBC	320	640	160	320	320	640
Asialo	0	0	0	0	0	0
Resialylated SA α 2,6Gal	400	400	100	200	200	200
Resialylated SA α 2,3Gal	320	160	160	320	320	320
Ratio SA α 2,6/ α 2,3	1.25	2.5	0.63	0.63	0.63	0.63

cells. G218¹W and P162¹L+D158²N found in HKMA-12E and -12B respectively appear to be responsible for the switch. Interestingly, the change in specificity observed due to G218¹W wasn't influenced by T156²N or N154²S present in HKMA-20C and -20D respectively (Table 2). The switch in binding could be clearly observed when the data was represented as a ratio (SA α 2,6/2,3). The only exception was HKMA-12A (D2¹Y+N246¹D) which showed a similar binding pattern as the parent and preferentially bound α 2,6 SA.

3.3.2. Receptor binding specificity of the parental and mouse adapted variants

Receptor binding ability of A/HK/1/68 and the mouse adapted variants was also examined with sialylglycopolymers as previously described (69). These polymers consisted of SA α 2,6Gal (6' Sialyl(N-acetyllactosamine) or SA α 2,3Gal (3' Sialyl(N-acetyllactosamine) linkages. These linkages were chosen since SA α 2,6Gal is abundant in the human respiratory tract while SA α 2,3Gal is predominantly expressed in bird intestine and mouse lung (34, 52). As expected, the parental virus preferentially recognized 6' Sialyl(N-acetyllactosamine) (6'SLN) over 3' Sialyl(N-acetyllactosamine) (3'SLN) (Fig 16). Comparable to wt, all

Fig 16. Receptor binding specificity of A/HK/1/68 and the Mouse adapted variants. The ability of these viruses to bind biotinylated ligands (6'SLN and 3'SLN) was assessed using streptavidin-peroxidase. Graphs represent Absorbance (450 nm) versus polymer concentration.



mouse adapted viruses preferred 6'SLN. However, a few of the mutants exhibited increased relative ability to bind 3'SLN. These viruses contained the G218¹W (HKMA-12E and -20C) and P162¹L+D158²N (HKMA-12B) mutations.

3.4. Mutations in the HA genes of 10 sister clones of A/HK/1/68 after an independent mouse adaptation experiment

An independent mouse adaptation experiment was performed to gain more insight into the nature of the mouse adapted mutations and to obtain a higher resolution adaptive map. Ten sister clones were first obtained from prototype A/HK/1/68 in order to derive 10 independent mouse adapted (MA) variant populations. Each clone was then independently subjected to 21 serial lung to lung passages. Three clones were then derived from each mouse adapted population. Based on sequence analysis, all 10 mouse adapted populations contained viruses with HA mutations (Table 3). Within these viruses 13 single, 12 double and 1 triple mutants were identified. Of all the mutations detected after passage 21, four (162¹, 210¹, 218¹ and 154²) showed evidence of convergent evolution (Table 3). This refers to the independent selection of similar mutations within different viral isolates (13). In this study, parallel evolution was apparent with respect to G218¹E and Q210¹R (Table 3), where identical amino acid changes were detected in different

Table 3: Mutations identified in the HA genes of 10 sister clones of A/HK/1/68 generated through independent mouse adaptations. Each population is designated as HK-sister clone number-MA-mouse passage number.

NTD refers to nucleotide.

Convergent mutations are shown in red.

- indicates identity with parental sequence.

Virus	clone	Ntd 669	HA1 (aa #)															HA2 (aa #)						
			124	162	165	210	218	220	223	231	238	242	244	262	268	29	117	124	154	160	175			
A/HK/1/68		G/A	G	P	N	Q	G	R	V	S	K	V	T	M	S	K	R	N	D	G				
HK1MA21	1	G	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-				
HK1MA21	2	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK1MA21	3	G	-	-	-	-	-	S	-	-	-	-	-	-	Y	-	-	-	-	-				
HK2MA21	1	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK2MA21	2	A	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK2MA21	3	A	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK4MA21	1	G	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK4MA21	2	G	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK4MA21	3	G	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-				
HK5MA21	1	A	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	D				
HK5MA21	2	A	-	-	-	-	E	-	-	-	-	-	-	V	-	-	-	-	-	-				
HK5MA21	3	A	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK6MA21	1	A	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK6MA21	2	A	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK6MA21	3	A	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK8MA21	1	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK8MA21	2	G	-	-	-	-	E	-	-	-	-	-	-	-	-	-	K	-	-	-				
HK8MA21	3	G	-	-	-	-	E	-	-	-	N	-	-	-	-	-	-	-	-	-				
HK9MA21	1	A	-	-	-	-	-	-	-	N	-	-	-	N	-	-	-	-	-	-				
HK9MA21	2	A	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-				
HK9MA21	3	A	-	-	-	-	-	-	I	N	-	-	-	N	-	-	-	-	-	-				
HK10MA21	1	G	-	-	-	R	-	-	-	-	-	-	-	-	-	-	K	-	-	-				
HK10MA21	2	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK10MA21	3	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK11MA21	1	G	D	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK11MA21	2	G	D	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HK11MA21	3	G	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-				
HK12MA21	1	G	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	N	-				
HK12MA21	2	G	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	N	-				
HK12MA21	3	G	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	N	-				

mouse adapted isolates. On the other hand, directional evolution was evident with respect to G218¹W/E, P162¹L/S and N154²S/K (Tables 1 and 3), where different amino acid substitutions were seen at the same position (13).

Following these studies the most common adaptive mutations included the G218¹E as well as Q210¹R (Table 3). G218¹E is observed in 4 different mouse adapted populations such as HK4MA21, HK5MA21, HK6MA21 and HK8MA21. The Q210¹R was detected in 3 of the 10 mouse adapted populations including HK2MA21, HK10MA21 and HK12MA21. Because the A/HK/1/68 stock utilized in the above experiments included viruses that have either A or G at position 669 (non-coding change) the resulting sister clones derived from this stock were identical except for variation at this position. Therefore, the mouse adapted populations contained either A669 or G669 which indicated that the mutations selected following these experiments did not result from contamination but instead represent independent mutational events (Table 3).

3.5. Growth of the parental A/HK/1/68 and mouse adapted variants in mouse lungs

The replicative potential of the parental and mouse adapted variants was investigated in CD-1 mice (n = 2). CD-1 was the mouse strain used to perform the various mouse adaptation experiments. These mice were

chosen for our studies since they are outbred and thus represent a genetically heterogeneous population that would be more similar to natural host populations. Following inoculation at 5×10^3 pfu (plaque forming units), lungs were harvested 2 d.p.i. and titrated to determine the yield. All the mutants replicated better than the parent (Fig 17). Three of the mutants (MA-20C, MA-411 and MA-531) grew significantly better, $p < 0.05$. MA-20C and MA-411 exhibited the highest yield.

The growth of the parental and mouse adapted variants was also determined in Balb/c mice ($n = 2$). Infection was first performed using the parental and mouse adapted variants at 5×10^3 pfu. Lungs were harvested 1 d.p.i. and titrated to quantify the yield. In contrast to the parental virus, most of the mouse adapted variants replicated 10 to 100 fold better (Fig 18). MA-20 and MA-411 were associated with the highest viral yield. The only exception was MA-511 which showed a similar growth pattern as the parental virus at 1 d.p.i. In conclusion, the mutants replicated better than the parent in both CD-1 and Balb/c except for MA-511 which grew better in CD-1 but not Balb/c mice.

3.6. Immunofluorescent staining of lungs infected with A/HK/1/68 and the mouse adapted Variants

In humans, influenza A infections primarily involve the upper airways. However, in fatal cases infection of the lower airways has

Fig 17. Growth of A/HK/1/68 and the Mouse adapted variants in CD-1 lungs. Mice ($n = 2$) were intranasally inoculated using 5×10^3 PFU. Lungs were then harvested 2 d.p.i. and sonicated prior to titration. The mouse adapted viruses were more efficient at replicating within the lung when compared to the parent. MA-20C, MA-411 and MA-531 replicated significantly better, $p < 0.05$ (indicated with an asterix).

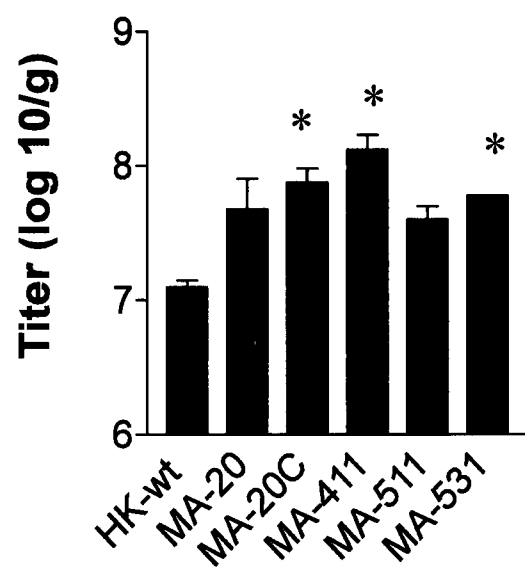
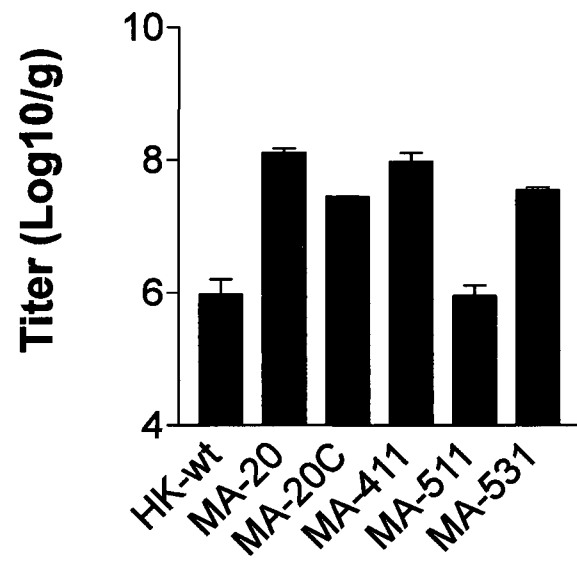


Fig 18. Growth of A/HK/1/68 and the Mouse adapted variants in BALB/c lungs. Mice (n = 2) were intranasally inoculated using 5×10^3 PFU. Lungs were then harvested 1 d.p.i. and titrated to quantify the yield. Most of the mouse adapted variants replicated significantly better than the parent.



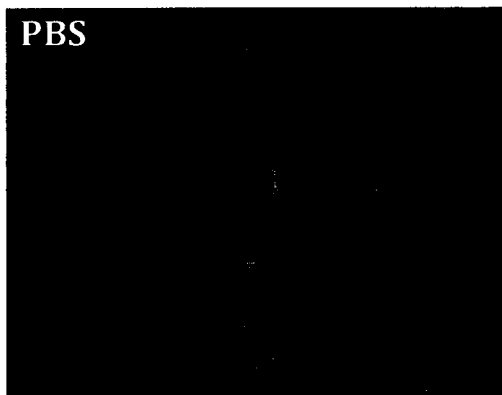
been observed (110). Human influenza strains exhibit restricted growth in mice. However, following mouse adaptation these viruses show a much more efficient replication (13). This was also apparent in this study as described above. Mouse adapted variants are known to replicate within bronchioles as well as alveoli (67). To determine the level of lung infection, CD-1 mice were inoculated using parental or mouse adapted variants at 5×10^3 pfu. After this lungs were harvested 2 d.p.i, frozen sectioned and stained using rabbit anti-HK antibody. The prototype virus appears to infect the bronchial epithelia and some alveolar cells. In contrast, all the mutants spread more efficiently within the bronchial epithelia and alveoli (Fig 19A and B). Within these mutants MA-20, MA-20C, MA-511 and MA-531 exhibited the strongest bronchial as well as alveolar staining.

The above experiment was also repeated using Balb/c mice. After inoculation of Balb/c mice using 5×10^3 pfu of the parental or mouse adapted variants, lungs were harvested 2 d.p.i. and stained using anti-HK serum. The staining pattern was similar to what was observed with CD-1 mice. In contrast to the parent, all the mouse adapted strains spread efficiently within the bronchial epithelia and alveoli (Fig 20A and B). Within these mutants MA-20, MA-20C and MA-411 were associated with the strongest bronchial as well as alveolar staining. MA-511 showed a more restricted staining pattern in comparison to the other

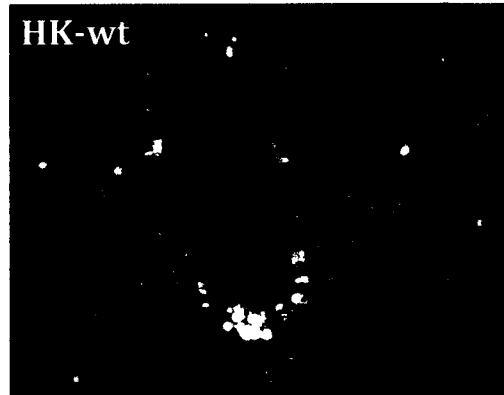
Fig 19. Immunofluorescent staining of CD-1 lungs infected with A/HK/1/68 and the mouse adapted variants. CD-1 mice were intranasally inoculated using 5×10^3 PFU. Lungs were then collected 2 d.p.i. and stained using anti-HK primary antibody. Sections were subsequently stained with Cy3-conjugated secondary antibody (red). Nuclei were also stained using Hoechst (blue). **(A)** Bronchioles, **(B)** Alveoli area. All the mouse adapted variants caused extensive infection of the bronchiolar epithelium and alveoli compared to the parent.

A

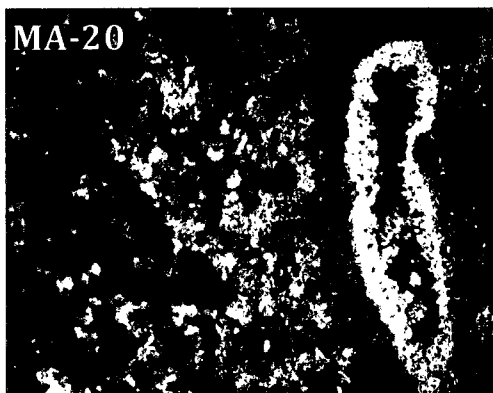
PBS



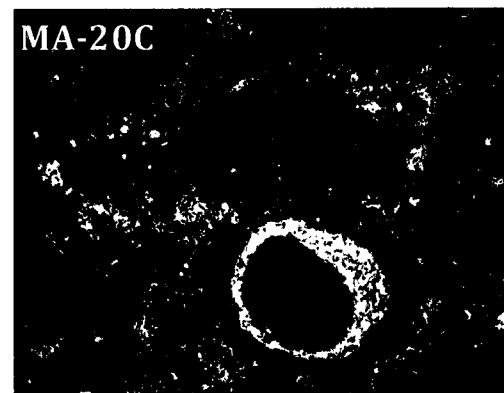
HK-wt



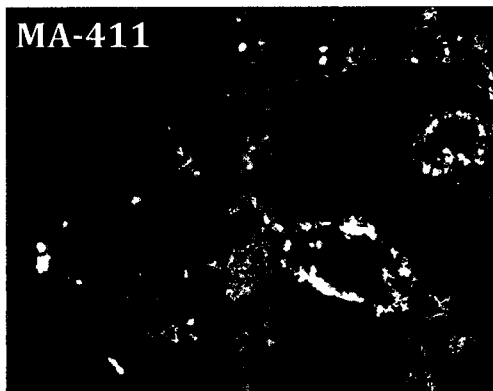
MA-20



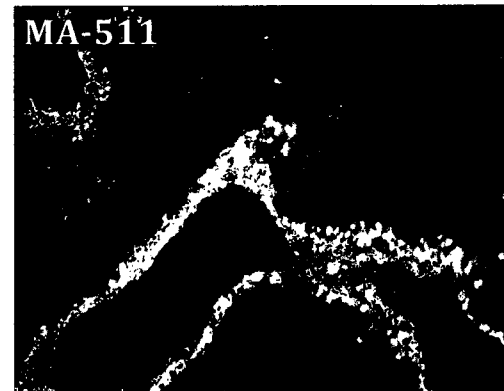
MA-20C



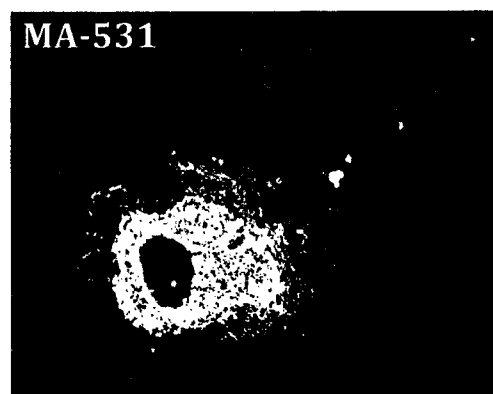
MA-411



MA-511



MA-531



B

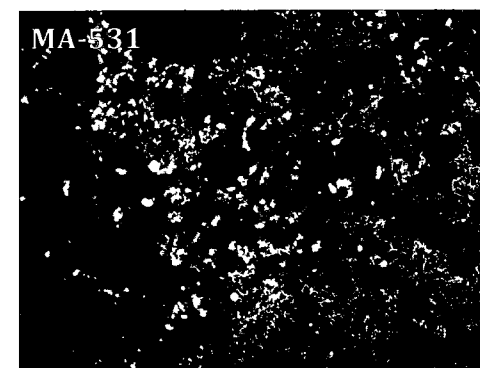
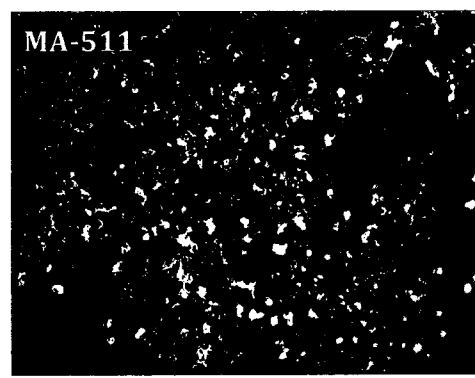
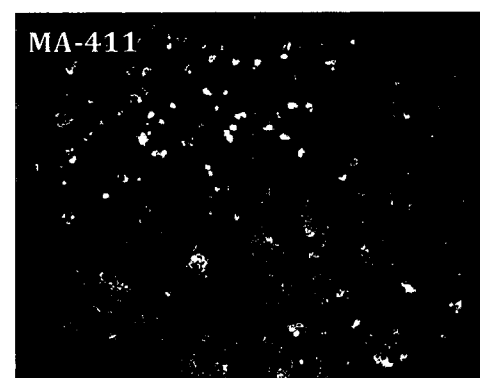
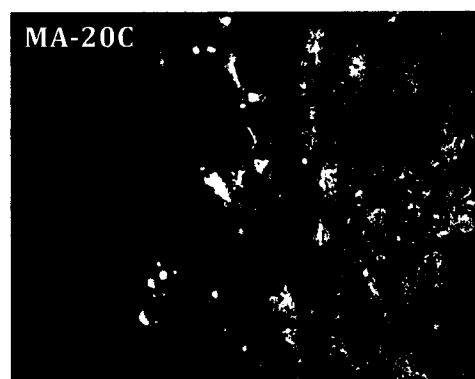
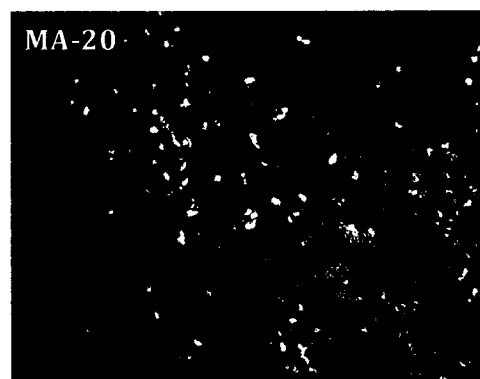
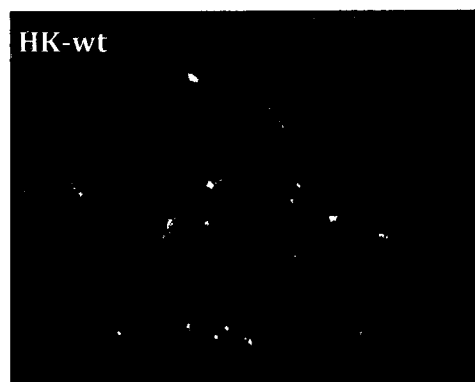
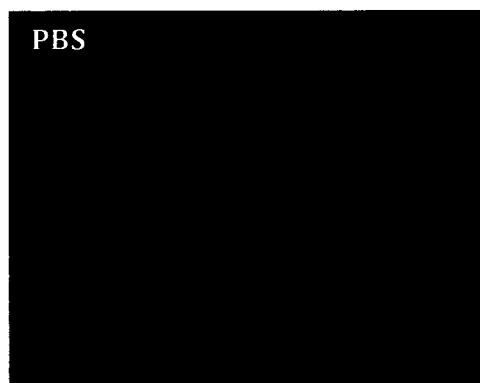
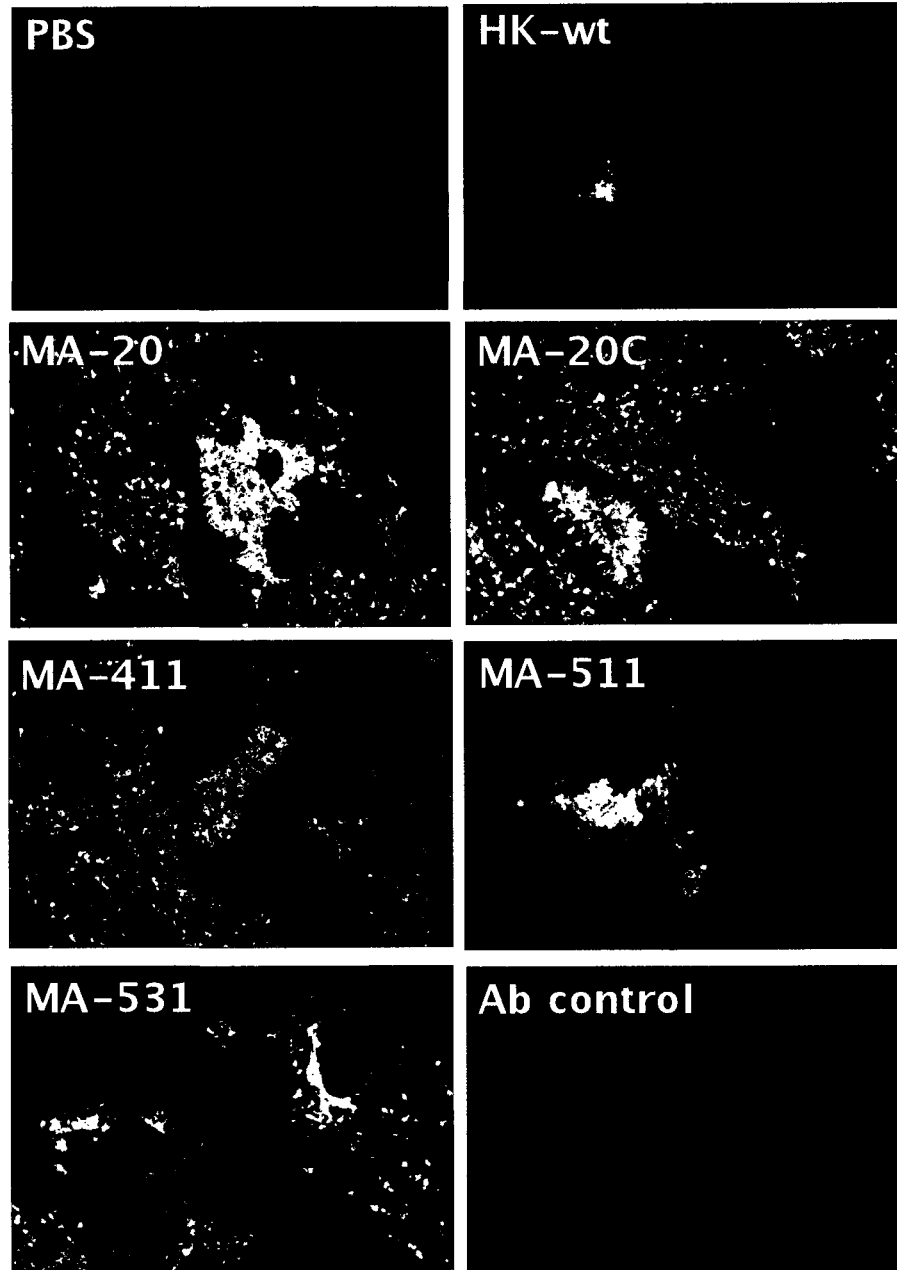
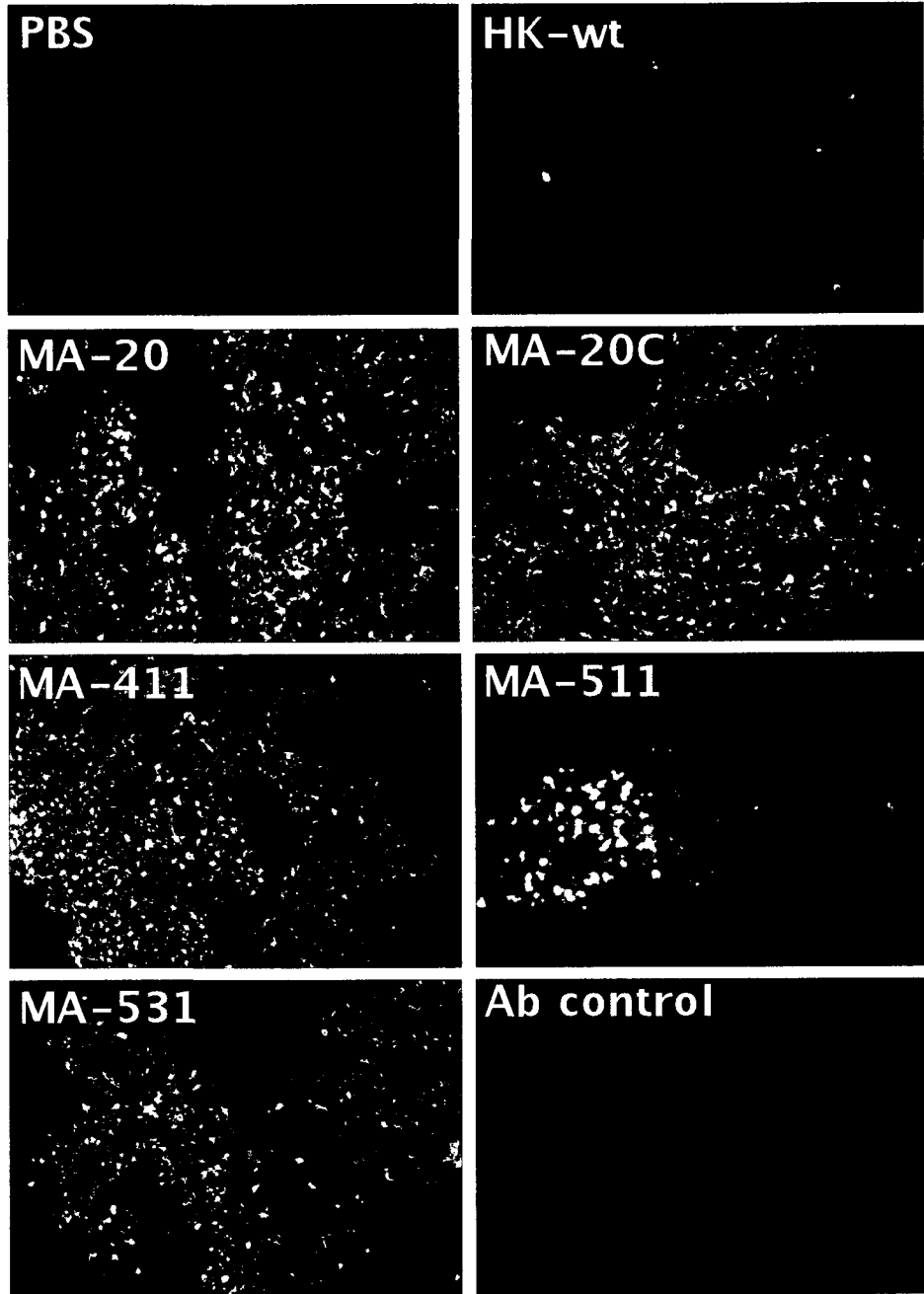


Fig 20. Immunofluorescent staining of Balb/c lungs infected with A/HK/1/68 and the mouse adapted variants. Balb/c mice were intranasally inoculated using 5×10^3 PFU. Lungs were then harvested 2 d.p.i., sectioned and stained using anti-HK primary antibody. Samples were then incubated in Cy3-conjugated secondary antibody (red). Nuclei were also stained using Hoechst (blue). **(A)** Bronchioles, **(B)** Alveoli regions. All the mouse adapted variants spread extensively within the bronchiolar epithelium and alveoli in contrast to the parent.

A



B



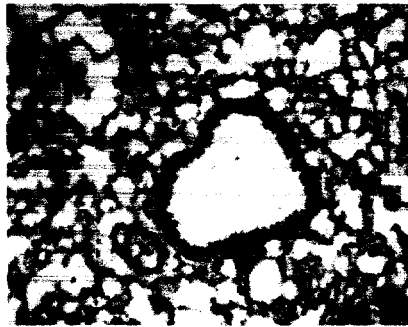
mouse adapted variants. This was consistent with the growth pattern that was observed with this mutant (Fig 18). In general, the level of infection within the bronchioles and alveoli was more extensive in CD-1 than Balb/c mice. In conclusion, mouse adapted isolates acquired the capacity to spread extensively within the bronchioles and alveoli causing pneumonia at low dosages.

3.7. Hematoxylin and eosin (H&E) staining of lungs infected with the parental and mouse adapted variants

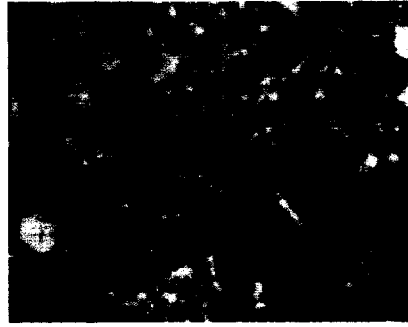
Balb/c mice were inoculated using the parental or mouse adapted variants at 5×10^3 pfu. Lungs were then harvested 2 d.p.i., sectioned and stained using Hematoxylin and eosin (H&E). This was performed to examine the level of injury within the infected lungs. In contrast to the parental virus, most of the mouse adapted variants (except MA-511) caused extensive lung damage (Fig 21). These lungs exhibited an increase in leukocyte infiltration and alveolar hemorrhage. In addition, desquamation of the bronchial epithelium was also observed. In some instances, the necrotic cells appear to end up within the lumen which is more evident in lungs infected with mouse adapted 20C, 411, and 531 where the airways are filled with exudate, debris and cells (Fig 21). There was also a large increase in inflammatory cells surrounding the bronchioles of the mouse adapted variants seen as darkly stained cells.

Fig 21. Hematoxylin and eosin (H &E) staining of infected Balb/c lungs. Balb/c mice were intranasally inoculated using 5×10^3 PFU. Lungs were then harvested 2 d.p.i, sectioned and stained with H&E. Infection with the mouse adapted viruses resulted in extensive lung injury compared to the parent.

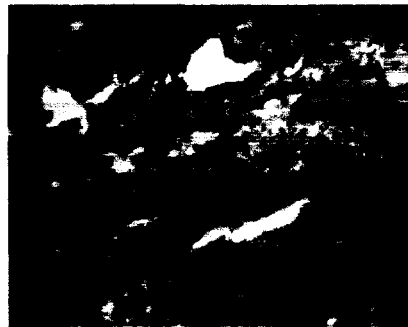
PBS



HK-wt



MA-20



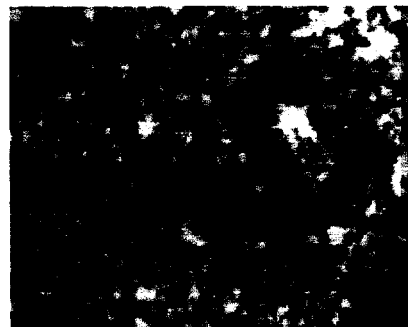
MA-20C



MA-411



MA-511



MA-531



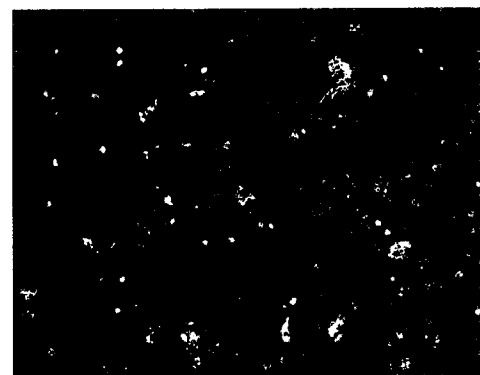
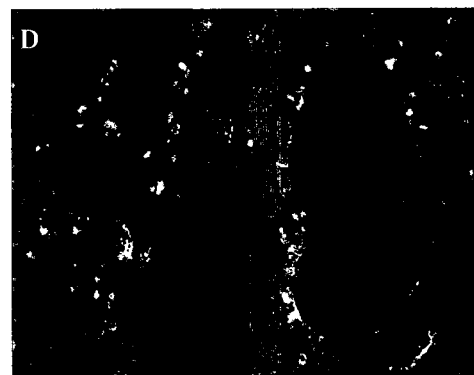
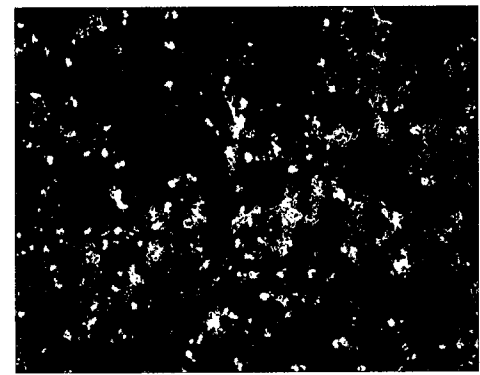
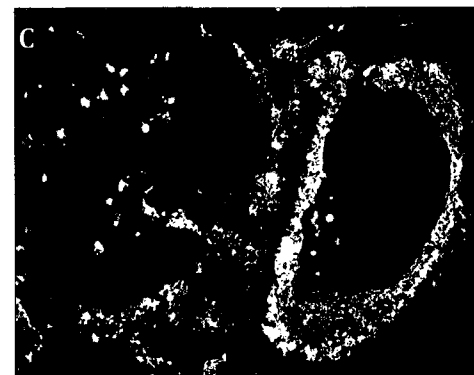
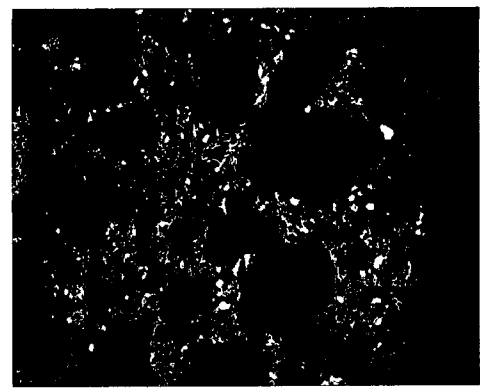
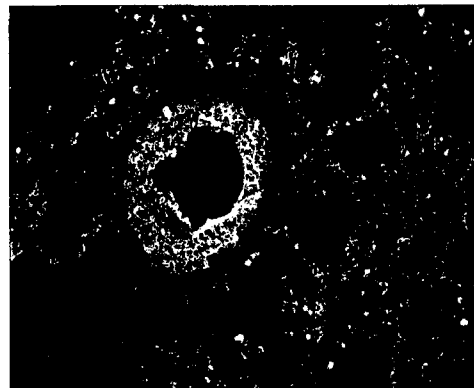
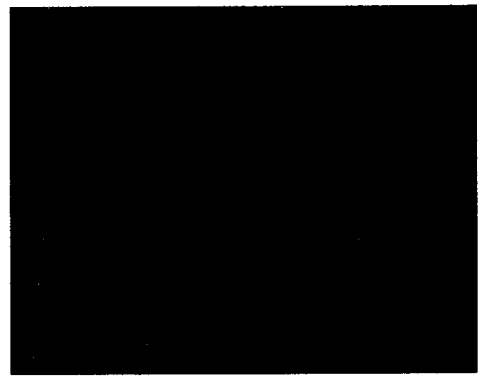
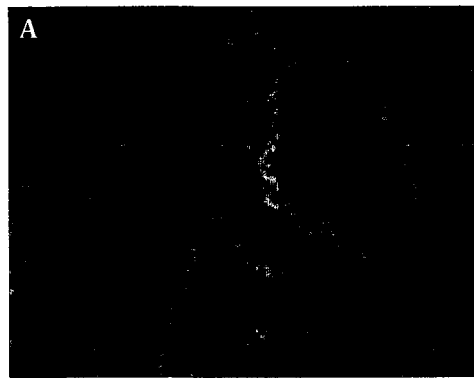
3.8. Immunofluorescent staining of lungs infected with A/Puerto Rico/8/34 (A/PR/8/34), Attenuated H5N1 vaccine strain and A/Turkey/Wisconsin/68 (H5N9)

The next step was to compare the pattern of infection seen with our viruses to other mouse adapted and avian viruses. Therefore, the staining pattern was investigated in lungs infected with A/PR/8/34 (H1N1), an attenuated H5N1 vaccine strain and A/Turkey/Wisconsin/68 (H5N9). Balb/c mice were first inoculated using 10^5 pfu of the appropriate virus before lungs were harvested 3 d.p.i. and stained. As expected, mouse adapted A/PR/8/34 spread efficiently within the bronchial and alveolar areas (Fig 22B). The pattern of infection seen with A/PR/8/34 was comparable to what was observed with our mouse adapted viruses, however in this study infection was performed with 10^5 pfu of virus. A similar pattern of infection was also seen for lungs infected with the attenuated vaccine strain (Fig 22C). This virus contains HA (lacking the multibasic cleavage site) and NA genes from a human H5N1 virus (A/Hong Kong/213/2003) with A/PR/8/34 internal genes. The H5N1 surface genes do not appear to change the ability of this virus to spread efficiently within the lung. In contrast a low pathogenic avian strain A/Turkey/Wisconsin/68, did not spread as extensively within the lung (Fig 22D). Interestingly, this virus shows preferential binding to the alveoli instead of the bronchial epithelium. As an avian virus

Fig 22. Immunofluorescent staining of infected lungs. BALB/c mice were inoculated using 10^5 PFU of virus. Lungs were then harvested 3 d.p.i. sectioned and stained using anti-H1N1 (A/PR8/34) or anti-H5N9 (attenuated H5N1 and A/Turkey/Wisconsin/68) primary antibodies. The samples were then stained with Cy3-conjugated secondary antibody (red) and Hoechst (blue). **(A)** PBS, **(B)** A/PR8/34, **(C)** Attenuated H5N1 vaccine strain and **(D)** A/Turkey/Wisconsin/68. The results indicate that A/PR8/34 and the Attenuated H5N1 vaccine strain are more effective at infecting the lung in comparison to A/Turkey/Wisconsin/68.

Bronchioles

Alveoli



A/Turkey/Wisconsin/68 preferentially binds α 2,3 linked glycans, the SA type that are exclusively expressed within the mouse lung (52). However, our later data indicates that different types of α 2,3 linked glycans appear to be present in the bronchial and alveoli regions.

3.9. Generation of recombinant viruses possessing convergent HA mutations

The role of the HA mutations on a fixed background was investigated by generating synthetic viruses as previously described by Neumann et al. This system employs 8 PHH21 plasmids expressing vRNA specific for each gene segment. In addition, 4 protein expressing plasmids (polymerase proteins and NP) were also included. All 12 plasmids were co-transfected into a confluent monolayer (293T+10% MDCK) to generate the virus of interest (80). The first set of recombinants were rescued using the HA and NA genes from A/HK/1/68 with the remaining six genes of A/WSN/33 (Table 4). These viruses differed solely due to mutations on their HA genes which contained convergent mutations that had been identified following mouse adaptation (Tables 1 and 3). The recombinant WSN viruses were designated as rWSN-HA-HK. In addition, we attempted to rescue the HA mutations using A/HK/1/68 backbone. However, only the wt and G218¹W mutant were successfully rescued on this backbone (Table 4). The recombinant HK viruses were designated as rHK-HA.

Table 4. The recombinant WSN (rWSN) viruses were generated using HK HA and HK NA on A/WSN/33 backbone, while the recombinant HK (rHK) viruses contained all eight genes from A/HK/1/68.

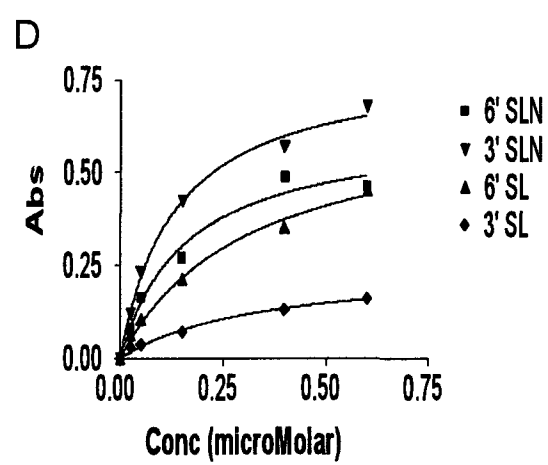
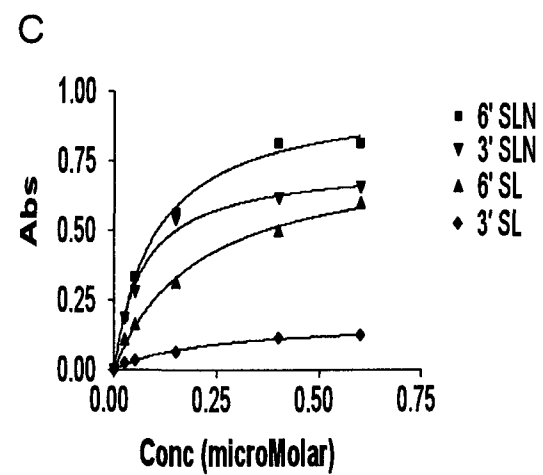
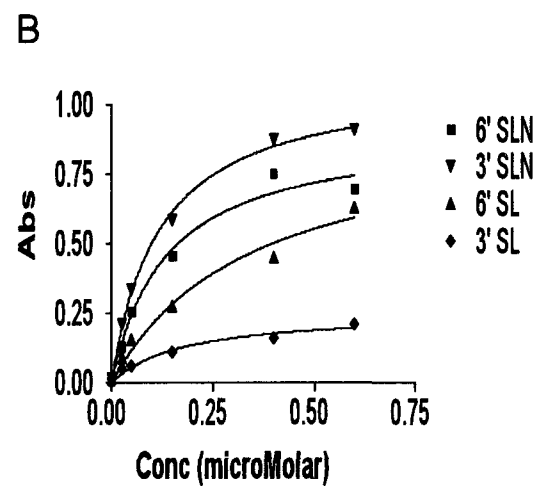
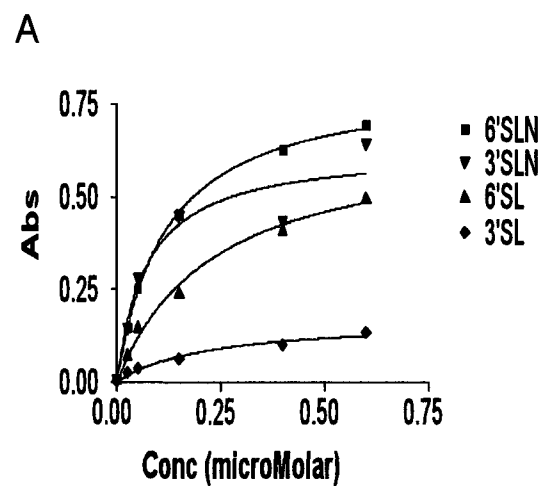
1. rWSN-HA-HK-wt
2. rWSN-HA-HK-G218¹W
3. rWSN-HA-HK-T156²N
4. rWSN-HA-HK-G218¹W+ T156²N
5. rWSN-HA-HK-G218¹E
6. rWSN-HA-HK-Q210¹R
7. rWSN-HA-HK-P162¹S
8. rHK-HA-wt
9. rHK-HA-G218¹W

3.10. Receptor binding specificity of the recombinant viruses

Receptor binding ability with respect to sialic acid linkage type was examined for the rWSN-HA-HK viruses containing G218¹W, T156²N or G218¹W+T156²N mutations. Several Sialyl(N-acetyl)lactosamine (SLN) and Sialyllactose (SL) polymers were utilized as previously described. These consist of SA α 2,6Gal (6'SLN, 6'SL) and SA α 2,3Gal (3'SLN, 3'SL) linkages. The polymers used in this assay contain biotin connected to a polyacrylamide linker (69). When the assay was performed using SL, HK-wt as well as the mutants exhibited a similar pattern. All the viruses tested preferentially recognized 6'SL instead of 3'SL (Fig 23). The G218¹W, T156²N or G218¹W+T156²N mutations do not appear to alter the binding specificity of the viruses for these polymers.

The assay was then repeated using SLN which differs due to an N-acetyl modification at the inner saccharide of the receptor. The structure of this receptor is Neu5Ac α 2,6 or α 2,3Gal β 1-4GlcNAc showing the modification on the inner sugar (glucose). This ligand was chosen since the identity of the third saccharide has been implicated in altering binding specificity. In accordance with this, certain human viruses prefer 6'SLN over 6'SL (32). When the assay was performed using 6'SLN and 3'SLN different binding patterns were observed. HKwt as well as the T156²N mutant showed strong binding to these polymers. However, these viruses still favored 6'SLN over 3'SLN

Fig 23. Receptor binding specificity of the rWSN-HA-HK viruses. The ability of these viruses to bind biotinylated ligands (6'SL, 3'SL, 6'SLN and 3'SLN) was assessed using streptavidin-peroxidase. Graphs represent Absorbance (450 nm) versus polymer concentration. **(A)** rWSN-HA-HK-wt, **(B)** rWSN-HA-HK-G218¹W, **(C)** rWSN-HA-HK-T156²N and **(D)** rWSN-HA-HK-G218¹W+ T156²N.

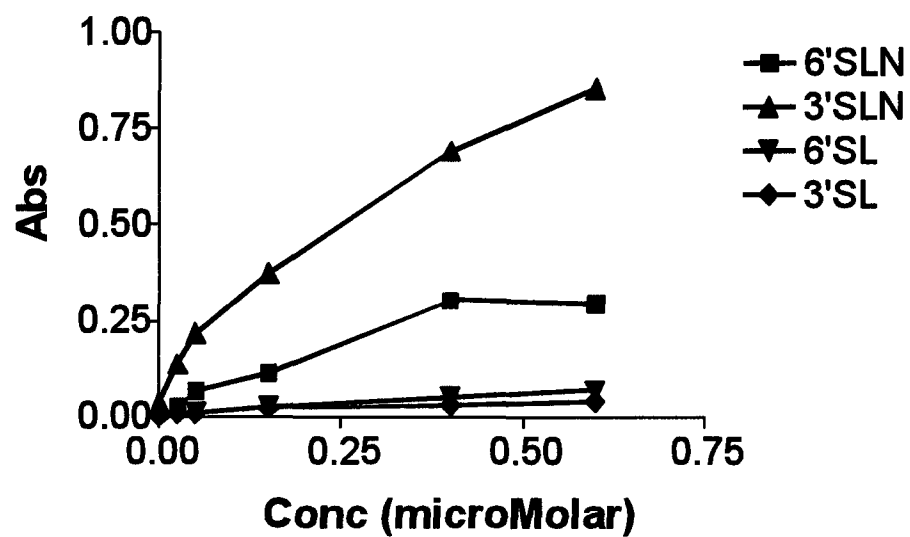


(Fig 23). In contrast, the G218¹W and G218¹W+T156²N mutants preferentially recognized 3'SLN. These mutants gained the ability to bind 3'SLN even though they retained binding to 6'SLN. This indicated a shift in receptor binding specificity towards SA α 2,3Gal containing ligands.

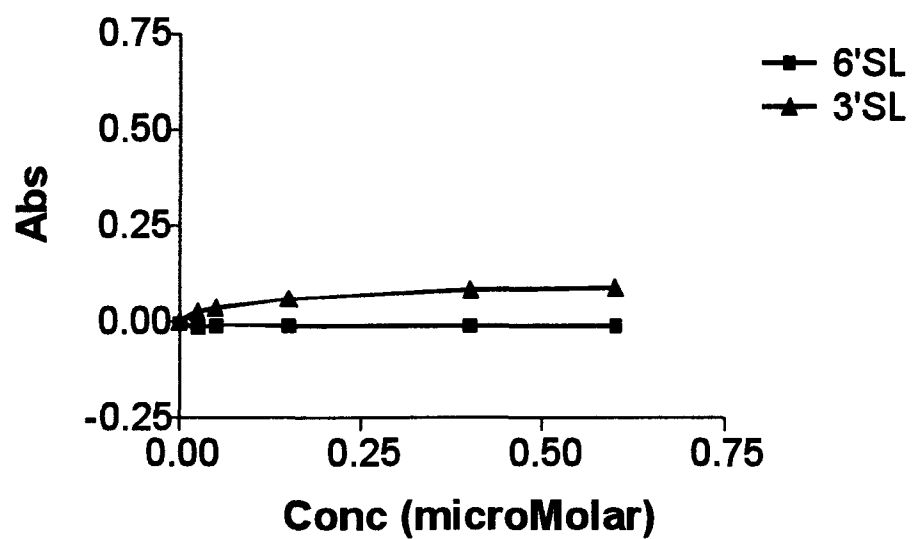
Receptor binding specificity of the attenuated H5N1 vaccine strain and A/Turkey/Wisconsin/68 (H5N9) was also examined. When the SL ligands were used, the attenuated H5N1 virus appeared to bind both 6'SL and 3'SL with similar affinity (Fig 24A). With respect to SLN, this virus preferentially recognized 3'SLN over 6'SLN. Previous studies indicate that most human H5N1 viruses isolated between 1997 and 2004 exhibit strong binding to SA α 2,3Gal. However certain viruses from 2003, such as A/HK/212/2003 and A/HK/213/2003, showed decreased affinity for SA α 2,3Gal. These viruses had gained the ability to bind SA α 2,6Gal, through the acquisition of certain HA mutations (32). In accordance with these results, the attenuated H5N1 vaccine strain bound both 3'SLN and 6'SLN, however this virus still favored 3'SLN. In comparison, A/Turkey/Wisconsin/68 bound 3'SL better than 6'SL (Fig 24B). This is consistent with the fact that avian viruses such as A/Turkey/Wisconsin/68 prefer α 2,3 linked receptors (34). Unfortunately, the binding specificity of this virus for the SLN ligands could not be elucidated due to technical difficulties.

Fig 24. Receptor binding specificity of the attenuated H5N1 vaccine strain and A/Turkey/Wisconsin/68 (H5N9). The ability of these viruses to bind biotinylated ligands (6'SL, 3'SL, 6'SLN and 3'SLN) was assessed using solid phase binding assay. Graphs represent Absorbance (450 nm) versus polymer concentration. **(A)** Attenuated H5N1 vaccine strain and **(B)** A/Turkey/Wisconsin/68.

A



B

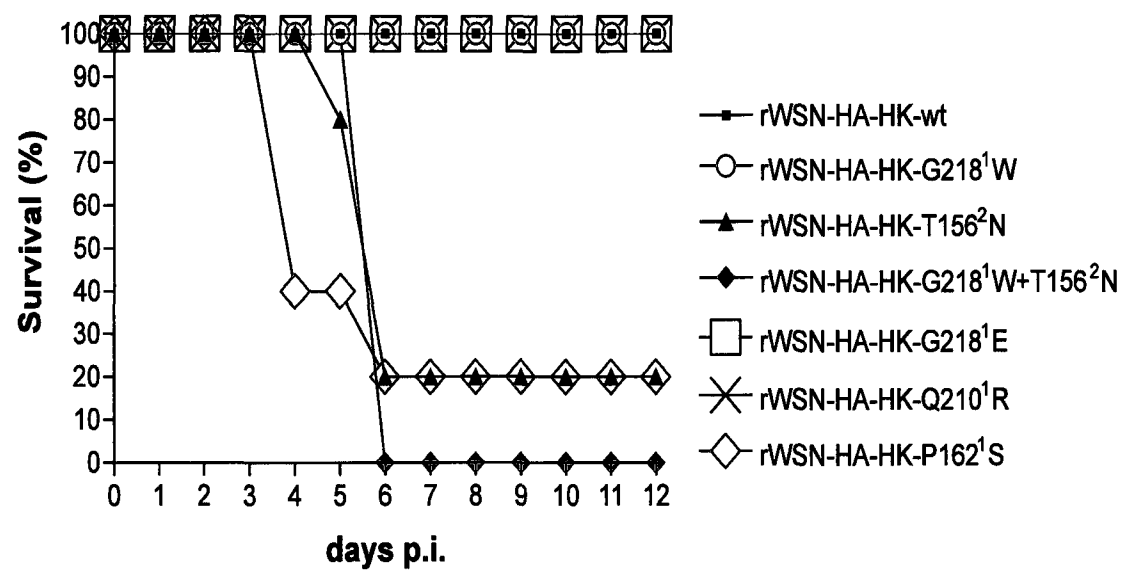


3.11. Median lethal dose (LD₅₀) of the rWSN-HA-HK viruses

Virulence of the rWSN-HA-HK viruses was evaluated in Balb/c mice (n=5). After inoculation with 10⁶ pfu, mortality was recorded over a 2 week period of observation. HK-wt and three of the mutants (G218¹W, G218¹E and Q210¹R) were not lethal at this dosage. As a result, these viruses have an LD₅₀ of >10^{6.5} PFU (Fig 25). On the other hand, three of the mutants (T156²N, P162¹S, G218¹W+T156²N) were more virulent and resulted in mortality. Two of them (T156²N, P162¹S) killed 80% at 6 d.p.i. Moreover, it appears that death from P162¹S happened earlier at 4 d.p.i. (Fig 25). LD₅₀ for the mutants was calculated to be 10^{5.7} pfu (>10^{0.8} fold increase in virulence). In comparison, the G218¹W+T156²N mutant killed 100% at 6 d.p.i. As a result, the infection was repeated using 10⁵ pfu which was not lethal for any of the 5 mice. Therefore, LD₅₀ for this mutant was 10^{5.5} pfu (>10¹ fold increase in virulence). In conclusion, three of our mutations (T156²N, P162¹S, G218¹W+T156²N) resulted in enhanced virulence however, the effect of G218¹W, G218¹E and Q210¹R could not be elucidated using this backbone.

During the survival study, morbidity was also examined by recording weight changes. All six mice infected with rWSN-HA-HK-wt exhibited some weight loss. Four of these mice lost 10% of their weight at 3 d.p.i. prior to weight gain. On the other hand, two of these mice

Fig 25. Virulence of the rWSN-HA-HK viruses. Balb/c mice (n = 5) were first inoculated using 10^6 PFU. Mortality was then recorded for 12 days. The T156²N, G218¹W+ T156²N and P162¹S mutations were involved in enhancing virulence.



lost 20 and 30% of their weight respectively at 6 d.p.i., however both regained the weight and survived (Fig 26A). Of the five mice infected with the G218¹W mutant, three of them lost ~10% of their weight at 3 d.p.i. before regaining it back. In contrast, two of them did not exhibit any weight loss (Fig 26B). Four of the five mice infected with the T156²N mutant exhibited considerable weight loss (~30%) and died by 6 d.p.i. The remaining mouse lost 15% of its weight at 3 d.p.i. followed by weight gain and survival (Fig 26C). All five mice infected with the G218¹W+T156²N mutant lost ~30% of their weight at 6 d.p.i. followed by death (Fig 26D). On the other hand, the weight of the five mice infected with the G218¹E mutant remained somewhat constant throughout the study (Fig 26E). Four of the five mice infected with the Q210¹R mutant lost 10% of their body weight at 3 d.p.i. followed by weight gain. In comparison, one mouse did not exhibit any weight loss for the duration of the study (Fig 26F). All five mice infected with the P162¹S mutant exhibited considerable weight loss. Four of the five mice lost ~30% of their weight followed by death. Of these, three mice died by 4 d.p.i. while one mouse died two days later. In contrast, the remaining mouse lost 20% of its weight at 5 d.p.i. followed by weight gain and survival (Fig 26G).

The body weights were also represented as an average (Fig 27). Some heterogeneity was observed with respect to HK-wt, G218¹W, G218¹E and Q210¹R. Even though none of these viruses were lethal

Fig 26. Weight change in mice inoculated with the rWSN-HA-HK viruses. During the survival study weights were recorded on a daily basis. The graphs represent body weights of individual mice within each group. **(A)** rWSN-HA-HK-wt, **(B)** rWSN-HA-HK-G218¹W, **(C)** rWSN-HA-HK-T156²N, **(D)** rWSN-HA-HK-G218¹W+ T156²N, **(E)** rWSN-HA-HK-G218¹E, **(F)** rWSN-HA-HK-Q210¹R and **(G)** rWSN-HA-HK-P162¹S.

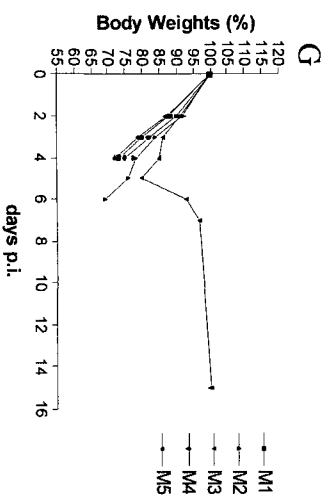
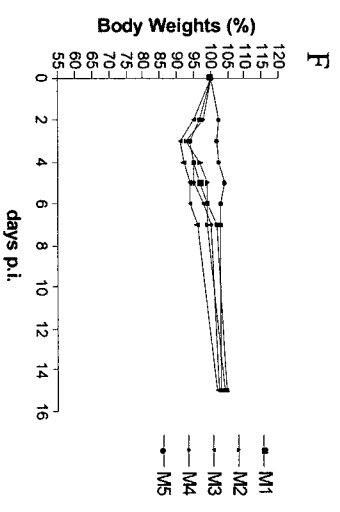
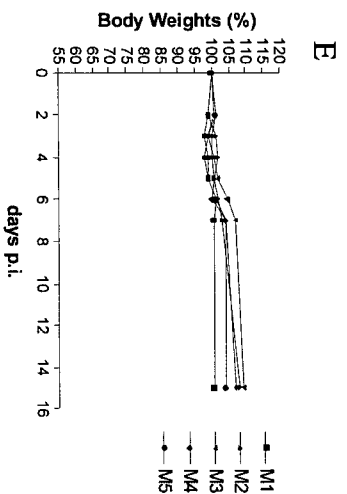
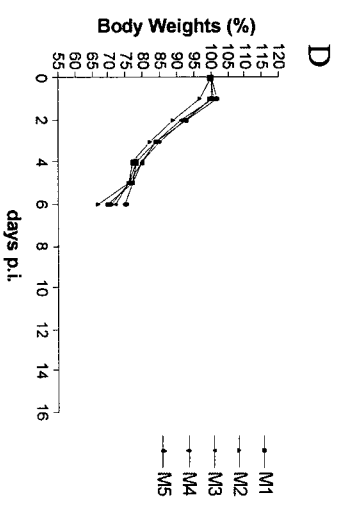
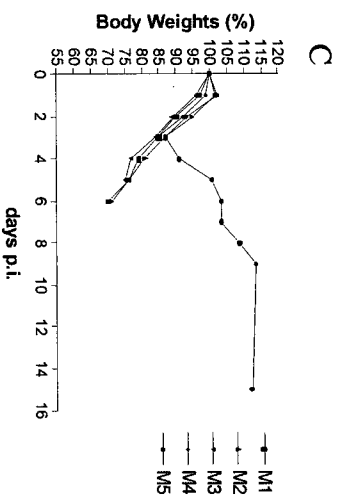
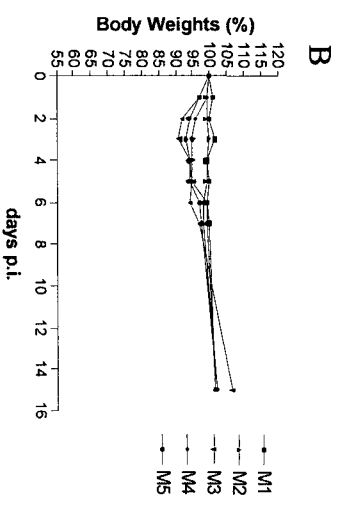
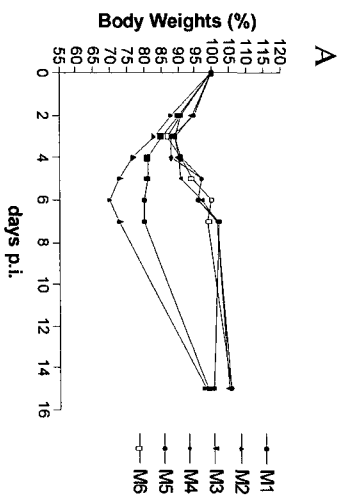
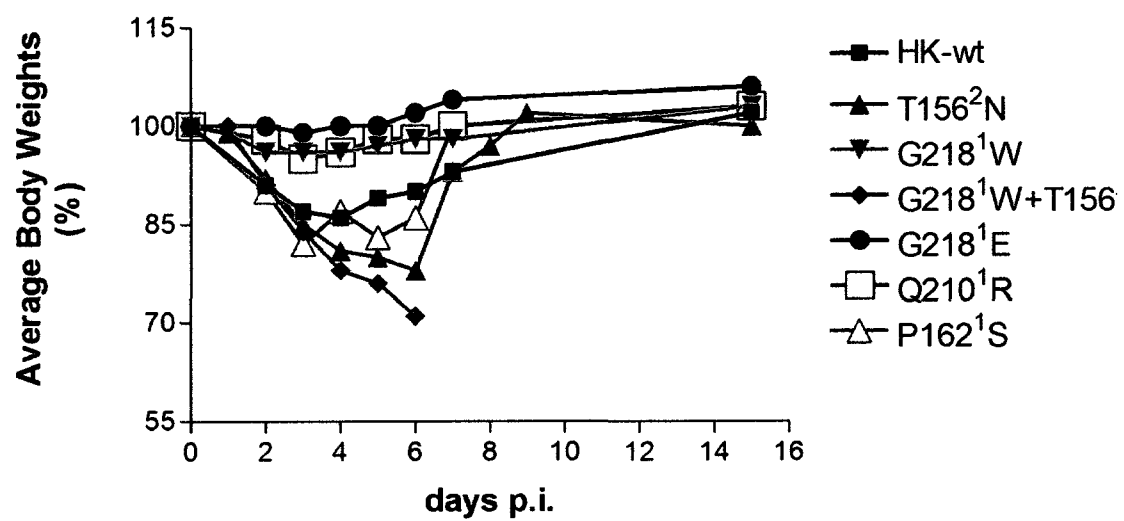


Fig 27. Average body weights of mice inoculated with the rWSN-HA-HK viruses. Each point represents the average weight of 5 mice per group.

B



(Fig 25) mice infected with HK-wt still lost on average 15% of their weight at 4 d.p.i. before regaining it back. In contrast, body weights of mice infected with the G218¹W, G218¹E and Q210¹R mutants remained relatively unchanged (Fig 27). In general, the body weights were consistent with the LD₅₀ data (Figs 25 and 27).

3.12. Median lethal dose (LD₅₀) of the rHK-HA viruses

Virulence of the rHK-HA viruses was also determined in Balb/c mice (n = 5) after inoculation of 10⁶ pfu. Surprisingly, rHK-HA-wt killed 20% at 6 d.p.i. showing an LD₅₀ of 10^{6.3} PFU (Fig 28). In contrast, the rHK-HA-G218¹W mutant was not lethal. Therefore this mutant has an LD₅₀ >10^{6.5} PFU (Fig 28).

During this study body weight was also recorded on a daily basis. Of the five mice infected with rHK-HA-wt, two lost ~15% of their weight at 5 d.p.i. prior to weight gain and survival. Another two lost ~20% of their weight at 4 d.p.i., at which point one mouse regained the weight and survived while the other died by 6 d.p.i. The remaining mouse lost ~5% of its weight at 2 d.p.i. before regaining it back (Fig 29A). Of the five mice infected with the rHK-HA-G218¹W mutant, two of them lost ~5% of their weight at 3 d.p.i. before regaining the weight. In contrast, the remaining three did not exhibit any weight loss (Fig 29A). The body weights were also represented as an average (Fig 29B).

Fig 28. Virulence of the rHK-HA viruses. Balb/c mice (n = 5) were inoculated at 10^6 PFU. Following infection, mortality was recorded for 12 days.

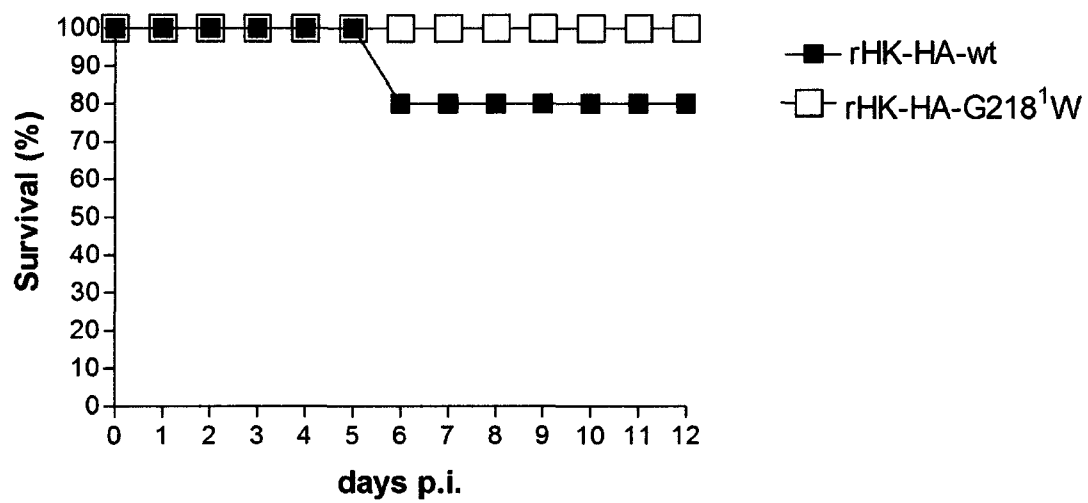
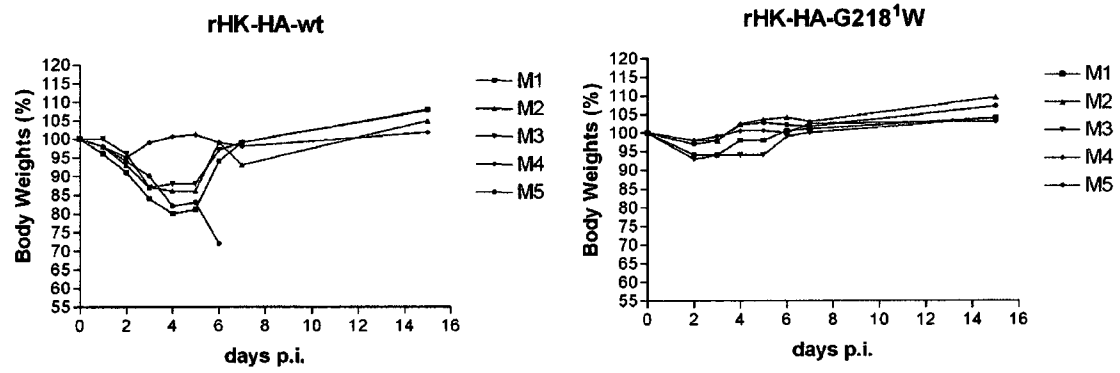
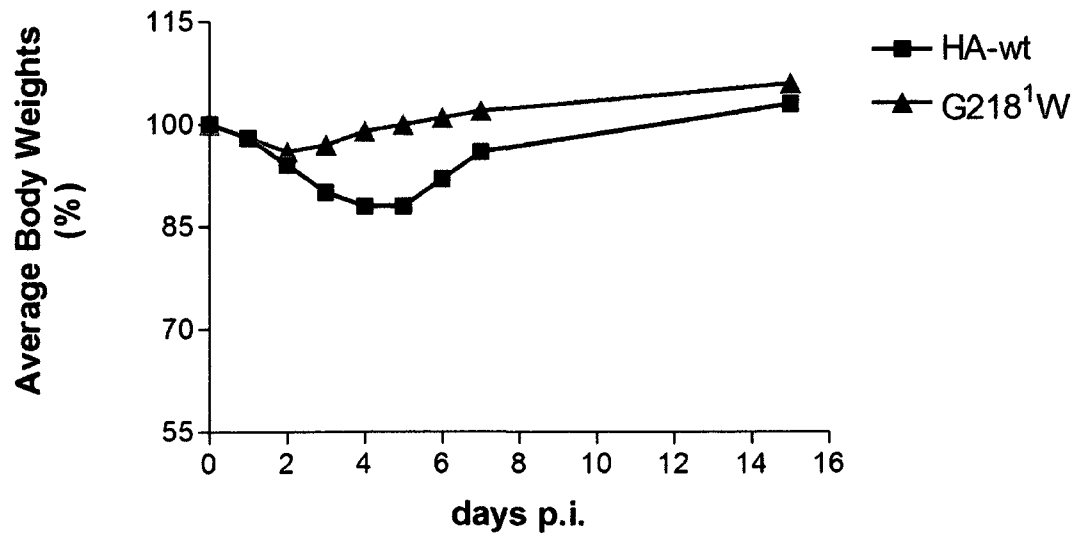


Fig 29. Weight change in mice inoculated with the rHK-HA viruses. During the survival study, body weights were recorded daily. **(A)** Body weights of individual mice within each group, **(B)** Each point represents the average weight of 5 mice per group.

A



B



The results observed here were consistent with the LD₅₀ data (Figs 28 and 29B).

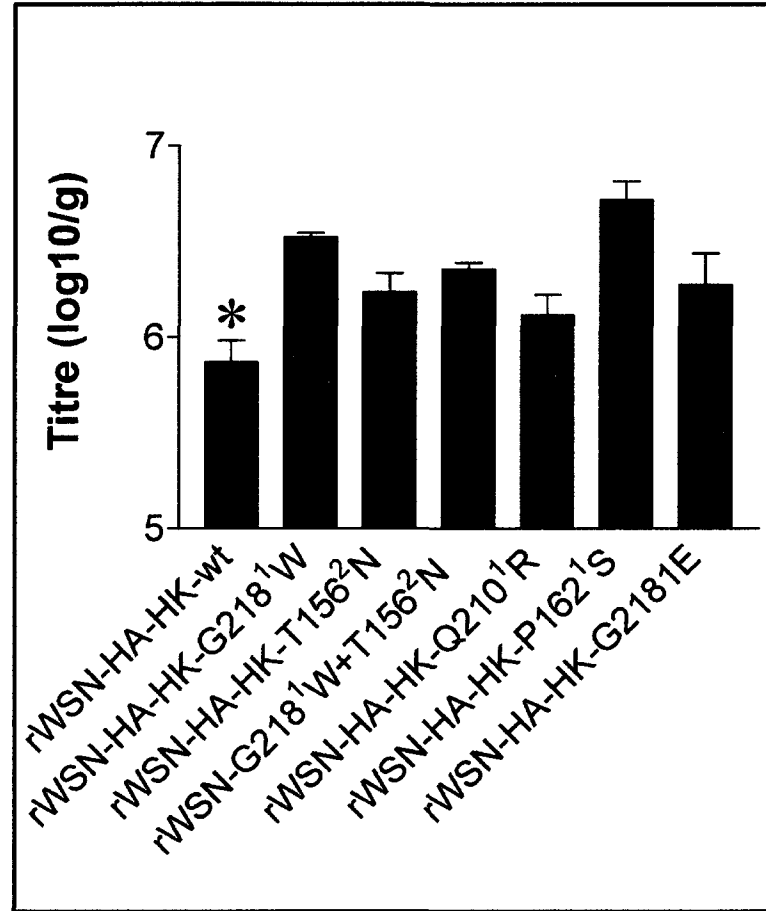
3.13. Growth of the recombinant viruses in mouse lungs

The effect of the HA mutations on lung replication was also investigated in Balb/c mice (n = 3) using the rWSN-HA-HK viruses. Infection was first performed using 5×10^3 pfu of virus. Lungs were then harvested 3 d.p.i. and the samples were titrated to determine the yield. All the mutants tested replicated significantly better than HK-wt, $p < 0.05$ (Fig 30). G218¹W, G218¹W+T156²N and P162¹S exhibited the highest titre. Two of these mutants (G218¹W+T156²N and P162¹S) are also more virulent than HK-wt. In comparison the G218¹W mutant did not kill at the dose used to assess virulence (Fig 25). In general, the mouse adapted HA mutations appear to provide these viruses with a replicative advantage.

3.14. Immunofluorescent staining of lungs infected with the rWSN-HA-HK viruses

The mouse adapted HA mutations increased the ability of the viruses to replicate within the lung. Next we examined the effect these mutations might have on tropism. The staining pattern was investigated in lungs infected with the rWSN-HA-HK viruses. Balb/c mice were first inoculated using 10^5 pfu. Lungs were then harvested 2 d.p.i. sectioned

Fig 30. Growth of the rWSN-HA-HK viruses in lungs. BALB/c mice (n=3) were intranasally inoculated using 5×10^3 PFU. Lungs were then harvested 3 d.p.i. and titrated to quantify the yield. All the mutants tested replicated considerably better than HK-wt ($p < 0.05$).



and stained using anti-HK antibody. HK-wt infected a small area within the bronchioles. In contrast, most of the mutants spread more efficiently within the bronchial epithelium. The only exception was Q210¹R which exhibited a similar staining pattern as HK-wt (Fig 31A). The results obtained from the staining were also quantified. Two frozen lung sections were analyzed for each sample. The different patterns of bronchial staining were counted and represented as percentages (Table 5). All the mutants spread efficiently within the alveoli when compared to HK-wt (Fig 31B). Interestingly, the virulent mutants resulted in the most extensive staining.

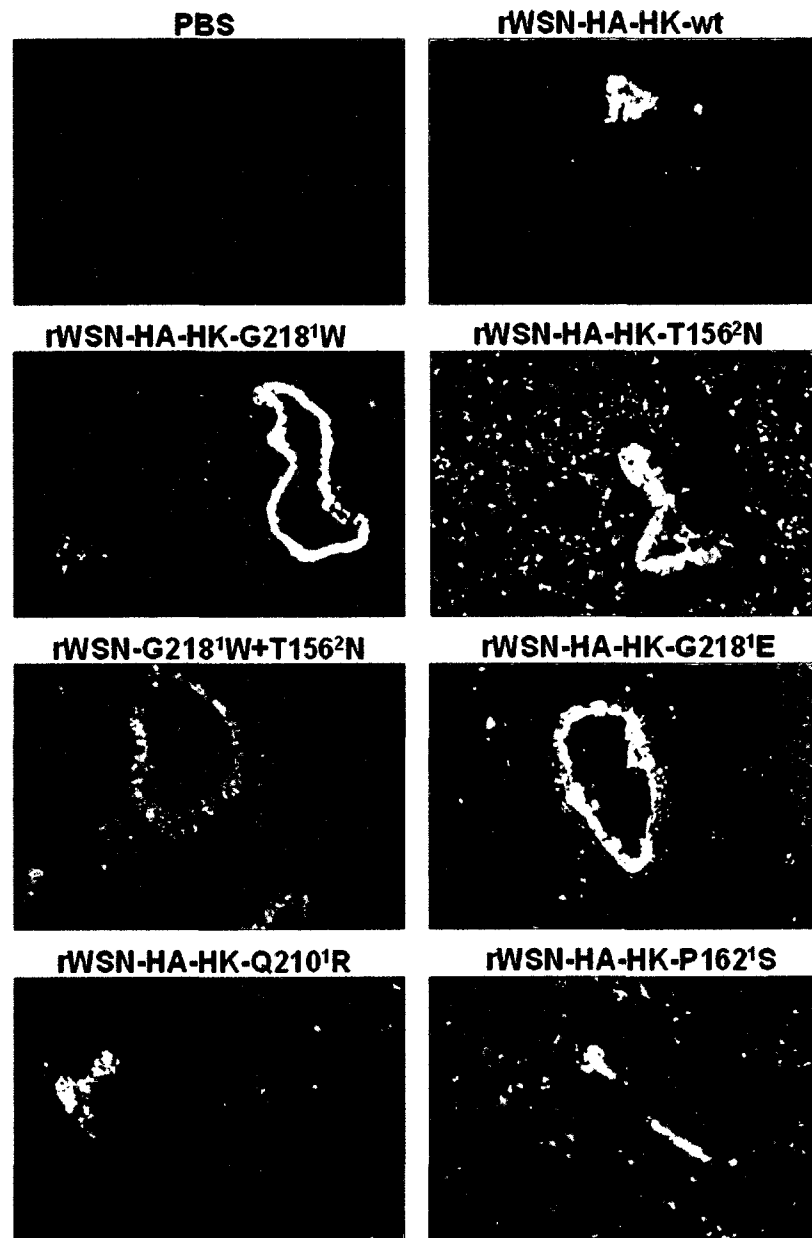
Lungs infected with the rWSN-HA-HK viruses were also stained with hematoxylin and eosin. As expected, these lungs were not as damaged as those infected with the mouse adapted viruses. However, most of the mutants (T156²N, G218¹W+T156²N and Q210¹R) still caused more damage within the bronchial epithelium compared to HK-wt (Fig 32).

3.15. Immunofluorescent staining of lungs infected with the rHK-HA viruses

The level of infection was also examined in mice inoculated with 10⁵ pfu of rHK-HA viruses using immunofluorescent staining for viral protein.

Fig 31. Immunofluorescent staining of lungs infected with the rWSN-HA-HK viruses. Balb/c mice were first inoculated using 10^5 PFU. Lungs were then harvested 2 d.p.i., sectioned and stained using anti-HK primary antibody. The samples were then stained with Cy3-conjugated secondary antibody (red). Nuclei were also stained using Hoechst (blue). **(A)** Bronchial epithelium, **(B)** Alveolar regions. Most of the mutants spread extensively within the bronchial epithelium and alveoli compared to HK-wt.

A



B

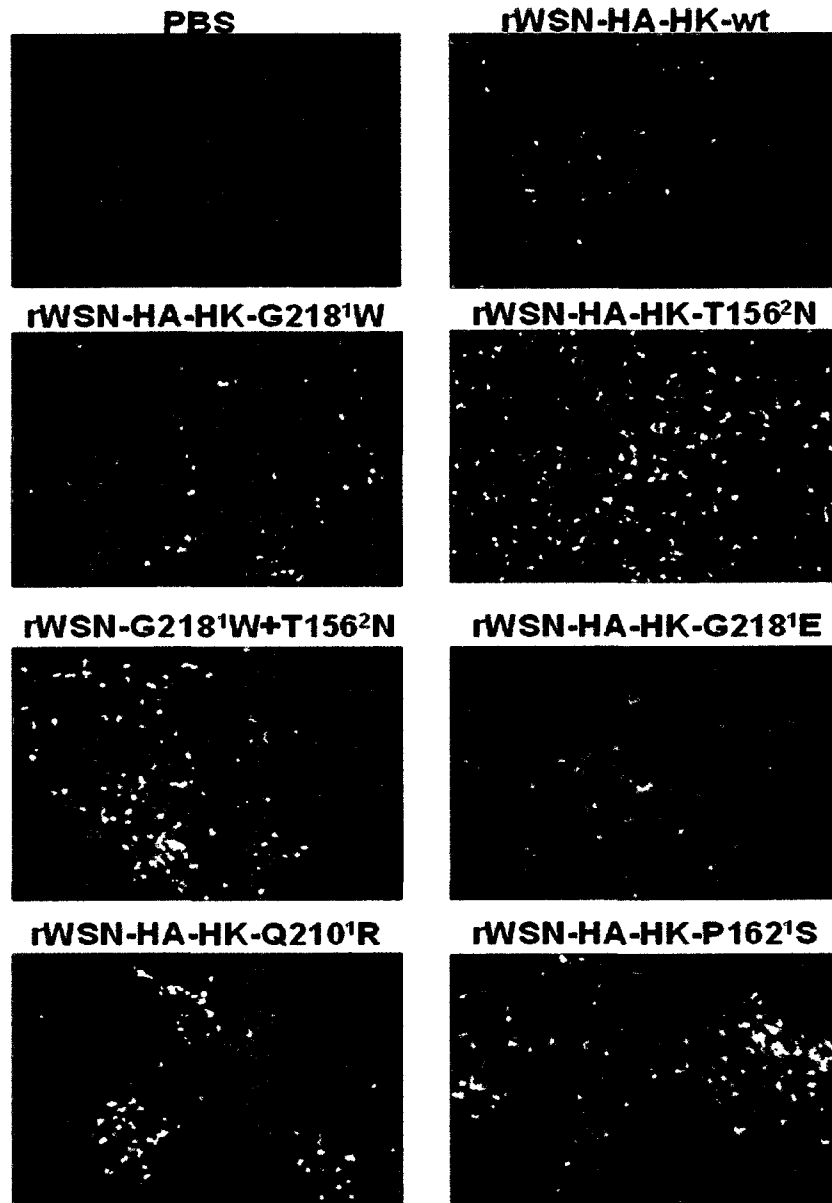
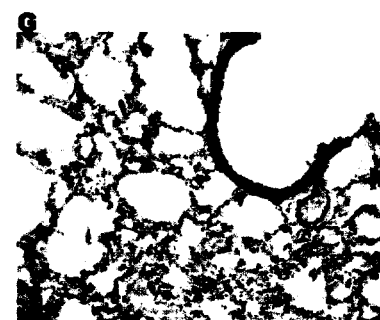
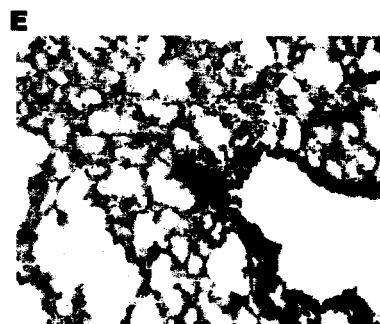
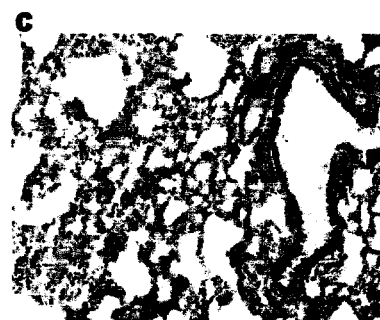


Table 5. Staining pattern of bronchioles seen in representative lungs infected with the rWSN-HA-HK viruses.

Recombinant viruses	Fully stained	$\frac{3}{4}$ stained	$\frac{1}{2}$.5 stained	$\frac{1}{4}$ stained	< $\frac{1}{4}$ stained	No staining
rWSN-HA-HK-wt	-	-	-	10 %	50 %	40 %
rWSN-HA-HK-G218¹W	9 %	-	-	-	64 %	27 %
rWSN-HA-HK-T156²N	7 %	-	-	13 %	40 %	40 %
rWSN-HA-HK-G218¹W + T156²N	6 %	12 %	-	40 %	24 %	18 %
rWSN-HA-HK- G218¹E	7%	-	-	14 %	7 %	72 %
rWSN-HA-HK- Q210¹R	-	-	-	9 %	36 %	55 %
rWSN-HA-HK- P162¹S	-	-	14%	14 %	43 %	29 %

- Not Applicable

Fig 32. Hematoxylin and eosin (H &E) staining of lungs infected with the rWSN-HA-HK viruses. BALB/c mice were intranasally inoculated using 10^5 PFU. Lungs were then harvested 2 d.p.i , sectioned and stained with H&E. (A) PBS, (B) rWSN-HA-HK-wt, (C) rWSN-HA-HK-G218¹W, (D) rWSN-HA-HK-T156²N, (E) rWSN-HA-HK-G218¹W+ T156²N, (F) rWSN-HA-HK-Q210¹R and (G) rWSN-HA-HK-P162¹S. Infection with the mutants caused more damage to the bronchial epithelium in comparison to HK-wt.



The rHK-HA-G218¹W mutant spread extensively within the bronchioles when compared to rHK-HA-wt that infected only a few cells (Fig 33).

With respect to the G218¹W mutant, two different bronchial staining patterns were observed as shown in Fig 33. Interestingly, the alveoli did not show much staining in lungs infected with either of the rHK-HA viruses.

3.16. Infection of Mouse tracheal epithelial cells (mTEC) with the rWSN-HA-HK viruses

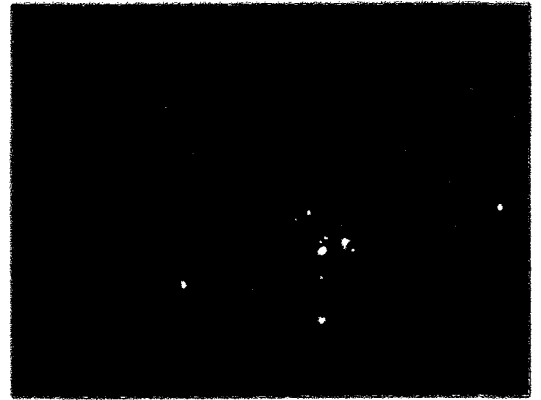
The ability of the rWSN-HA-HK viruses to replicate within primary Mouse tracheal epithelial cells (mTECs) was investigated. The cells were first differentiated at the air-liquid interface as explained by Ibricevic et al (52). The apical (top) side of the monolayer was then inoculated with the synthetic viruses using MOI of 3. After a 20 hr incubation period, the mTECs were stained with anti-HK antibody to determine the extent of infection. The experiment was done in duplicate for each sample. Infected cells were then counted for several microscopic fields (n = 4 to 8). In comparison to HK-wt, most mutants infected considerably higher percentage of cells ($p < 0.0001$) (Fig 34A and B). Of these mutants, Q210¹R and P162¹S were identified as the most efficient. The only exception was the G218¹E mutant which infected a lower percentage of cells compared to HK-wt.

Fig 33. Immunofluorescent staining of lungs infected with the rHK-HA viruses. CD-1 mice were inoculated using 10^5 PFU of virus. Lungs were then harvested 3 d.p.i. and stained using anti-HK primary antibody. Antigen was then detected by using Cy3-conjugated secondary antibody (red) and Hoechst (blue) to stain the nuclei. rHK-HA-G218¹W spread extensively within the bronchial epithelium in comparison to rHK-HA-wt.

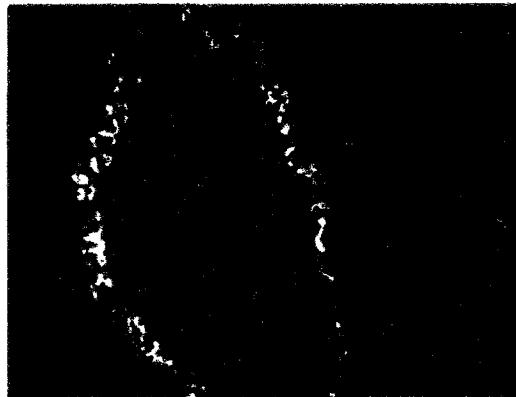
PBS



rHK-HA-wt



rHK-HA-G218¹W



rHK-HA-G218¹W

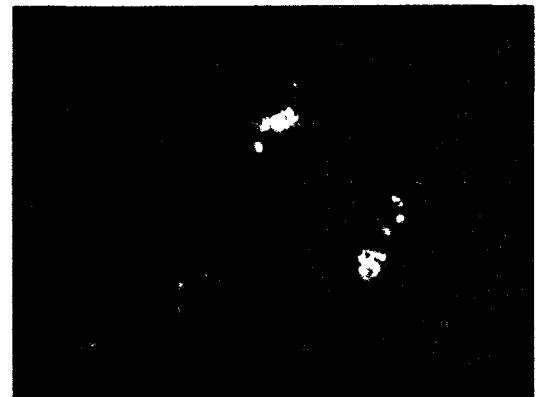
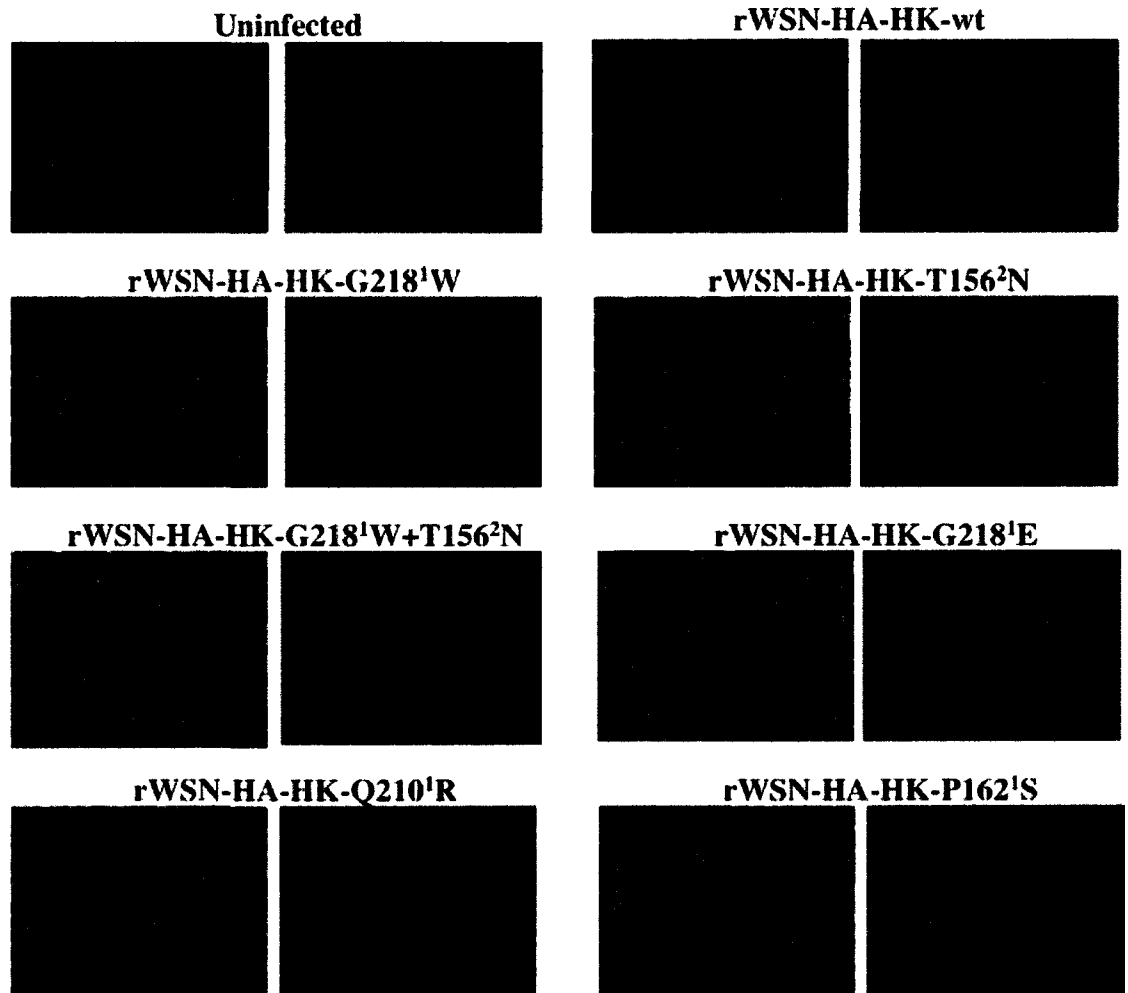
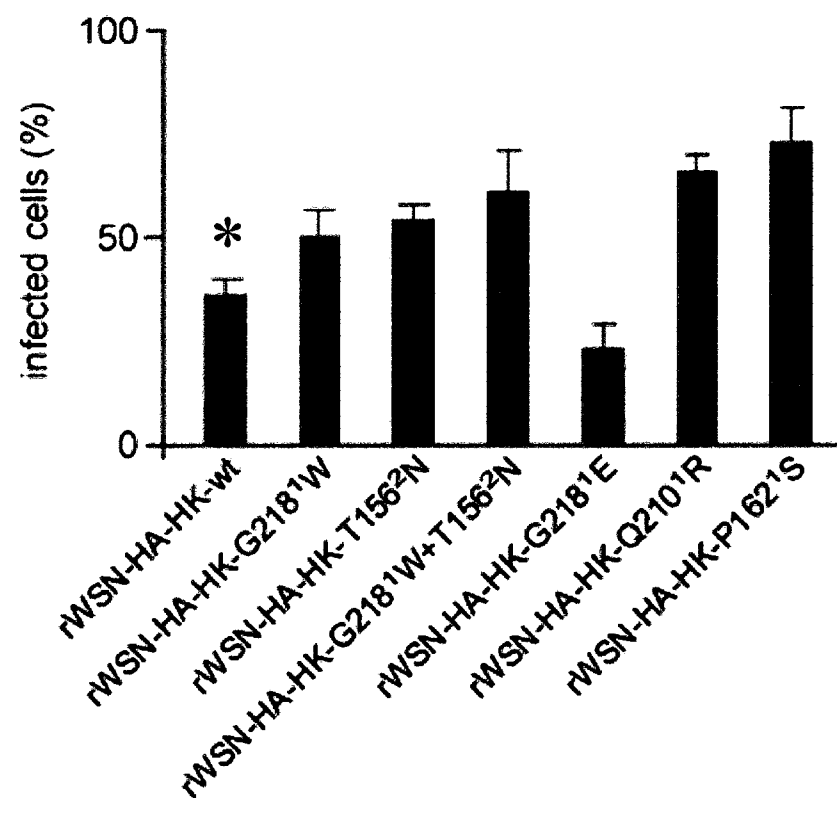


Fig 34. Infection of Mouse Tracheal Epithelial cells (mTECs) using the rWSN-HA-HK viruses. Cells were grown and differentiated under air-liquid interface. The mTECs were then infected at MOI of 3, followed by a 20 hr incubation period. **(A)** The infected cells were stained using anti-HK antibody (red). The nuclei were also stained using 4',6-diamidino-2-phenylindole (DAPI) (blue). **(B)** Percentage of infected cells. Infections were performed in duplicate and positive cells were counted for several microscopic fields ($n = 4$ to 8). Most of the mutants, with the exception of G218¹E, infected higher percentage of mTECs compared to HK-wt ($p < 0.0001$).

A



B

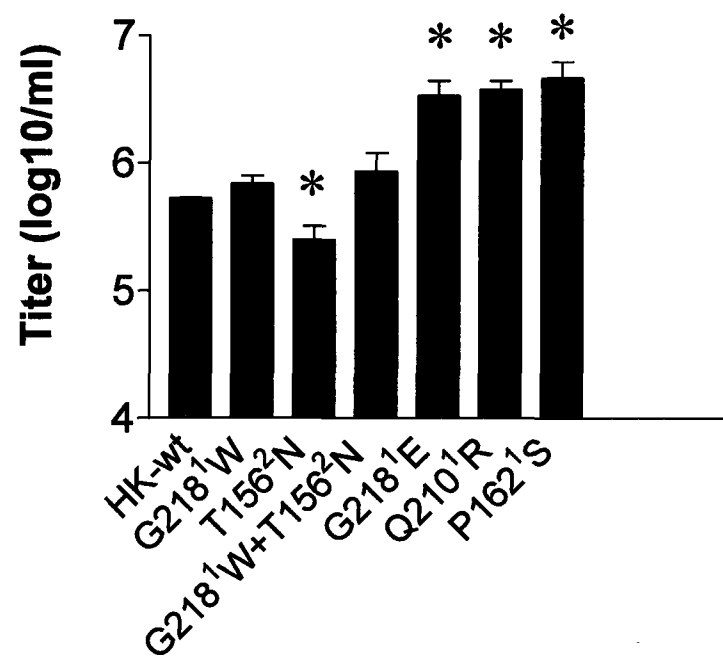


In addition, the amount of virus released after infection was quantified by a plaque assay. In comparison to HK-wt, most of the mutants replicated to a higher titre with three of them (G218¹E, Q210¹R and P162¹S) exhibiting a significant increase ($p < 0.05$) (Fig 35). The increase in titre associated with Q210¹R and P162¹S was comparable to the level of infection observed in Fig 34A. In contrast, the high titer acquired from G218¹E is inconsistent with the level of infection. These results suggest that G218¹E has a reduced ability to infect cells and may be inhibited during entry but replicates much more efficiently once inside the cell. In addition, T156²N exhibited significantly lower yield than HK-wt despite illustrating an extensive ability to infect these cells (Fig 34A). Based on the results, this mutant appears to be efficient at entering the cells but is hindered at the final stages of the replication cycle, possibly during assembly or release. Furthermore, decrease in yield observed with T156²N appears to be rescued by G218¹W since the double mutant (G218¹W+ T156²N) exhibits an increase in titre (Fig 35).

3.17. Infection of Mouse tracheal epithelial cells (mTEC) with the rHK-HA viruses

The ability of the rHK-HA viruses to infect mTECs was also investigated. In accordance with the above results, the G218¹W mutant infected higher percentage of cells compared to the wt (Figs 36A and B). However, there was a difference with respect to the different backbones,

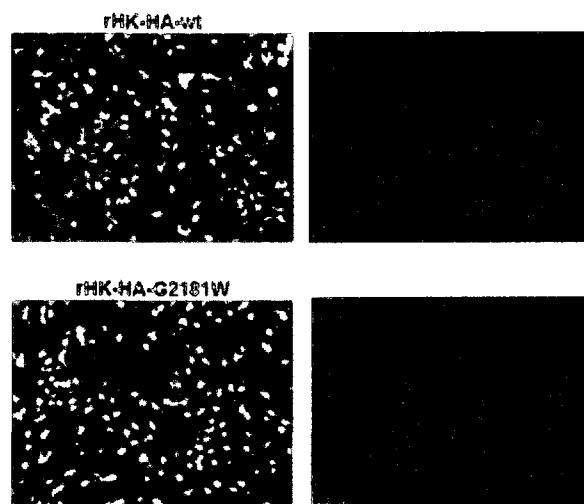
Fig 35. Growth of the rWSN-HA-HK viruses in mTECs. After infection of the mTECs the apical media was collected for titration. The experiment was done in duplicate and the values on the graph represent the average. Some mutants were significantly different than HK-wt, $p < 0.05$ (shown with asterisk).



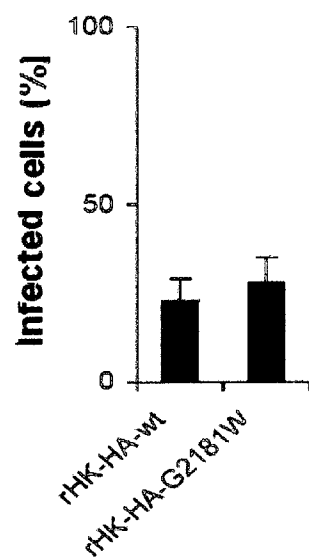
rWSN-HA-HK viruses

Fig 36. Expression and growth of the rHK-HA viruses in mTECs. Cells were grown and differentiated under air-liquid interface. The mTECs were then infected at MOI of 3, followed by a 20 hr incubation period. **(A)** The infected cells were stained using anti-HK antibody (red). The nuclei were also stained using 4',6-diamidino-2-phenylindole (DAPI) (blue). **(B)** Percentage of infected cells. **(C)** After infection of the mTECs the apical media was collected for titration. The experiment was done in duplicate and the values on the graph represent the average.

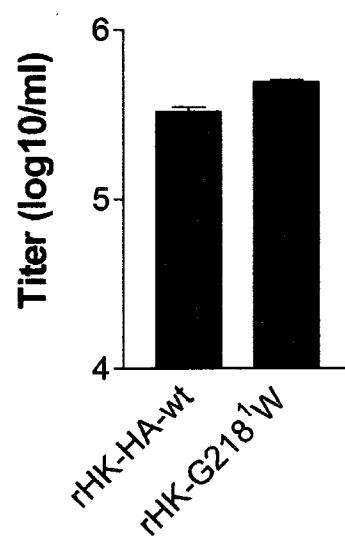
A



B



C

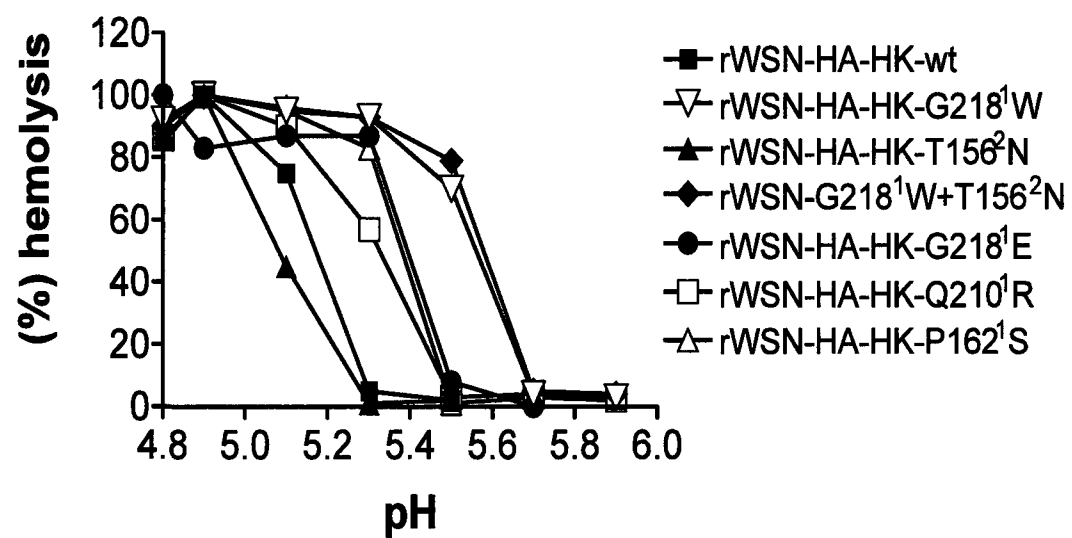


rWSN-HA-HK viruses were much more efficient at infecting these cells compared to rHK-HA viruses (Figs 34A and 36A). After infection with the rHK-HA viruses, the apical media was also quantified using a plaque assay. Based on the results, the G218¹W mutant replicated significantly better than the wt ($p < 0.05$) (Fig 36C).

3.18. The pH dependence of hemolysis by the recombinant viruses containing HA Mutations

Hemolysis assays were performed to determine the pH optimum of fusion as previously described (100). The results indicated that most of the mutants exhibited an increased pH of fusion compared to HK-wt, which fuses at pH 5.2 (Fig 37). Distinct groupings of pH optima for fusion were observed with respect to these mutants. First there was Q210¹R which fused at pH 5.35, 0.15 units more than HK-wt. The next group includes G218¹E and P162¹S which fuse at pH 5.4, 0.2 units more than HK-wt. The last group includes G218¹W and G218¹W+T156²N which fuse at pH 5.6, 0.4 units more. This was consistent with the pH of fusion that was observed for the mouse adapted variants containing G218¹W or G218¹W+T156²N mutations (Fig 1). This indicated that the increase in pH observed with the mouse adapted viruses was due to the HA mutations. The only exception was T156²N which fuses at pH 5.1 (0.1 units less) despite being more virulent (Figs 25 and 37). These results indicate that elevated pH might play some role in enhancing

Fig 37. The pH dependence of hemolysis by the rWSN-HA-HK viruses. Hemolysis was assessed at 0.2 pH increments and 37°C. The assay was done in duplicate and the values were averaged. Most of the mutants, with the exception of T156²N, exhibited an elevated pH.



virulence but it does not appear to be a prerequisite for increased virulence.

3.19. Location of mutations within the HA molecule following passages 12, 20 and 21

The mutations acquired after passages 12, 20 and 21 are spread out within the primary structure. However, these mutations are localized in two distinct domains within the monomer (Fig 38 A-C). One adaptive region resides at the top of the molecule where mutations are found behind the RBS or within subunit interfaces. The second adaptive region identified through mouse adaptation was located at the membrane proximal end of the structure. Convergent mutations (162¹, 210¹, 218¹ and 154²) acquired from the two independent experiments are indicated in Fig 38D. These represent the most common changes that were elucidated from our studies.

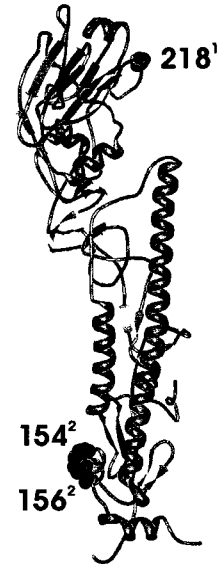
Some of the mutations identified after passage 21 are in close proximity to the secondary binding site mentioned above. These include residues 238¹, 262¹ and 268¹ shown in Fig 39. Additional studies are required to determine the possible effect of these mutations in receptor binding specificity. Furthermore, the location of the HA2 mutations within the low pH conformation was investigated. Most of the mutations (154, 156, 158 and 160) acquired after mouse adaptation appear to

Fig 38. Location of the passage 12, 20 and 21 mutations within the HA trimer. The figures were created with PyMOL software using a monomer of the 1HGD H3 hemagglutinin structure. Individual mutations are represented by a different colored sphere. **(A)** Passage 12 mutations, **(B)** Passage 20 mutations, **(C)** Passage 21 mutations and **(D)** Convergent mutations identified after the independent experiments.

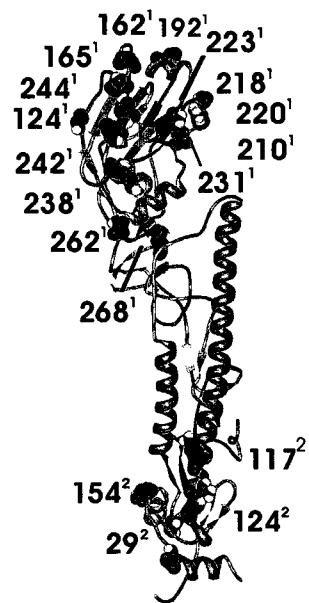
A



B



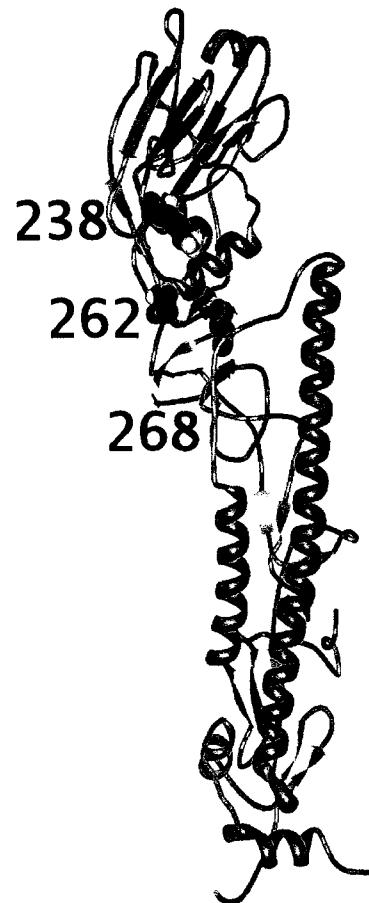
C



D

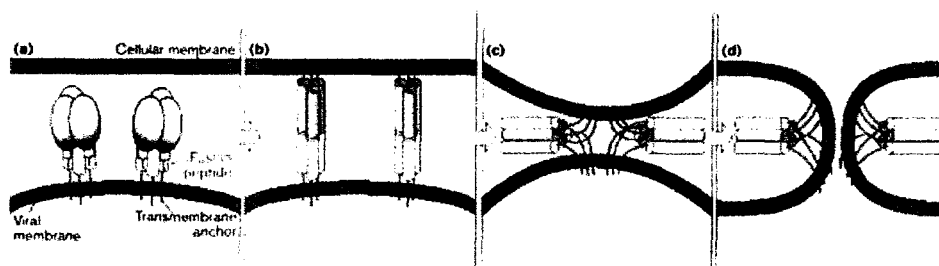
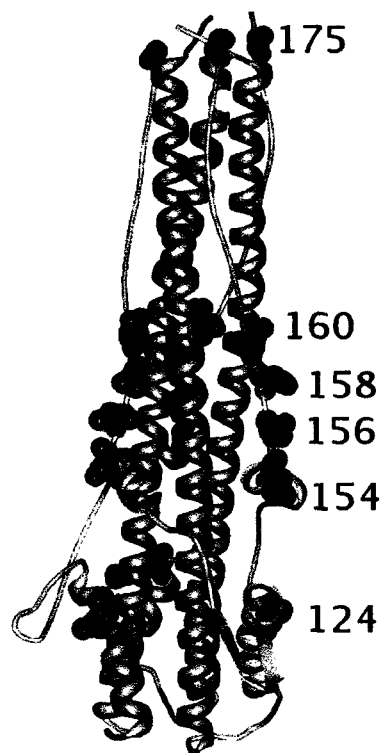


Fig 39. Mutations located within the Secondary binding site of the HA trimer. The figures were created with PyMOL software using a monomer of the 1HGD H3 hemagglutinin structure.



cluster in the middle of the structure (Fig 40). These changes might have an effect on the stability of the structure or transition to this form.

Fig 40. Location of mutations within the low pH conformation of HA2. The image was created with PyMol software using 1 QU1. Most of the mutations appear to be located in the middle of this conformation.



4. Discussion

4.1. Mouse adaptation

Molecular evolution of influenza A is not fully understood (49). However, our mouse adaptation studies offer a valuable theoretical and practical model for evolution. The approach involves serial passaging large population sizes. As a result, we have variants that possess all feasible single nucleotide changes. The mutation rate for Influenza A is about 1 substitution for each genome. For instance, A/PR/8/34 (H1N1) possesses 13,588 nucleotides. This translates to 40,764 ($3 \times 13,588$) single nucleotide variants. Because of the mutation rate, populations that possess all of these mutations ie population of $> 40,764$ infectious particles will possess all single nucleotide polymorphism (14). This allows competition among all available variants and selects the most adaptive mutants which represent the fittest variants. On the other hand, passaging small population sizes would have generated deleterious mutations because of the random amplification of a subset of variants (13). Using the above approach (ie strong selection with large population sizes), we have identified adaptive mutations (Tables 1 and 3). After the first mouse adaptation study, G218¹W was the most adapted change identified (Table 1). Following an independent experiment (10 mouse adapted populations), Q210¹R and G218¹E appear to be the most adapted (Table 3). If we compare the two studies (11 populations), convergent

mutations are detected in eight populations. These mutations are located at positions 210¹, 218¹, 162¹ and 154².

The G218¹E mutation has been identified by Narasaraju et al following mouse adaptation of A/Aichi/2/68 (H3N2). These mouse adapted variants were highly pathogenic and caused systemic infection (78). The G218¹E mutation was also detected in cell adapted X-31 (H3N2) viruses. These mutants appear to fuse at an elevated pH when compared to the wild type (66). Based on these observations, G218¹E appears to be a common substitution upon adaptation of influenza viruses to different hosts such as mouse and MDCK cells. In addition, G218¹E/R was also detected in antibody escape mutants. These variants displayed a shift in receptor specificity (from α 2,6 to α 2,3 receptors) and fused at an elevated pH. Hence, certain substitutions appear to control antigenicity, receptor binding and membrane fusion (119). Interestingly, many mutations affect receptor specificity or fusion but only a subset do both.

4.2. Replication and infection abilities of A/HK/1/68 and the mouse adapted variants

High virulence associated with the mouse adapted variants depends on significant viral growth as well as enhanced tropism. The replicative potential of these viruses was investigated in CD-1 and Balb/c mice. The two strains were compared to determine host specific

effects. The growth studies indicated significant mouse strain specific differences. All the mouse adapted variants replicated better than the parent in CD-1 mice (Fig 17). This is consistent with the fact that these viruses were generated in the CD-1 strain. In contrast, most of the variants (except MA-511) replicated better than the parent within Balb/c mice (Fig 18). The restricted growth observed with MA-511 may be attributable to the antiviral effects of interferon or a difference in host resistance (73).

The infection pattern was also investigated by immunochemical staining in CD-1 and Balb/c mice inoculated with the prototype and mouse adapted variants. A similar staining pattern was generally observed in both strains of mice (Figs 19 and 20). However, the staining was more extensive in CD-1 as compared to Balb/c mice. The prototype infects the bronchi and some alveolar cells. In comparison, mouse adapted variants cause much broader and extensive infection of the bronchioles and alveoli. These variants appear to exhibit greater extent of infection as well as high viral yield per cell. In addition, the results also indicated that alveolar infection is more severe than bronchioles with respect to these mutants. In general, our adapted isolates have acquired mutations that allow efficient replication within the bronchioles and alveoli. Each of these mutations enhanced infection in one or more cell type. Hence, these can be categorized as being specialists or

generalists. In conclusion, the mouse adapted viruses can better exploit the lung thereby causing pneumonia (13).

4.3. Generation of recombinant viruses containing convergent mutations

In general, the mouse adapted isolates exhibited enhanced replication and caused extensive infection within the lung. However, these viruses have mutations in most of the gene segments, hence it was hard to elucidate the effect of the HA mutations on different backgrounds. As a result, we generated synthetic viruses to determine the role of these mutations on a constant background. Two different backbones were used to rescue these viruses, the mouse adapted A/WSN/33 (H1N1) and the human prototype A/HK/1/68 (H3N2). All our convergent mutations were successfully rescued on the WSN backbone, however only wt HA and G218¹W were rescuable on the HK backbone (Table 4). The rescue system used at that time was not optimal. Recent studies in our laboratory conducted using a more optimal system indicated that these mutations can indeed be rescued on this backbone. The new system uses eight (one for each gene) bidirectional plasmids and involves longer transfection incubation times (changed from 5 hrs to 16 hrs) before replacing the DNA-transfection reagent complex with fresh media containing Trypsin.

rWSN-HA-HK viruses generated for our experiments possess HA/NA from A/HK/1/68 and the remaining segments from A/WSN/33. These gene combinations were chosen since HA and NA have to be compatible. A successful infection depends on a balance between the activities of the two glycoproteins (5, 114). Results obtained by Wagner et al indicated that synthetic WSN viruses containing mismatched HA and NA were attenuated (114).

4.4. Changes in receptor binding specificity

The HA mutations acquired through adaptation affected receptor binding specificity. The receptor binding assay was initially performed using resialylated chicken erythrocytes. The mouse adapted isolates containing P162¹L+D158²N (HKMA-12B) or G218¹W (HKMA-12E and -20C) exhibited a shift in receptor specificity. In contrast to the prototype, these mutants preferentially recognized SA α 2,3Gal resialylated erythrocytes (Table 2). In some instances (HKMA-12A and HKMA-12E), higher HA titre was obtained when using native blood instead of the modified cells. This indicates that sialic acid moieties are not completely restored on the surface of the neuraminidase treated cells (35).

In addition, receptor binding was also examined by utilizing sialylglycopolymers as previously described (69). Interestingly, the prototype as well as the mouse adapted isolates preferred 6'SLN over

3'SLN. However, viruses possessing G218¹W (HKMA-12E and -20C) or P162¹L+D158²N (HKMA-12B) showed an enhanced relative ability to bind 3'SLN (Fig 23). Even though a shift in binding was not observed using this assay, these mutations still extended the binding ability of the viruses for different linkages. Since these isolates contain background mutations in other segments including NA, the binding assay was repeated using recombinant viruses that differ solely on their HA mutations. In the above binding studies, the mutants containing G218¹W exhibited an increased effect. As a result, this mutation along with the corresponding T156²N mutation was used for further studies.

The rWSN-HA-HK viruses possessing G218¹W exhibited altered binding. In contrast to HK-wt, these mutants preferentially recognized 3'SLN instead of 6'SLN. However, this shift did not occur when the assay was performed using SL ligands (Fig 24). Based on these results, G218¹W increases binding to α 2,3 linked glycans through its interaction with the inner saccharide. These ligands differ due to an N-acetyl modification within the inner sugar (glucose) of SLN. The inner saccharide has previously been shown to play an essential role in receptor binding specificity (32).

The difference in binding observed between the mouse adapted and rWSN-HA-HK viruses possessing the same HA mutation was very surprising (Figs 16 and 24). One difference between these viruses relates to their NA genes. The mouse adapted viruses possess mutant NA genes

while the recombinant viruses contain wild type NA. This protein cleaves terminal sialic acids (SA) found on the surface of cells and viral glycoproteins. Mutations within the NA gene may alter its substrate specificity (59). Hence, the NA genes of the mouse adapted and recombinant viruses may prefer different linkages. In addition, the NA genes of the mouse adapted viruses may have cleaved off the bound sialic acid during the binding assay. However, this shouldn't have happened since Zanamivir (NA inhibitor) was used in the assay to inhibit NA activity.

4.5. Membrane pH of fusion

The second property affected by the HA mutations was pH of fusion. Hemolysis assay was conducted on the passage 12 and 20 isolates as described by Smeenk et al. Almost all the mouse adapted variants (except HKMA-12A) exhibited elevated pH of fusion (Fig 1). This indicated that variants which fuse at a higher pH are generally selected upon adaptation but not always. Other mouse adapted viruses such as A/Philippines/82/BS/ML10 (H3N2) have also been shown to fuse at an elevated pH (46). In addition, MDCK-adapted variants including X-31 (H3N2) and A/Japan/305/57 (H2N2) exhibited an identical pattern. This effect was independent of cell type since Vero-adapted X-31 exhibited an elevated pH (66). In general, adaptation to different hosts (mouse versus cell monolayer) appears to select variants

that fuse at a higher pH. These variants might have a replicative advantage since uncoating could happen at lesser levels of acidification in endosomes and thus could occur at a faster rate (46).

4.6. Loss of carbohydrate within the globular head domain

Two of our mouse adapted isolates (HK11MA21-1 and HK11MA21-2) possess the N165¹D mutation which removes a conserved glycosylation site (Table 3). This residue is located within the globular head domain. Other mouse adapted variants including A/NWS/33 (H1N1) have also exhibited loss of carbohydrate side chains. This indicates that it could be a common feature of mouse adaptation. Removal of carbohydrate near the RBS is associated with resistance to β inhibitors (116). Beta inhibitors are lectins that bind oligosaccharides on the globular head domain. As a result, these lectins shield and thus inhibit access to the RBS. Since these inhibitors appear to be an essential part of the host defence system, loss of carbohydrate is one way these viruses can bypass it (3, 46). Previous studies in our laboratory have also indicated that mouse adapted isolates of A/HK/1/68 (H3N2) and A/FM/1/47 (H1N1) are resistant to β inhibitors (13, 100). Hence, β inhibitor resistant variants appear to be selected upon adaptation (3, 13).

A Serum resistant variant of Mem71_H-Bel_N (H3N1) had acquired the T167¹N mutation that removes the same glycosylation site (N₁₆₅-V₁₆₆-T₁₆₇). Studies indicated that these mutants exhibited altered receptor

binding specificity. It appears that removal of the oligosaccharide would allow better contact with the RBS (3). In our case, further studies will have to be performed to corroborate these observations. Interestingly, both isolates possessed an additional mutation, G124¹D (Table 3). This indicates that the mutations might be functionally dependent on one another.

4.7. Ability of the rWSN-HA-HK viruses to infect Mouse lung (Balb/c) and Mouse tracheal epithelial cells (mTECs)

The mouse adapted mutations increased the ability of the virus to infect the various cell types within the lung and/or mTECs cultured in vitro. However, the extent of infection varied depending on the cell type (Table 6). Most of the mutants were more efficient at infecting the bronchi than HK-wt. The only exception was Q210¹R which was comparable to HK-wt (Fig 31a). In contrast, all the mutants spread more effectively within the alveoli (Fig 31b). The virulent mutants (T156²N, G218¹W+T156²N and P162¹S) caused the strongest alveolar staining (Figs 25 and 31b). Hence, virulence seems to correlate more with how well these mutants can infect the alveoli versus tracheal cells (mTECs). The mouse adapted mutations that increased virulence resided within HA1 and HA2 domains. This suggests virulence could be affected in a receptor dependent and independent fashion. Unfortunately, the effect of G218¹W, G218¹E and Q210¹R on virulence could not be elucidated

Table 6. Infection and growth patterns observed in the mouse lung and Mouse tracheal epithelial cells (mTECs) using rWSN-HA-HK viruses

With respect to wild type

	Bronchiolar staining	Alveoli staining	Yield in Lung	mTEC staining	mTEC apical titer	Virulence
G218 ¹ W	↑↑	↑	↑↑	↑	=	
T156 ² N	↑↑	↑↑	↑	↑	↓	↑
G218 ¹ W +T156 ² N	↑↑	↑↑	↑	↑	=	↑
G218 ¹ E	↑↑	↑	↑	↓	↑	
Q210 ¹ R	=	↑	↑	↑	↑	
P162 ¹ S	↑	↑↑	↑↑	↑	↑	↑

using the WSN background, where even the rWSN-HA-HK-wt was avirulent at dosage of 10^6 pfu. However, we did see increased growth in viruses containing the G218¹W, G218¹E and Q210¹R mutations indicating an increased ability to spread throughout the lung. In the future this issue can be addressed using a stronger backbone such as A/PR/8/34.

In contrast to HK-wt, most mutants (except G218¹E) infected a higher proportion of mTECs (Fig 34). Of these mutants, Q210¹R and P162¹S were associated with the most extensive staining. Consistent with the staining, these two mutants replicated significantly better than HK-wt (Fig 35). Interestingly, infection with the G218¹E mutant was also associated with high viral yield even though the staining was very weak relative to HK-wt (Figs 34 and 35). This suggests that the virus might have problems at the attachment stage but replicates efficiently once it enters the cell. It also suggests a more efficient usage of proteins since more virus resulted from G218¹E infected cells. Previous studies indicate that α 2,3 receptors are abundant in mice (52). However, our data show discrete staining within the various cell types (Table 6). Hence, different α 2,3 linked sialic acids appear to be present in the trachea, bronchial as well as alveoli regions with different relative binding among these cells by the individual mutants. This was also corroborated using lungs infected with A/Turkey/Wisconsin/68 (H5N9) (Fig 22d). As an avian virus A/Turkey/Wisconsin/68 favors α 2,3 linked receptors (34)

however it replicated better within the alveoli when compared to the bronchial epithelium.

4.8. HA1 domain

Sequence analysis indicated that a total of 24 sites were affected within the HA gene upon mouse adaptation of A/HK/1/68. This was elucidated from a total of 11 independently adapted populations (Tables 1 and 3). Of these sites, 16 were located within HA1 while 8 resided in the HA2 domain. The mutations were clustered in two distinct areas within the trimer. One of the adaptive regions resides within the globular head domain (Figs 38 and 41). In this domain, several mutations including Q210¹R are located within the eight-stranded beta sheet. This is the most prominent central feature within the globular head and supports the RBS (119). On the other hand, most of the other mutations such as G218¹W/E reside at trimer interfaces. Based on previous studies, mutations that increase pH of fusion reside within subunit interfaces. These changes weaken subunit interactions involved in activating the exposure of its N-terminal fusion peptide (24, 46, 119). In addition, mutations which elevate the pH of fusion also reside within or surround the hydrophobic fusion peptide (66).

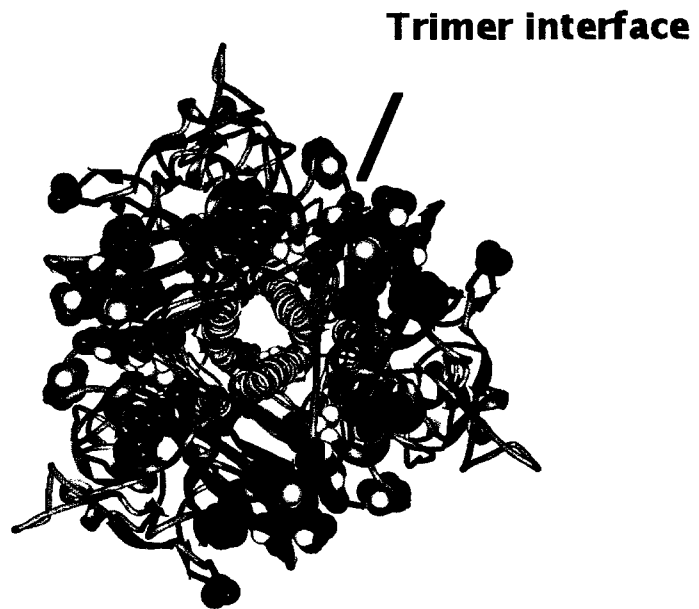
None of our mutations involve residues that directly contact the receptor. However, these substitutions could cause rearrangements within the RBS thereby altering binding specificity. Studies conducted

Fig 41. Location of the mutations acquired after the longitudinal and parallel adaptation experiments. (A) Side view. (B) Top view

A



B



with the 1918 (H1N1) HA indicate that mutations which allowed enhanced binding of the virus to SA α 2,6Gal linkages did not involve residues that interact with the receptor. In this case, the mutations caused rearrangements which allowed binding to bulky SA α 2,6Gal moieties (34). As stated above, amino acid 218 is located within the HA1-HA1 interface near the RBS (24, 98, 119). Hence, substitutions at this site would disrupt monomer interactions. The change from Glycine (smallest) to Tryptophan or Glutamic acid which are larger could push the HA1 subunit outwards. In turn these changes can potentially alter the RBS, more specifically the 220 loop that contacts SA at positions 225 and 226 (24, 34). Two other mutations, V223¹I found within this loop as well as R220¹S might also alter its structure which in turn can affect receptor binding to this loop. The T192¹A mutation resides within the 190 helix which also forms part of the RBS (119). This substitution can potentially affect the conformation of the helix and change receptor specificity. Most of the amino acid changes observed within the turns of the beta sheets involved acquisition of charged residues. These included N165¹D, Q210¹R, V242¹E, V244¹E and N246¹D. The only exception was S231¹N present in two isolates of HK9MA21. As stated above, the N165¹D mutation removes carbohydrate within the globular domain which could alter binding specificity (3). Mutations at residues 231 and 244 have been identified in A/PR/8/34 mutants selected using anti-HA monoclonal antibody cocktails (119). Viruses containing either of these

mutations were associated with increased receptor affinity. A substitution at amino acid 231 would modify binding due to its proximity to the RBS. Amino acid 244 is located within the HA1-HA1 interface, hence changes at this position could disrupt subunit interactions (119). Amino acid 246 which is also part of the interface was mutated in mouse adapted bovine serum (BS) resistant A/Philippines/82/BS/ML10 (H3N2). These mutants exhibited elevated pH as well as altered receptor specificity (46). Further studies need to be conducted in order to verify the role of each mutation in receptor binding. However, the binding studies we have already conducted indicate that the G218¹W mutation causes changes that may narrow the RBS. The consequence of this was enhanced binding to α 2,3 linked receptors. Studies indicate that the H3 avian RBS, which recognizes SA α 2,3Gal moieties, appears to be narrow. In contrast, human RBS is wider to accommodate the SA α 2,6Gal moiety (42).

Some HA1 mutations resided near the secondary binding pocket. These included K238¹N, T262¹N and M268¹V (Fig 39). Two of these mutations (K238¹N and M268¹V) occurred in combination with the G218¹E mutation (Table 3). This suggests a possible collaborative effort between these mutations. On the other hand, the T262¹N mutation is accompanied by V223¹I or S231¹N. However, isolate HK9MA21-2 possesses just the T262¹N mutation which indicates that it was selected

first. In the future, recombinant viruses containing these mutations could be generated in order to elucidate the role of this site.

4.9. HA2 domain

The second adaptive region is located in the membrane proximal end of the structure (Fig 38). Most HA2 mutations detected following mouse adaptation had a corresponding HA1 mutation suggesting structural linkage between these domains. Some of the mutations (S29²Y, K117²R, R124²K, G175²D) surround the five stranded beta sheet located at the centre of the base of HA. Of these, residue 117 was also mutated in cell adapted transfectant viruses (66). These variants exhibited an increase in the pH of fusion. Residue 117 resides close to N-termini (fusion peptide) therefore the mutation could destabilize this region (66, 119). Most of the beta sheet is comprised of the carboxy terminus of the HA2 subunit. However, its middle strand consists of the N-terminal domain of HA1 (119). The D2¹Y mutation is located at the tip of this middle strand. The remaining mutations (N154²S/K, T156²N, D158²N, D160²N) are in close proximity to one another. These mutations reside within the helix-loop-helix region found near the membrane. As stated above, N154²S/K as well as T156²N eliminate the same glycosylation site N₁₅₄-G₁₅₅-T₁₅₆. These residues are also located in close proximity to the cleavage loop. Removing carbohydrate near this site has been shown to enhance cleavage as well as virulence. However,

this was not observed in our mouse adapted isolates since cleavage was not detectable without trypsin (13). The location of these substitutions within the low pH conformation was also examined. N154²S/K, T156²N, D158²N and D160²N appear to cluster in the middle of this structure (Fig 40). Further studies have to be performed in order to elucidate the effect of these mutations on the low pH structure. These substitutions might play a role in the stability of this conformation. The mutations could also affect intermediate conformations that occur during transition to the stable low pH fusion structure.

4.10. Independent selection of identical mutations in other highly virulent viruses

Mutations selected through our mouse adaptation studies parallel several of those observed in the recently adapted canine viruses (H3N8) (83) as well as the highly pathogenic avian H5N1 viruses (51). Canine influenza has been isolated since 2004 in USA. This virus has high sequence similarity with circulating equine viruses (H3N8). It was transmitted from horses to dogs followed by adaptation in the new host. Sequence analysis of the HA genes identified several canine adapted mutations. Of these mutations, five (N54¹K, N83¹S, W222¹L, I328¹T, N154²T) were present in all the dog isolates tested (83). In addition, one canine virus also contained G218¹E, the same change detected within four of our mouse adapted populations (Table 3 and 7) (83). Hence,

Table 7. Sequence alignment of the HA gene of the Canine isolates
(Payungporn et al 2008)

Virus	Amino acid positions in mature HA																		
	7†	29	54	78	79	83	92	107	118	159	218	222	261	328	479	483	492	541	
A/equine/Kentucky/5/2002	G	I	N	V	F	N	S	S	L	N	G	W	K	I	G	N	R	K	
A/equine/Massachusetts/213/2003	.	.	.	A	.	.	.	.	.	S	.	.	.	.	.	.	.	.	
A/equine/Ohio/1/2003	.	.	.	A	.	.	.	.	.	S	.	.	.	.	.	.	.	.	
A/equine/Bari/2005	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A/canine/Florida/242/2003	D	.	K	A	.	S	.	.	.	S	.	L	.	T	.	T	.	.	
A/canine/Florida/43/2004	.	M	K	A	.	S	N	.	.	S	.	L	.	T	.	T	.	R	
A/canine/Texas/1/2004	.	M	K	A	.	S	.	.	V	S	.	L	N	T	E	T	.	.	
A/canine/Iowa/2005	.	M	K	A	L	S	.	.	V	S	E	L	N	T	E	T	.	.	
A/canine/Miami/2005	.	M	K	A	.	S	.	.	V	S	.	L	N	T	E	T	K	.	
A/canine/Jacksonville/2005	.	M	K	A	.	S	.	P	V	S	.	L	N	T	E	T	K	.	

identical mutations and sites (G218¹E, W222¹L and N154²T) appear to be affected upon adaptation of influenza to these animals (Fig 42).

Certain highly pathogenic avian H5N1 isolates differ in pathogenicity. Studies conducted using recombinant viruses indicated that several substitutions within the HA gene controlled increased virulence in chickens. Two of the substitutions, E216¹K and P221¹S (H3 numbering) reside in close proximity to our mouse adapted mutations (Fig 43) (51). In addition, E216¹D was detected in mouse adapted A/black duck/New Jersey/1580/78 (H2N3) (39). Thus, similar areas appear to be affected upon mouse adaptation regardless of the HA subtype. Taken together, the above observations indicate that the mouse model is a useful tool in identifying adaptive mutations that are host specific and non host specific. Hence, these mutations are general determinants of virulence (13).

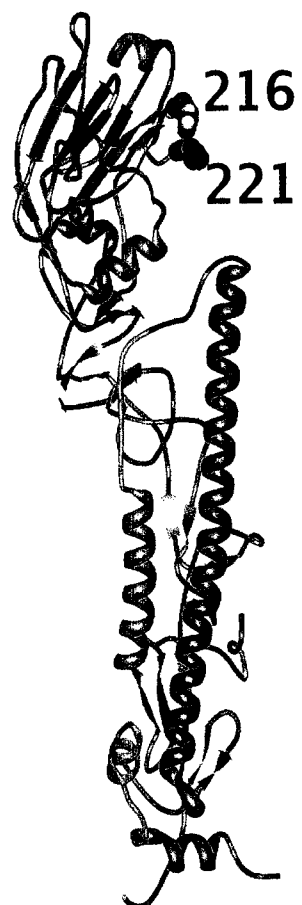
4.11. Conclusion

In general we found that mouse adaptation generates virulent variants that are more efficient at exploiting the new host (13). Upon sequence analysis of 11 independent populations, 24 HA mutations were identified. The HA mutations clustered within two areas of the trimer, globular head domain or membrane proximal end. Four of these

Fig 42. Location of mutations detected within the Canine adapted virus



Fig 43. Location of the mutations detected in the Highly pathogenic Avian H5N1 influenza virus



mutations (210¹, 218¹, 162¹ and 154²) showed evidence of convergent evolution.

The most adaptive HA mutations increased infection and replication in several cell types and locations within the lung. However, virulence correlated with the enhanced ability to infect alveolar cells. The data indicates that HA mutations which increase tropism for alveolar cells appear to be selected upon adaptation. In addition, different staining patterns were observed in the mouse lung and mTECs. This indicated that distinct 2,3 linked receptors might be present in the upper and lower respiratory tracts of mice.

Some of the mouse adapted mutations have been identified in other virulent viruses. This identifies them as important determinants of virulence. Hence, these mutations can serve as predictors of virulence for newly emerging variants. Even though this study focuses on the HA gene, we have previously identified virulence determinants in all other genes including NS1 (13). These results further reiterate the fact that mouse adaptation is a very valuable tool for understanding the genetic basis of virulence. In particular our studies provide a practical and theoretical basis for performing studies of mouse (or any alternative host) adaptation using other influenza viruses from various hosts. In addition, mutations identified following mouse adaptation can also be useful for generating novel therapeutic drugs that can be directed against influenza A viruses (13).

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Appendix I

Identification of residues involved in the interaction between the Hemagglutinin (HA) and Matrix (M1) proteins

Introduction and Rationale

The M1 protein of Influenza A is an abundant and highly conserved viral protein. It is known to be essential in assembly and budding. This protein forms a shell beneath the host derived lipid envelope separating it from the core of the virus. As a result, it is involved in an interaction with ribonucleoproteins (RNPs) as well as glycoproteins (HA and NA) (1, 2). Studies conducted using Triton X-100 demonstrated the association between M1 and the glycoproteins. Both HA and NA are known to concentrate within lipid rafts. These microdomains are resistant to detergents such as Triton X-100. M1 does not associate with rafts. Hence, it will only become resistant to solubilization by interacting with a raft associated protein. In these studies, M1 expressed alone was sensitive to this detergent. On the other hand, M1 in addition to HA or NA exhibited resistance to solubilization and co-purified with rafts. The ability of these glycoproteins to make M1 detergent resistant provides proof of their interaction. In addition, M1 was shown to associate with the cytoplasmic domain of HA (1). However, residues involved in this interaction are currently unknown.

The goal of this project was to identify residues that are involved in the HA-M1 interaction. The approach involved the following steps:

- A) To introduce attenuating mutations within the cytoplasmic tail of HA
- B) To generate recombinant viruses containing these mutant HA genes
- C) To serial passage these viruses in MDCK cells in order to derive biological revertants
- D) Sequence analysis of the HA and M1 genes

This study relied on the fact that adaptation would result in competition and selection of viruses that have acquired compensating mutations. These mutations could be located within the HA and M1 genes thereby potentially identifying residues involved in this interaction. Three residues were chosen to be mutated within the cytoplasmic tail of HA. These mutations included G213²N, C217²S and C220²S. Amino acid 213 was chosen because it is located near the edge of the transmembrane domain. On the other hand, amino acids 217 and 220 are known to be conserved (Fig 44) (6). In addition, the mutagenic oligonucleotides (KC1 to KC3) used for site directed mutagenesis contained two nucleotide changes to introduce a single amino acid mutation (Fig 45). This was done to minimize the chance of reversion.

Figure 44. Primary structure of the Hemagglutinin showing the cytoplasmic domain
(Chen et al 2005)

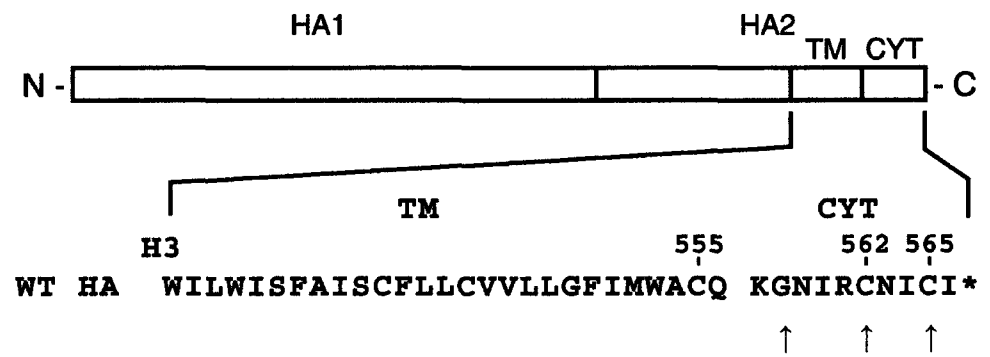


Figure 45: List of mutagenic oligonucleotides used for site directed mutagenesis

KC1:GGGCCTGCCAAAGAAACAACATTAGGTGCAAC
GGC

KC2:AGGCAACATTAGGTCAAACATTTGCATTTG
TGC

KC3:TAGGTGCAACATTTCAATTTGAGTGTATTAG
TGC

Methodology

Introduction of mutations in the cytoplasmic tail of HA

Mutations were introduced into the cytoplasmic tail of the HA gene by using the GeneEditor in vitro site directed mutagenesis system (Promega) in accordance with the manufacturer's instructions.

Virus rescue

Recombinant viruses containing the HA mutations were generated using the 12 plasmid system as previously described (8). These viruses contained HA and M1 genes of A/HK/1/68 (H3N2) on A/WSN/33 (H1N1) backbone. Prior to passaging the HA and M1 genes were sequenced. The HA genes had the expected mutations while the M1 genes for these viruses were clean.

Adaptation in MDCK cells

Three clones were isolated from mutant 1A (1A1 to 1A3) and mutant 2A (2A1 to 2A3) following plaque purification. Each clone was serially passaged 20 times in MDCK cells. The viral yield after each passage was quantified using a plaque assay. Different passages were also selected for sequence analysis of the HA and M1 genes.

Location of mutations in the HA trimer

The figures were created using PyMOL software and a monomer of the A/HK/1/68 H3 hemagglutinin structure (PDB ID: 1HGD).

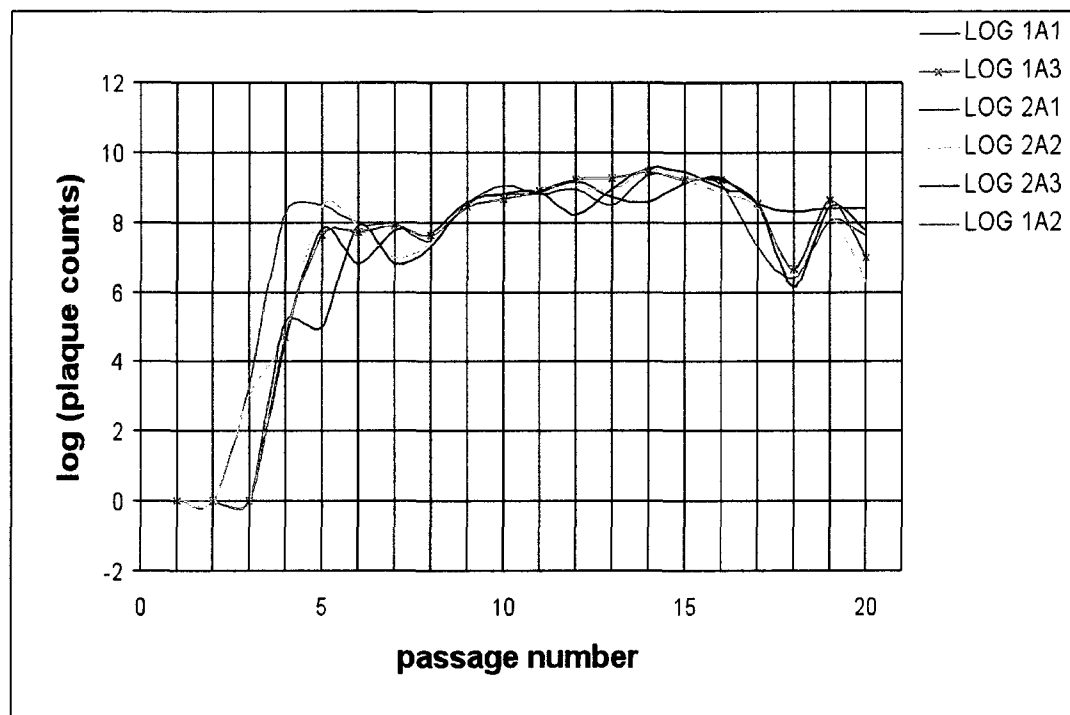
Results

Replication abilities of the cell adapted isolates

The recombinant viruses possessing the G213²N (mutant 1A) or C217²S (mutant 2A) mutations were successfully rescued on the A/WSN/33 background. However, the virus containing the C220²S mutation could not be rescued. Previous work indicates that viruses containing this exact mutation can be successfully rescued. These mutants contained X-31 HA (H3) with the remaining gene segments from A/WSN/33 (6). Therefore, this suggests that our rescue system might not have been optimal for rescuing this mutation. Three clones were first derived from mutant 1A (1A1-1A3) and mutant 2A (2A1-2A3). Each of these clones were then serially passaged 20 times in MDCK cells. The next step was to investigate the replicative ability of these viruses after each passage. Therefore, the isolates were titrated to quantify the yield.

The results indicated that the starting viruses were indeed attenuated (Fig 46). After passage two, none of the viruses had detectable virus. Two of the isolates (2A1 and 2A2) showed an increase in titre ($\sim 10^3$ PFU/ml) after passage three. The remaining mutants exhibited detectable virus ($\sim 10^5$ PFU/ml) after passage 4. Most of the isolates exhibited high titre ($\sim 10^8$ PFU/ml) after passage five, with the exception of isolate 2A3 which required an additional passage to reach this

Fig 46. Replication abilities of the cell adapted viruses



titre. Interestingly, isolate 2A1 attained this high titre ($\sim 10^8$ PFU/ml) earlier at passage 4. After passage five, all the isolates replicated at a somewhat constant rate until a dip in titre was observed at passage eighteen. This is probably due to the existence of defective viruses. In conclusion, the cell adapted isolates have acquired mutations which are advantageous for growth in the new environment.

Sequence analysis

In contrast to the starting viruses, the cell adapted variants exhibited a much more efficient replication. Hence, we were interested in identifying the changes acquired through adaptation. The passage three and five isolates were chosen for sequence analysis of the HA and M1 genes. Passage three was chosen since this was the first time differences were apparent with respect to the various isolates. On the other hand, passage five was the earliest passage to exhibit high titre for most of the isolates (except 2A3). Following passage three, the HA genes of five isolates (1A1, 1A2, 1A3, 2A1 and 2A2) were partially sequenced. With the exception of 1A3 and 2A2 the rest showed wild type sequence (Table 8). The 1A3 mutant possessed the Y178¹F, S219¹T and N248¹S mutations. In contrast, the 2A2 mutant contained just the Y178¹F and S219¹T mutations. In addition, Y178¹F and S219¹T show evidence of convergent evolution since these are independently selected within different adapted isolates (3).

Table 8. Mutations in the HA genes of the cell adapted viruses

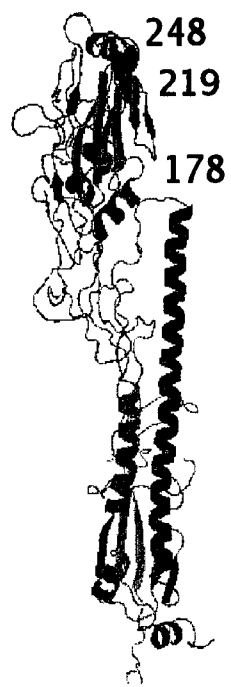
		HA1		
	178	218	219	248
WT	Y	G	S	N
1A1 Pass 3	-	-	-	-
1A2 Pass 3	-	-	-	-
1A3 Pass 3	F	-	T	S
2A1 Pass 3	-	-	-	-
2A2 Pass 3	F	-	T	-
1A1 Pass5	-	E	-	-
1A3 Pass 5	-	-	-	-
2A1 Pass5	-	W	-	-
2A3 Pass 5	-	R	-	-

After passage five, the HA genes of four isolates (1A1, 1A3, 2A1 and 2A3) were partially sequenced. Three of the four viruses (except 1A3) possessed mutant HA genes. All these isolates (1A1, 2A1 and 2A3) exhibited a mutation at residue 218 (G218¹E/W/R) indicating convergent evolution (Table 8). In addition, the M1 genes were fully sequenced for all the passage three and five isolates. However, no mutations were detected within the M1 genes.

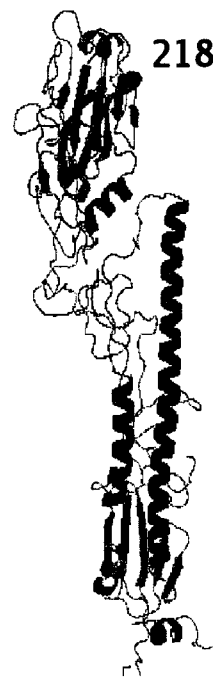
Location of the cell adapted mutations within the HA monomer

With respect to the HA monomer, the passage three and passage five mutations appear to reside within the globular head domain (Fig 47). Three mutations (Y178¹F, S219¹T and N248¹S) were identified following passage three (Fig 47A). Of these substitutions, Y178¹F is located within the eight-stranded anti-parallel beta sheet. The S219¹T mutation is proximal to both the G218¹W/E mutation identified following our mouse adaptation studies and the P221¹S substitution found in the highly pathogenic avian H5N1 viruses (12). Amino acid 219 resides close to the 220 loop (forms RBS) as a result this substitution could affect the conformation of the loop and indirectly alter binding. On the other hand, the N248¹S mutation is close to N246¹D which was detected in one of our mouse adapted isolates (HKMA-12A). As previously described, amino acid 246 is also mutated in mouse

Figure 47. Location of the cell adapted mutations within the HA monomer



A



B

adapted A/Phil82/BS/ML10 (H3N2). These mutants showed elevated pH of fusion as well as altered binding (5).

In contrast to the passage three isolates, only one residue was mutated (G218¹W/E/R) in all the passage five isolates tested (Fig 47B). This mutation has also been detected in our mouse adapted viruses (G218¹W/E), canine adapted viruses (G218¹E) (9), cell adapted X-31 viruses (G218¹E) (6) as well as antibody escape mutants (G218¹E/R) (11). As previously stated, amino acid 218 resides within the HA1-HA1 interface near the RBS. Therefore, substitutions at this site could disrupt monomer interactions (4, 10, 11). Based on our results, a mutation at this site appears to alter receptor binding and pH of fusion. This has also been observed by Wiley et al with respect to their antibody escape mutants (11).

Discussion

The HA genes of the passage three and five isolates were partially sequenced due to technical difficulties. As a result, it was impossible to see if the cytoplasmic tail mutations reverted back to wild type or not. Since the M1 genes of these viruses did not contain any mutations, the attenuation observed with the starting viruses appears to be compensated for by the HA mutations. Three sites (178¹, 219¹ and 248¹) were mutated following passage three. Two of these mutations (Y178¹F and S219¹T)

showed evidence of parallel evolution (Table 8). These mutations occur together in both isolates (1A3 and 2A2) which indicates that they might be functionally dependent on each other. Interestingly, both these mutations disappear by passage five, at least with respect to the 1A3 isolate (unable to sequence 2A2). These mutations undoubtedly play a role in adaptation but they do not appear to be the most fit. Amino acid 218 was mutated in three (1A1, 2A1 and 2A3) of the four passage five isolates sequenced. This mutation showed evidence of directional evolution. Based on several studies, this residue appears to be affected upon adaptation of influenza to mice (our mouse adaptations studies and Narasaraaju et al 2008), MDCK cells (this study and Lin et al 1997) or dogs (9). In general, similar regions are affected upon adaptation of influenza to different hosts such as mice, MDCK cells or dogs. These data indicate that the 218 site is a strongly adaptive site that can increase HA activity to overcome the species barrier or attenuating mutations.

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Appendix II

Solutions

Denaturing solution

4 M guanidinium thiocyanate
25 mM sodium citrate (pH 7)
0.5% sarcosyl (w/v)
0.1 M 2-mercaptoethanol

Luria-Bertani (LB) Media

Per litre
10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
Adjust pH to 7.5 with NaOH and autoclave

LB Agar plates

Prepare LB media as described, just before autoclaving add 15g agar/litre. Autoclave, allow to cool to 55°C add then add 500 µg/µl ampicillin. Pour plates and allow to harden

SOC Medium

Per litre
20 g Bacto-tryptone
5 g Yeast extract
0.5 g NaCl
Adjust pH to 7.5 with potassium hydroxide and autoclave. Just before use add 20 ml of sterile 1M glucose. Sterilize by filtration through 0.22 µm filter

TE Buffer

10 mM Tris-HCl pH 7.4
1 mM EDTA pH 8
Autoclave

STE

100 mM Tris-HCl (pH 8)
1 M NaCl
10 mM EDTA (pH 8)
Autoclave

Lysozyme Buffer

50 mM glucose

25 mM Tris (pH 8)

10 mM EDTA

Autoclave and add lysozyme (4 mg/ml) before use

1.54 M NaCl

Add 90 g to 1 litre distilled water

0.5 M Dibasic sodium phosphate (500 ml stock)

Add 35.49 g to 500 ml distilled water

0.5 M monobasic sodium phosphate (500 ml stock)

Add 35 g to 500 ml distilled water

200 mM phosphate buffer

Add approximately 50 ml monobasic sodium phosphate to 150 ml dibasic sodium phosphate, until pH equals 7.2. monitor pH while adding monobasic sodium phosphate, if less than 50 ml of monobasic has been used adjust volume to 200 ml by adding distilled water. If more than 50 ml of monobasic was used discard excess mono/dibasic solution. Bring volume to 500 ml by adding distilled water

10 mM Phosphate Buffered Saline (PBS)

Add 50 ml 200 mM phosphate buffer to 100 ml NaCl (1.54 M) bring to 1 litre with distilled water

Antibody buffer

Add 3g bovine serum albumin to 100 ml of 10 mM PBS while stirring.

Add 0.3 ml 100% Triton-X with stirring. Bring pH to 7.2 with NaOH.

Appendix III

Primers

Universal for segments 1,2, 3 and 6

10232 CCGCTAGCGAAAGCAGG

RTG GGGGAGCGAAAGCAGG

Universal for segments 4,5,7 and 8

10231 CCGCTAGCAAAAGCAGG

RTA GGGGAGCAAAAGCAGG

Segment 4 specific primers

10238 (-) CCGCAGTAGAAACAAGGGTG

KC 7 (-) GGTTATTAGTAGAAACAAGGGTGTTTTTAATTAC

KC 15 (+) GGGGAGCAAAAGCAGGGGATAATTC

Appendix IV

Curriculum Vitae

Degree

B.Sc., Honors in Biochemistry, University of Ottawa, 2001

Scholarship

Dulketvich Memorial Trust, 2007

Oral Presentations

American Society of Virology, McGill University, Montreal, 2004

RNA Club, University of Ottawa, 2006

RNA Club, University of Ottawa, 2008

American Society of Virology, University of British Columbia, Vancouver, 2009

Poster Presentation

Ottawa Centre for Research and Innovation (OCRI) held at the University of Ottawa, 2007

Publications

Keleta L, Ibricevic A, Bovin NV, Brody SL, Brown EG. Experimental evolution of human influenza virus H3 hemagglutinin in the mouse lung identifies adaptive regions in HA1 and HA2. *J Virol.* 2009; 83(7): 3417.

Wang S, Liu Q, Pu J, Li Y, Keleta L, Hu YW, Liu J, Brown EG. Simplified recombinational approach for influenza A virus reverse genetics. *J Virol Methods.* 2008;151(1):74-8

Nielsen K, Yu WL, Lin M, Davis SA, Elmgren C, Mackenzie R, Tanha J, Li S, Dubuc G, Brown EG, Keleta L, Pasick J. Prototype Single Step Lateral Flow Technology for Detection of Avian Influenza Virus and Chicken

Antibody to Avian Influenza Virus. Journal of Immunoassay and Immunochemistry. 2007;28(4):307-18

Volunteer Work

Tutoring, Bell High School (1996-1997)

Organizing radiology forms and serving at the cafeteria and gift shop, Queensway Carleton Hospital (1997-1999)

Participated in a fundraiser for children living with AIDS, Ethiopian Community (2002-2003)

Registration Assistant, Northern Lights, Infection and Immunity Meeting of the Canadian Federation of Biological societies (CFBS) (2006)