
Receptor binding properties, cell tropism and transmission
of influenza A virus

Submitted for the degree of
Doctor of Philosophy

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To Ben, Paul and Wiety

I confirm that the work presented within this thesis is my own.

All other data are appropriately referenced.

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Abstract

The first influenza pandemic of the 21st century was caused by the influenza A (H1N1) 2009 virus (A(H1N1)pdm09) that emerged from a swine-origin source. Although human infections with swine-origin influenza have been reported intermittently in the past decade, none went on to cause a pandemic or sustained human-to-human transmission. In previous pandemics, specific residues in the receptor binding site of the haemagglutinin (HA) protein of influenza have been associated with the ability of the virus to transmit between humans. In this thesis the effect of mutations at residue 227 in HA on cell tropism and transmission of A(H1N1)pdm09 is described. In A(H1N1)pdm09 and previous seasonal H1N1 viruses this residue is glutamic acid (E), whereas in swine influenza it is alanine (A). Using human airway epithelium, a differential cell tropism of A(H1N1)pdm09 compared to A(H1N1)pdm09 E227A and swine influenza was shown suggesting this residue may alter the sialic acid conformer binding preference of the HA. Furthermore, multi-cycle viral growth of both A(H1N1)pdm09 E227A and swine influenza was found to be attenuated in comparison to A(H1N1)pdm09 in human airway epithelium. However this altered tropism and viral growth in human airway epithelium did not abrogate respiratory droplet transmission of A(H1N1)pdm09 E227A in ferrets. This suggests that acquisition of 227E was not solely responsible for the ability of A(H1N1)pdm09 to transmit between humans. Because the work with the E227A mutant showed that small differences in cell tropism that may affect influenza virus transmissibility could be detected in human airway cells, a receptor binding

assay was developed for laboratory surveillance using commercial human airway epithelium cultures, to screen for zoonotic influenza strains of particular concern for human health.

To further investigate adaptations by influenza virus for infection of the human host, the cell tropism determined by the HA protein of an avian H7N7 and human H7N3 isolate was compared. Binding to non-ciliated human airway cells was increased for the human isolate. This human case of H7N3 infection yielded two isolates from different sites (eye and throat) from the same patient on the same day. A whole genome sequencing assay was designed for H7 isolates and both eye and throat isolate were fully sequenced. One synonymous nucleotide change was found in the NS gene segment and one synonymous and two non-synonymous nucleotide changes were found in the PB2 gene segment. Comparison of the non-synonymous changes in the protein sequence of PB2 to available avian and human virus PB2 sequences revealed that the substitutions in the eye isolate were comparatively uncommon. Interestingly, these changes resulted in an increased viral growth in human airway epithelial cells at 32°C when compared to the throat isolate, a phenomenon which was not observed at 37°C.

Finally, the use of a lung model maintained by the ex-vivo lung perfusion (EVLP) technique for study of virus infection was tested. This technique allows the use of both human and porcine lungs up to 24 hours after abstraction and is a potential model for respiratory pathogens and novel treatments. Porcine lungs were infected with A(H1N1)pdm09. Physiological and virological parameters were measured in two separate experiments and infection was demonstrated by increased viral loads in samples obtained at late time points after infection.

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Abbreviations

ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BSA	Bovine Serum Albumin
CDC	Centre for Disease Control and Prevention
CO ₂	Carbon Dioxide
CPE	Cytopathic Effect
cRNA	Complementary Ribonucleic acid
DMEM	Dulbecco's Modified Eagles Media
DNA	Deoxyribonucleic Acid
<i>E. coli</i>	Escherichia coli
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked Immunosorbance Assay
ESPRIT	Expression of Soluble Proteins by Random Incremental Truncation
EU	European Union
EVLP	<i>Ex Vivo</i> Lung Perfusion
EVPLTS	<i>Ex Vivo</i> Porcine Lung Tissue Sections
FAO	Food and Agriculture Organization

FBS	Foetal Calf Serum
GFP	Green Fluorescent Protein
GISRS	Global Influenza Surveillance and Response System
h.p.i.	Hours Post Infection
HA	Haemagglutinin
HAE	Human Airway Epithelium
HPA	Health Protection Agency
HPA MS	Health Protection Agency Microbiology Services
HRP	Horseradish Peroxidase
IC	Imperial College
IFN	Interferon
ILI	Influenza-like illness
IOBA-NHC	IOBA - Normal Human Conjunctival tissue
LAP	Left Atrial Pressure
LB	Luria Broth
LRT	Lower respiratory tract
M	Matrix
MAA	Maackia amurensis
MDCK	Madin-Derby Canine Kidney Cells
MOI	Multiplicity of Infection
mRNA	Messenger RNA

NA	Neuraminidase
NEAA	Non-essential amino acids
NEP/NS2	Nuclear Export Protein
NICs	National Influenza Centres
NLS	Nuclear localization signal
NP	Nucleoprotein
NS1	Non-structural Protein 1
OIE	World Organisation for Animal Health
PA	Acidic Polymerase
PAGE	Polyacrylamide Gel Electrophoresis
PAP	Pulmonary Artery Pressure
PB1	Basic Polymerase 1
PB2	Basic Polymerase 2
PBS	Phosphate Buffered Saline
PCLS	Precision-Cut Lung Slices
PCR	Polymerase Chain Reaction
PFU	Plaque-forming Units
PKR	Protein Kinase R
RBS	Receptor Binding Site
RD	Respiratory Droplet
RdRp	RNA-dependent RNA polymerase

RNA	Ribonucleic Acid
RNP	Ribonucleoprotein particle
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulphate
SF9	Spodoptera frugiperda
SNA	Sambucus nigra
SOC	Super Optimal Broth with Catabolite repression
TLIS	Total Lung Injury Score
TRIG	Triple-Reassortment Internal Gene
UK	United Kingdom
URT	Upper respiratory tract
vRNA	Viral Ribonucleic Acid
WB	Western Blot
WGS	Whole Genome Sequencing
WHO	World Health Organization

Amino Acid Abbreviations

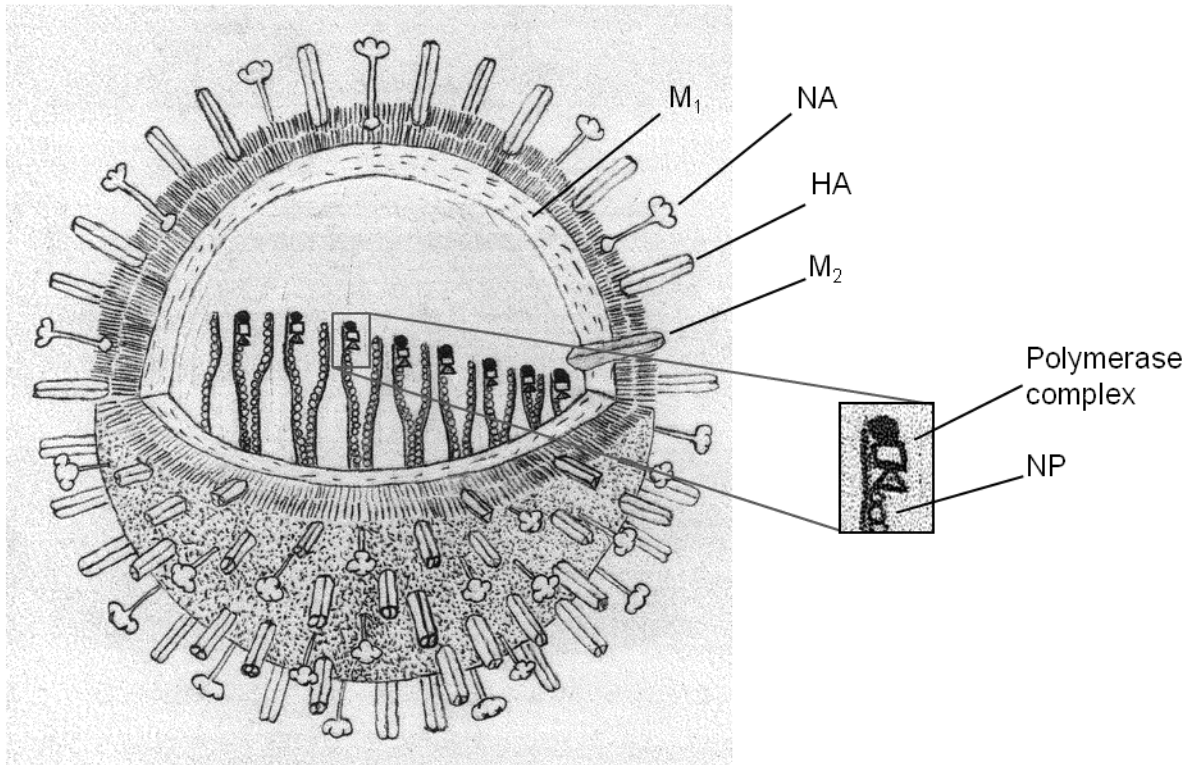
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan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

1 Introduction

1.1 Taxonomy and nomenclature

Influenza viruses are members of the family *Orthomyxoviridae* (from the Greek *orthos*, 'straight, correct' and *myxa*, 'mucus') of which influenza A, B and C constitute three separate genera. These genera are distinguishable by antigenic differences in the nucleoprotein (NP) and matrix (M) proteins. Genus influenza A virus can be further divided into subtypes based on its surface proteins haemagglutinin (HA), of which currently 17 different subtypes are defined, including one recently discovered in little yellow-shouldered bats (Fouchier et al. 2005, Rohm et al. 1996b, Tong et al. 2012) and neuraminidase (NA), of which currently 9 different subtypes are defined. Theoretically, this would amount to a total of 153 influenza A subtypes. All HA subtypes but H17 are found within the wild bird population, which forms the natural reservoir of influenza virus. However, only the HA subtypes H1, H2 and H3 as well as NA subtypes N1 and N2 have been associated with epidemics of disease in human hosts (Hilleman 2002). Individual influenza strains are named according to genera, host of origin (if non-human), geographical origin, strain number and year of isolation (WHO 1980). For example, the first pandemic H1N1 2009 (A(H1N1)pdm09) virus isolated in England was named A/England/195/2009.

a.



b.

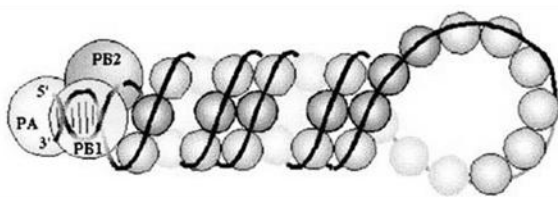
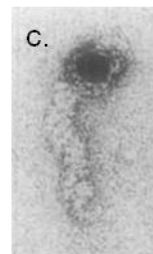


Figure 1.1 Schematic structure of influenza A particle and RNPs

a) Three types of proteins – receptor-binding protein HA, receptor-destroying protein NA and ion channel M₂ – are inserted through the lipid bilayer of the virus. Structural protein M₁ is found underneath the lipid bilayer and interacts with the RNPs. Image adapted from textbook 'Fields Virology', Chapter 46 'Orthomyxoviruses' b) An RNP is formed of one RNA segment (black line) encapsidated by NP protein, which is coiled around itself with both ends anchored to a polymerase complex consisting of proteins PB1, PB2 and PA. Image adapted from the paper by Portela *et al.* (Portela and Digard 2002). c/d) Immunogold labelling targeting PB1 and PB2 c) and PB1 and PA d) on purified RNPs shows binding of the polymerase complex to one side of the RNP. Image adapted from the paper by Murti *et al.* (Murti, Webster and Jones 1988) with permission of Elsevier.

1.2 The structure of influenza virus

The genetic information of influenza virus is coded in negative (-) sense, single-stranded RNA (vRNA) divided into eight (influenza A and B) or seven (influenza C) segments. For influenza A virus, these segments vary from 890 nucleotides (segment 8) to 2341 nucleotides (segment 1 and 2); laboratory-adapted influenza virus A/Puerto Rico/8/1934 (PR8) has a total genome length of 13588 nucleotides (Lamb and Krug 1995). The eight different segments of influenza encode a total of 13 proteins. Segment 1 encodes basic polymerase 2 (PB2), segment 2 encodes basic polymerase 1 (PB1), PB1-F2 and PB1-N40, segment 3 encodes the acidic polymerase protein (PA) and the recently discovered PA-X, segment 4 encodes haemagglutinin (HA), segment 5 encodes the nucleoprotein (NP), segment 6 encodes the neuraminidase (NA), segment 7 encodes the matrix protein (M1) and ion channel protein (M2) and segment 8 encodes the non-structural protein a (NS1) and the nuclear export protein (NEP or NS2). All RNA segments serve as templates for mRNA and antigenome (+) strand synthesis (cRNA). Within the virus, the RNA segments are encapsidated by NP protein and folded, resulting in helicoidal pseudocircular structures (*Figure 1.1b*). The packaged RNA segment is associated with one heterogeneous polymerase complex, RNA-dependent RNA polymerase (RdRp), consisting of polymerase proteins PB1, PB2 and PA, which simultaneously binds both termini of the RNA segment (*Figure 1.1c/d*). The subviral particle containing vRNA, NP proteins and the polymerase complex is known as the ribonucleoprotein particle (RNP). Each RNP behaves as an individual replication and transcription unit. The RNPs are encapsidated in a shell of M₁

proteins lined by a lipid bilayer derived from the plasma membrane of the host cell in which the virus replicated. This plasma membrane contains three proteins; ion channel M₂, receptor-binding protein HA and receptor-destroying protein NA, of which the latter two can be observed as spikes protruding from the viral membrane (*Figure 1.1*).

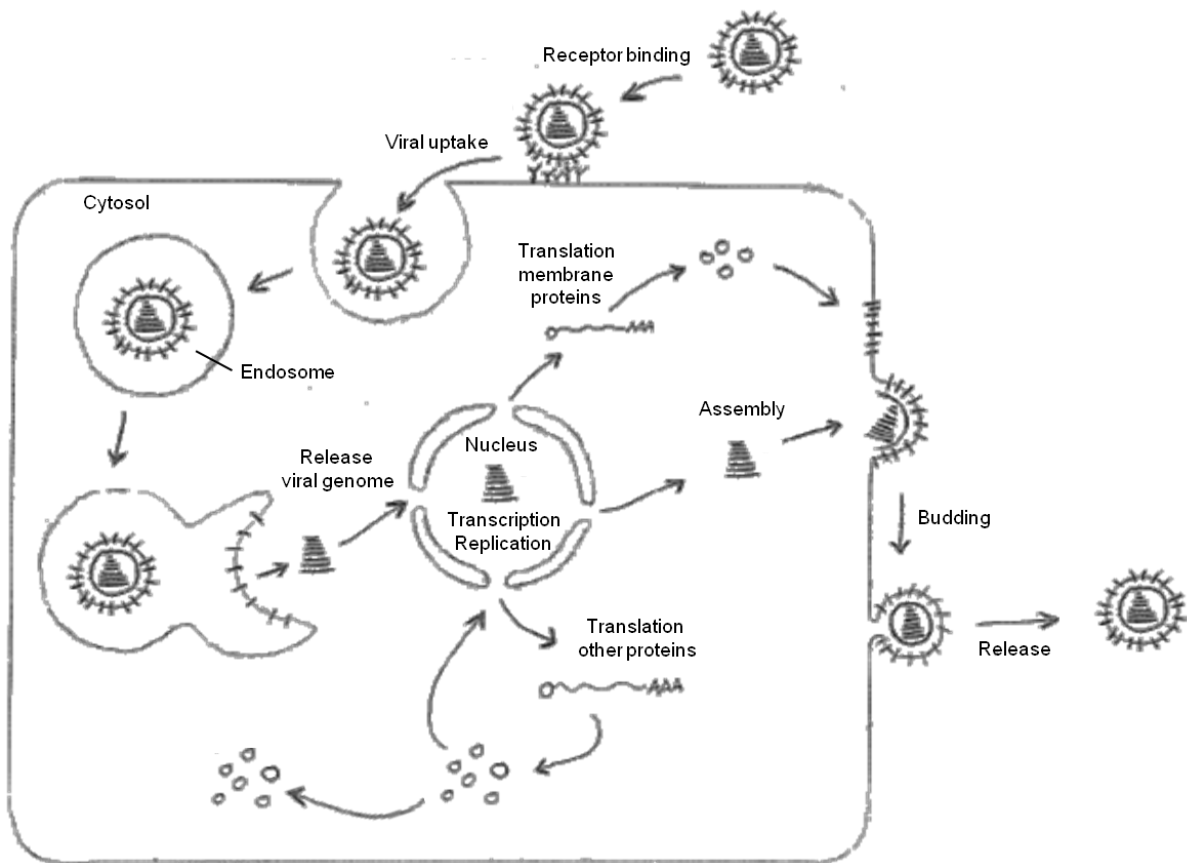


Figure 1.2 Schematic diagram of the viral life cycle

Please see text for details on the viral life cycle. Figure adapted from textbook 'Fields Virology', Chapter 45 'Orthomyxoviridae: The Viruses and Their Replication'.

1.3 The replication cycle of influenza A virus

The HA protein is translated within the host cell as a precursor (HA0). Cleavage of HA0 by host proteases upon virion release results in the formation of two subunits, HA1 and HA2, of which the former is important in receptor binding, whereas the latter is important in membrane fusion upon entry (Steinhauer et al. 1995). HA then binds to the host cell receptor, sialic acid, and the virus enters the cell via receptor-mediated endocytosis (Lamb and Krug 1995). The pH within these endosomes is particularly low, typically around pH 5-6. This low pH results in a conformational change of the HA protein, preserving subunit HA1 but exposing the HA2 subunit fusion peptide (Bullough et al. 1994). Consequently, the fusion peptide inserts into the membrane of the endosome, thereby fusing the viral and endosomal membrane. The low pH in the endosome also opens up the M2 ion channel, which lowers the intra-viral pH. Upon acidification of the viral core, the viral RNPs are released from the protein shell formed by M1, and enter the host cell's cytoplasm (Martin and Helenius 1991a, Martin and Helenius 1991b, Zhirnov 1990). Transcription and replication of the influenza viral genome occurs in the nucleus rather than in the cytoplasm in contrast to most other RNA viruses, and therefore the RNPs require nuclear translocation. The viral proteins within RNPs (PB1, PB2, PA and NP) all have nuclear localisation signal (NLS) regions, however the NLS on the NP protein is both sufficient and necessary for nuclear import of RNPs using the classical importin α/β route (O'Neill et al. 1995, O'Neill and Palese 1995, Cros, Garcia-Sastre and Palese 2005). Within the nucleus, three different types of RNA are produced during influenza infection; 1) viral messenger RNA

(mRNA), 2) positive sense complementary RNA (cRNA) which serves as a template for 3) negative sense viral RNA (vRNA), destined to be incorporated into the eventual progeny virion.

Cellular mRNA contains a poly-A tail and a 5' methylated cap. Influenza virus does not have internal capping or methylation mechanisms (Plotch, Tomasz and Krug 1978). Ingeniously, the influenza virus RdRp acquires the 5' methylated cap of a host cellular mRNA by cleaving 10-13 nucleotides downstream of the cap structure using PB2 cap-binding and PA endonuclease activity (Fodor et al. 2002, Hara et al. 2006, Dias et al. 2009, Blaas, Patzelt and Kuechler 1982). This provides the RdRp PB1 subunit with a primer to initiate mRNA synthesis as well as ensuring host cell translation by ribosomes of viral mRNAs by including the 5' methylated cap. Moreover, viral mRNA contains a poly-A tail obtained by stuttering of the RdRp on the poly(U)₅₋₇ region at the 5' end of vRNA (Luo et al. 1991). Therefore, host and viral mRNAs are structurally indistinguishable.

As influenza is a negative sense RNA virus, genome replication is preceded by the production of full-length, positive sense cRNA, which is most likely coated with NP protein and associated with polymerases during replication to prevent degradation (Vreede and Brownlee 2007). This cRNA then functions as a template for negative sense vRNA replication. For replication of both cRNA and subsequent vRNA, no primers are necessary and no poly-A tail is made (Deng, Vreede and Brownlee 2006).

Newly synthesized viral proteins PB1, PB2, PA and NP are imported into the nucleus for the assembly of viral RNPs (Fodor and Smith 2004, Mukaigawa and Nayak 1991, Neumann, Castrucci and Kawaoka 1997). The nascent viral RNPs are then transported out of the nucleus

into the cytoplasm. It is hypothesized that the export of viral RNPs is achieved by binding of RNPs to the C-terminal part of the M1 protein (Baudin et al. 2001), which subsequently binds the viral NEP protein with its N-terminal domain (Akarsu et al. 2003), thereby blocking the N-terminal NLS found on M1 (Ye, Robinson and Wagner 1995). NEP then binds to nuclear export protein Crm1, which binds RanGTP resulting in nuclear export of the viral RNPs complex through the nuclear pores (Akarsu et al. 2003).

After the membrane proteins HA, NA and M2 are synthesized, they are inserted into the plasma membrane of the host cell. HA and NA are both preferentially inserted into lipid rafts, whereas insertion of the M2 ion channel occurs throughout the entire membrane (Leser and Lamb 2005). M1 binds to the plasma membrane where HA and NA are inserted and virions are formed by a process called budding, during which the membrane bulges from the cell and is eventually pinched off (reviewed in (Nayak et al. 2009)). Recently, a specific role of the M2 protein has been suggested for the scission of influenza virions from the host membrane using its amphipathic helix to modify membrane curvature (Rossman et al. 2010).

Finally, NA cleaves sialic acids on the host cell to prevent aggregation at the cell membrane (Palese and Compans 1976, Liu et al. 1995) and the newly formed virions are released to infect new host cells and repeat the life cycle.

1.4 Haemagglutinin, the receptor binding protein of influenza virus

HA is the most abundant protein on the influenza virion, covering about 80% of the viral surface (Lamb and Krug 1995). It plays a major role in three different phases throughout the influenza virus life cycle. Firstly, HA is the receptor-binding protein and initiates viral entry. Secondly, during endocytosis pH-induced conformational changes within the HA protein expose a small peptide which allows fusion of the viral and cellular membrane resulting in the release of the viral genome into the host cell. Finally, due to its prominent position on the influenza viral surface, HA is the major antigen of influenza viruses. Neutralising antibodies are produced in the human host against specific antigenic regions (epitopes) on the HA molecule and epidemics are associated with antigenic drift due to mutations within or close to these regions.

The HA protein is encoded on RNA segment 4 and is named after its ability to agglutinate red blood cells by binding to sialic acid residues on the cell surface (Hirst 1941). The genomic sequence of HA was the first influenza gene to be fully sequenced (Porter et al. 1979). It encodes a signal peptide, receptor-binding region HA1, fusion-mediating region HA2 and the basic cleavage site between HA1 and HA2. The structure of the HA protein consist of a small hydrophilic domain on the inside of the virion, a small, uncharged hydrophobic peptide which spans the membrane, and a large hydrophilic glycosylated domain on the external surface of the virion. The latter domain constitutes a long, helical chain composed primarily of HA2 topped by a large globular head made exclusively of HA1. This globular head contains the receptor-

binding site (RBS) as well as the antigenic epitopes. The HA1 and HA2 domains are covalently linked via a disulfide bond (Wilson, Skehel and Wiley 1981). The HA protein is expressed as a homotrimer (*Figure 1.3a*).

1.4.1 Cleavage of haemagglutinin

Initially, a precursor of HA (HA0) is synthesised in the host cell via the secretory pathway. HA0 is glycosylated (Daniels et al. 2003) and trimerized in association with chaperones in the endoplasmic reticulum (Copeland et al. 1986, Tatu, Hammond and Helenius 1995, Hebert, Foellmer and Helenius 1996). It is then transferred through the Golgi apparatus and integrated into lipid rafts in the plasma membrane (Skehel and Wiley 2000).

Cleavage of the precursor HA0 into HA1 and HA2 is necessary to allow fusion of viral and cellular membranes upon host cell entry (Klenk et al. 1975, Lazarowitz and Choppin 1975). Cleavage leads to a conformational change in which the fusion peptide (amino acids 1-10 of HA2) is inserted into a conserved cavity formed by residues of both HA1 and HA2 located adjacent to the cleavage site, most likely attributable to electrostatic forces (Chen et al. 1998). Cleavage occurs in two stages. First, an endoprotease cleaves HA at the carboxyl terminus of the arginine (R) residue located between HA1 and HA2 comprising the cleavage site, which creates the N-terminus of HA2. This is followed by the removal of amino acid R by a carboxypeptidase, thereby generating the C-terminus of mature HA1 (Garten and Klenk 1983). Cleavage of influenza virus within the human host is generally believed to be completed by

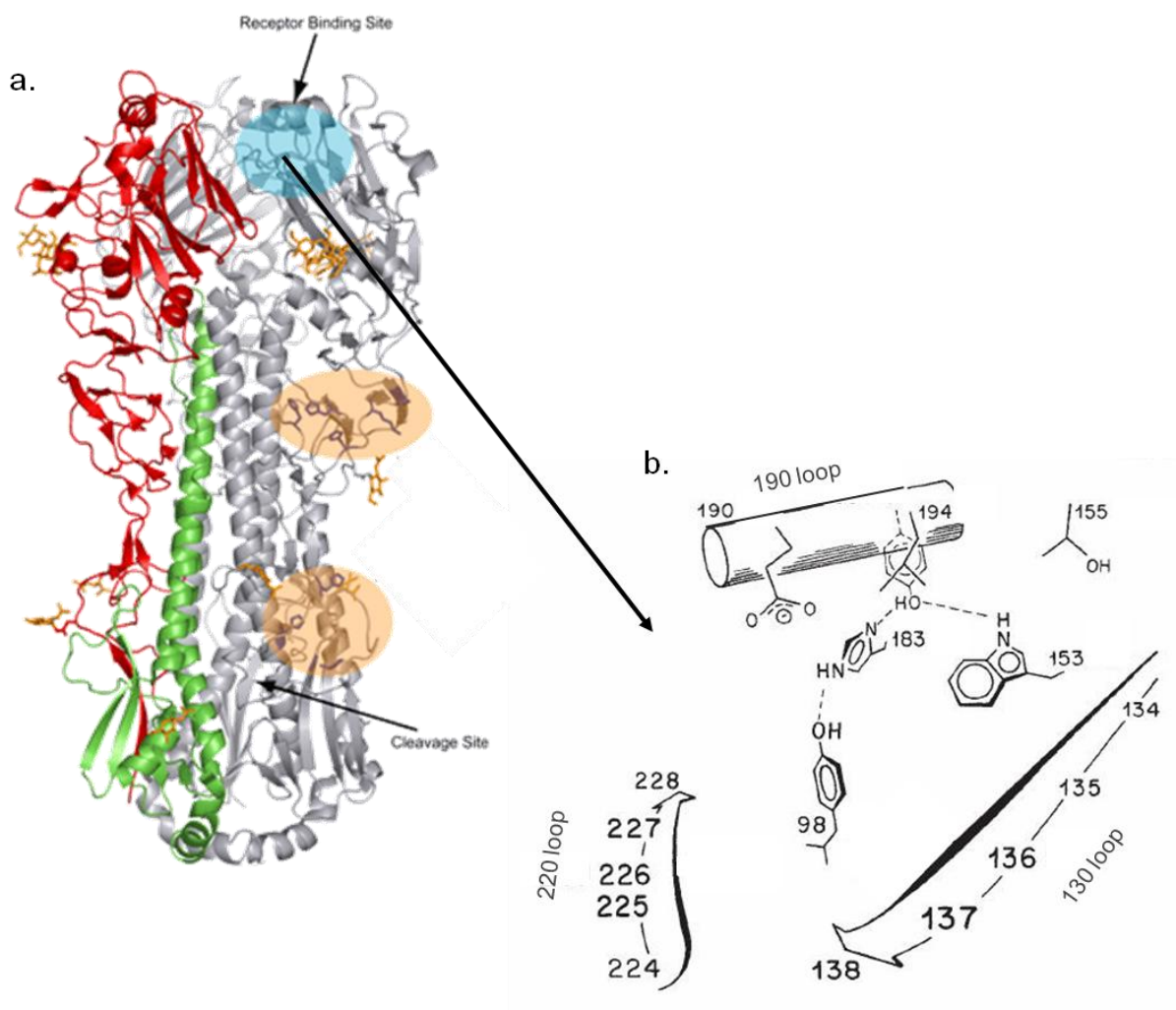
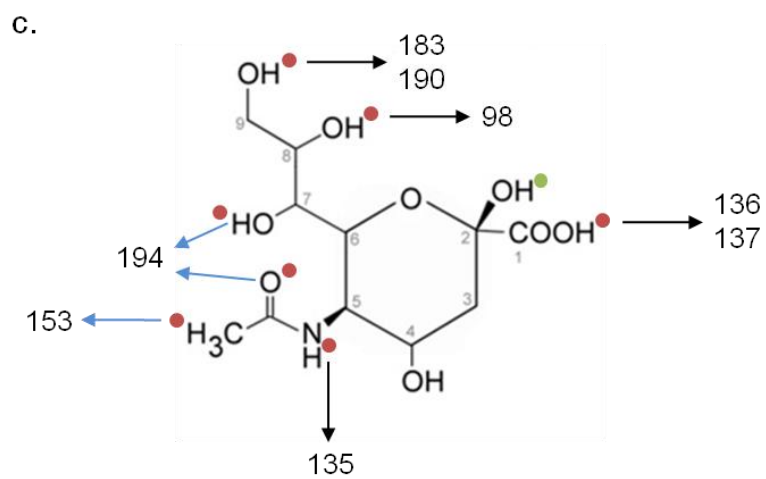


Figure 1.3 Receptor binding protein haemagglutinin (HA)

a) The ribbon presentation of the crystal structure of the uncleaved HA0 trimer of 1918 influenza virus adapted from Stevens *et al.* (Stevens and Wilson 2004) displays the receptor binding site (RBS) on the globular head (blue) and the cleavage site (black arrow). HA1 residues of one HA unit are coloured red, HA2 residues of one HA unit are coloured green. The orange shades represent 1918 influenza virus specific basic patches. b) The RBS pocket was adapted from Weis *et al.* (Weis et al. 1988). The base of the RBS is formed by tyrosine 98 and the aromatic ring of tryptophan 153. Residues 134 to 138 (the 130-loop) form the right side of the RBS, whereas residues 224 to 228 (part of the 220 loop) form the left side of the RBS. Residues 155, 183, 190 and 194 form the rear of the RBS. Of these, 190 and 194 are part of the 190 loop (Weis et al. 1988). c) Sialic acid is illustrated, showing all interactions with the RBS. Red dot, interaction with HA amino acid; Green dot, linkage to glycan; Black arrow, Hydrogen bond; Blue arrow, Van der Waals bond; Numbers represent the residue of HA1 with which interactions is found.

tryptase Clara, a soluble protease excreted by Clara cells located in the human respiratory tract, although other proteases can also cleave HA0 (reviewed in (Bertram et al. 2010)).

When influenza A virus spreads within poultry, occasionally a string of basic amino acids are introduced in the cleavage site of HA via either substitution or insertion, thereby creating a multibasic cleavage site (Horimoto et al. 1995, Mutinelli et al. 2003, Pasick et al. 2005). Multibasic cleavage sites have been associated with high pathogenicity and are recognised by endogenous proteases other than tryptase Clara, such as furin. The distribution of these proteases plays an important role in the increased pathogenicity of viruses with a multibasic cleavage site. Expression of tryptase Clara is restricted to the upper respiratory tract, so HA cleavage and subsequently viral replication is normally limited to the respiratory tract (Sakai et al. 1993). However, furin is expressed ubiquitously meaning the acquisition of a multi-basic cleavage site facilitates expanded cellular tropism and systemic spread of the virus (Walker et al. 1994, Stieneke-Grober et al. 1992). Insertion of a multibasic cleavage site into subtype H3N2 did not result in high pathogenicity in ferrets (Schrauwen et al. 2011), whereas insertion of a multibasic cleavage site in H1, H3, H10, H11 and H15 did not convert the low pathogenic influenza viruses to high pathogenic influenza viruses when investigated in chickens (Lee et al. 2009b). This site is therefore not the sole reason for a high pathogenicity profile. Furthermore, the virulence of viruses containing a multi-basic cleavage site in HA varies among mammalian hosts; whereas the presence of a multibasic-site in A/Vietnam/1203/2004 was associated with increased pathogenicity in mice and ferrets, this phenotype was absent in nonhuman primates (Suguitan et al. 2012).

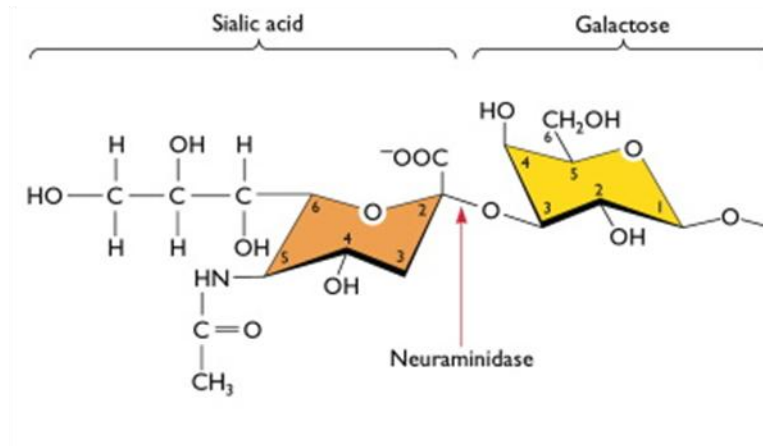
1.4.2 Receptor binding of HA

Glycolipids and glycoproteins can be found on the surface of cells in the human body. The glycans linked to these structures can carry a specific monosaccharide on the outermost capping position; the sialic acid. These are bound by conserved amino-acids in a pocket on the globular head of HA; the receptor binding site (RBS). The base of the RBS is formed by tyrosine 98 and the aromatic ring of tryptophan 153. Residues 134 to 138 (the 130-loop) form the right side of the RBS, whereas residues 224 to 228 (part of the 220 loop) form the left side of the RBS. Residues 155, 183, 190 and 194 form the rear of the RBS and of these, 190 and 194 are part of the 190 loop (Weis et al. 1988) (*Figure 1.3b*).

Upon receptor binding, one side of the pyranose ring found in sialic acid faces the base of the RBS, and the axial carboxylate, acetamido nitrogen and the 8- and 9-hydroxyl groups face into the RBS and form hydrogen bonds. Residues directly involved in receptor binding are 98, 135, 136, 137, 153, 183, 190 and 194 (Skehel and Wiley 2000) (*Figure 1.3c*).

Sialic acid can be linked to galactose at either the carbon-3 or carbon-6 position forming an α 2,3-linked or α 2,6-linked sialic acid respectively (*Figure 1.4*). The specificity of HA to either of these sialic acids forms a governing factor in cell tropism and consequently host specificity. Early binding studies with de-sialylated human red blood cells that had been re-sialylated using sialyltransferases specific for either α 2,3-linked or α 2,6-linked sialic acids showed human H3 influenza viruses have a preference for α 2,6-linked sialic acid, whereas avian H3 influenza viruses bound α 2,3-linked sialic acid (Rogers and Paulson 1983). This preference seems to correlate with the expression of sialic acids at the initial site of infection; α 2,3-linked sialic acid is

a.



b.

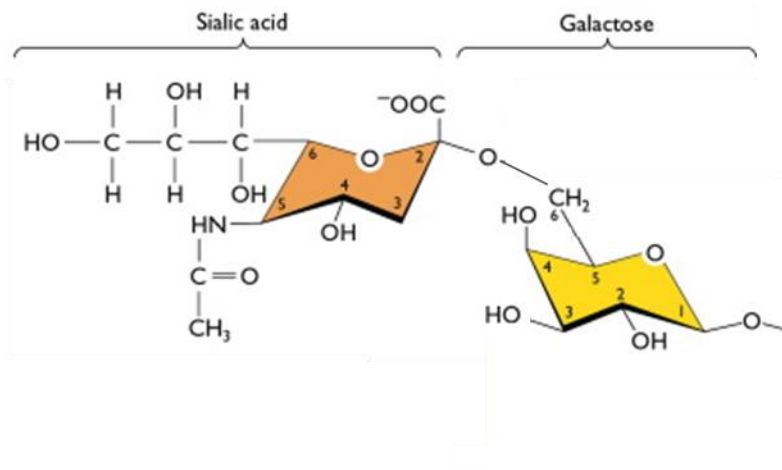


Figure 1.4 Two different linkages of sialic acid to galactose

Sialic acids can be linked to the adjacent sugars via either a) carbon-3 (α 2,3-linked sialic acid) or b) carbon-6 (α 2,6-linked sialic acid). α 2,3-linked sialic acid is commonly used as a receptor by avian influenza viruses, whereas α 2,6-linked sialic acid is the human influenza virus receptor. The α -ketosidic linkage between sialic acid and galactose cleaved by NA is depicted in panel a. Image adapted from textbook 'Principles of Virology' Chapter 5 'Attachment and Entry'.

found abundantly in the avian gut, while α 2,6-linked sialic acid is expressed at the apical surface of the human airway epithelia (Shinya et al. 2006, van Riel et al. 2010). Furthermore, mucins in the sialylated secretions of the human upper respiratory tract, which are important in the initial defence against influenza virus, contain mostly α 2,3-linked sialic acid (Couceiro, Paulson and Baum 1993).

In 1918, an H1N1 virus caused the first well-documented influenza pandemic of the 20th century. Two mutations in the receptor binding site (RBS) of the H1 HA are thought to have played a vital role in enabling the transition from birds to man; residue 190 changed to D from E which is commonly found in bird viruses, and residue 225 changed to D from G. These residues significantly affect the sialic acid preference of the H1 HA protein; human-like D at position 190 allows for stabilization of the human HA- α 2,6 sialic acid receptor interaction (Gamblin et al. 2004) for which avian-like E is too large (Glaser et al. 2005), whereas avian-like G at position 225 narrows the receptor binding pocket, which will then not fit α 2,6-linked sialic acid (Tse et al. 2011). Both humanizing changes were experimentally shown to be required for efficient virus transmission between ferrets (Tumpey et al. 2007). Similarly, for the influenza A (H3N2) subtype, whose HA gene was introduced from an avian source to cause a pandemic in 1968, two other residue changes in the HA RBS have been linked to sialic acid preference and transmissibility; namely Q226L and G228S (Matrosovich et al. 2000, Vines et al. 1998, Rogers et al. 1983, Martin et al. 1998, Roberts et al. 2011, Connor et al. 1994). Changing receptor-binding preference is thought to be critical in the initiation of a pandemic when the virus originates from a non-human host.

1.4.3 Membrane fusion mediated by HA

Upon receptor binding, the influenza virus particle is taken into the host cell by endocytosis and exposed to a low pH (pH 5-6) environment. This leads to an irreversible conformational change within the HA protein, exposing the fusion peptide and relocating it about 10 nm closer to the plasma membrane of the host cell (Bullough et al. 1994). Electron microscopy studies have shown that HA2 can be bound to the virion membrane via the N- and C-termini in the absence of a target membrane (Wharton et al. 1995). It is therefore hypothesised that during viral entry HA2 stays anchored in the viral membrane via its transmembrane domain and is inserted in the target membrane via its fusion protein. Subsequent structural rearrangements control the fusion of both membranes, revealing the inner virus to the cytoplasm and, in combination with acidification of the viral core by ion channel M2, resulting in the transfer of genetic material into the host cell (Weissenhorn et al. 1997, Hughson 1997).

1.4.4 Response of the immune system to HA

The third important role HA plays in the viral life cycle is as the major surface antigenic protein. Due to the prominence of HA on the virion's surface, neutralising antibodies against HA are commonly found in individuals following influenza infection. Five antigenic sites have been located on the HA protein of influenza. Three of these sites (A, B and D) are located on the globular head, in near proximity to the RBS. A fourth antigenic site (site C) is located 60 Å from the distal tip, whereas the location of the fifth antigenic site (site E) is on the lower part of the

globular head (Wiley, Wilson and Skehel 1981). Neutralisation of virus infectivity via antibodies occurs either due to direct binding to amino acids in the RBS thereby preventing sialic acid binding (Bizebard et al. 1995) or binding close to the RBS, preventing receptor binding indirectly through steric hindrance by the antibody (Fleury et al. 1999). Antigenic drift, which occurs in clusters (Smith et al. 2004), is driven by these neutralising antibodies. Mutations within the globular head of HA can directly prevent antibody-binding (Knossow et al. 1984), or can introduce a glycosylation site which shields the antigenic site from the antibody (Skehel et al. 1984). It has recently been proposed that receptor avidity and escape from antibodies are correlated. Using lab-adapted A/Puerto Rico/8/1934 (PR8)-immunised mice, PR8 was serially passaged and sequenced for antibody escape mutants. Interestingly, the resulting mutant viruses were able to escape antibody responses in assays in which virus, cells and antibody were used, but the binding of antibody to HA was not altered, suggesting a role for increased receptor binding in the evasion of antibody interference. Continued passage in naïve mice led to mutant viruses with decreased receptor binding avidity and consequently decreased antibody escape. In this model, influenza infection in immune individuals specifically selects single point mutant viruses with increased receptor binding avidity and decreased antigenicity, whereas subsequent transmission to a non-immune individual selects mutants with decreased receptor binding avidity (Hensley et al. 2009)

Segment	Length vRNA (nt)	Length mRNA (nt)	Encoded protein	Length protein (aa)	Molecular weight protein (kDa)	Approximate number of molecules/virion
1	2341	2320	PB2	759	85.7	30-60
2	2341	2320	PB1	757	86.5	30-60
			PB1-F2	87	13	ND
			PB1-N40	718	NK	NK
3	2233	2211	PA	716	84.2	30-60
			PA-X	231-251*	29	NK
4	1778	1757	HA	566	61.5	500
5	1565	1540	NP	498	56.1	1000
6	1413	1392	NA	454	50.1	100
7	1027	1005	M1	252	27.8	3000
		315	M2	97	11.0	20-60
8	890	868	NS1	230	26.8	ND
		395	NEP/NS2	121	14.2	130-200

Table 1.1 Influenza A virus genome RNA segments and proteins

Adapted from Fields Virology (Lamb and Krug 1995), based on influenza virus PR8 and Shaw *et al.* (Shaw *et al.* 2008), based on influenza virus A/WSN/33. NK = Not known, ND = Not detected, * Estimation based on (Oh *et al.* 2009).

1.5 The remaining twelve proteins of influenza A virus

To date, thirteen proteins have been identified which are produced by influenza A virus, including structural proteins and immune evasion proteins. RNA segments two, three, seven and eight encode multiple proteins, using molecular mechanisms such as mRNA splicing and overlapping reading frames (*Table 1.1*).

1.5.1 Influenza A virus RNA-dependent RNA polymerase

The heterotrimeric RdRp found attached to every RNP in influenza A virus particles consist of PB1, PB2 and PA which have an aggregate molecular mass of 250 kDa. PB1 forms the core of the polymerase, PA is bound to PB1's N-terminal region via its C-terminal region and PB2 is bound to PB1's C-terminal region via its N-terminal region (He et al. 2008, Obayashi et al. 2008). The influenza RdRp does not have proofreading activity and the resulting high mutation rate of approximately one error per replicated genome (Drake 1993) plays an important role in the continual evolutionary changes observed in influenza virus including adaptation to new hosts, evasion of the host immune response and development of antiviral resistance.

1.5.2 Basic polymerase protein 2

PB2 is encoded on RNA segment 1 of influenza A virus, and forms the polymerase complex together with PB1 and PA. The main function of PB2 is so-called "cap-snatching" of host pre-mRNA, a prerequisite of mRNA transcription (reviewed in (Fechter and Brownlee 2005)). This

process can be divided into three different steps. First the RdRp binds to the virion RNA promoter formed by the double-stranded RNA panhandle structures produced by circularization of the 5' and 3' ends of vRNA (Flick et al. 1996), followed by binding of the PB2 subunit to the cap structure of host mRNAs (Blaas et al. 1982), which is then cleaved off at 10-13 nucleotides downstream from the cap structure by endonuclease activity of the PA (Dias et al. 2009).

PB2 has no significant homology to other proteins and cannot be expressed in a soluble state. Therefore, no structural basis for the function of PB2 could initially be investigated. Following the development of ESPRIT (Expression of Soluble Proteins by Random Incremental Truncation), an automated, library-based method for mapping and soluble expression of protein domains from challenging targets, a soluble region within the PB2 protein was identified spanning amino acids 241-483 (Guilligay et al. 2008) and including residues 363F and 404F, which had been found to be crucial in the cap-binding function of PB2 in earlier research (Fechter et al. 2003). Using m⁷GTP-Sepharose as a replacement of the 5' mRNA cap, intrinsic cap-binding activity of this peptide was confirmed. Most of the amino acids in contact with m⁷GTP are completely conserved in all influenza strains and point mutations diminish cap-dependent transcription (Guilligay et al. 2008).

A second soluble C-terminal domain (amino acids 678-759) of PB2 identified using ESPRIT contained a nuclear localization signal (NLS) (⁷³⁶KRKR₁₂KRIR⁷⁵⁵, previously thought to be monopartite (Mukaigawa and Nayak 1991)) and was found to form a complex with importin α (Tarendeau et al. 2007). Previous research on RdRp had already shown that the individual polymerase subunits are translated in the cytoplasm, followed by importation into the nucleus to

assemble the heterotrimeric polymerase (Smith et al. 1987, Jones, Reay and Philpott 1986, Akkina et al. 1987). Importin α is involved in the importation of proteins into the cell nucleus (Imamoto et al. 1995) by recognizing and binding the NLS on a cargo protein (Weis, Mattaj and Lamond 1995) followed by binding of importin β which subsequently binds to the fibrils of the nuclear pore complex and translocates the cargo protein-importin complex (Bayliss, Littlewood and Stewart 2000).

As importin α plays such an important role in the viral life cycle, improving PB2 binding of importin α could be an important host adaptation mechanism. In non-influenza related research, a correlation has been found between the affinity of proteins for importin α and the import rate into the nucleus (Hodel et al. 2006, Hu and Jans 1999). Moreover, in studies using TAP-tagged PB2 pull-downs, PB2 proteins of avian influenza origin showed decreased binding to human importin α compared to those from human influenza (Resa-Infante et al. 2008), suggesting transport of avian-origin PB2 into the nucleus would be decreased in human cells. Furthermore, increased binding of mammalian importin α and PB2 was found in a mouse-adapted avian H7N7 influenza virus. This adaptation was associated with the D701N mutation in PB2 (Gabriel, Herwig and Klenk 2008). Interestingly, it has been observed that in H5N1 viruses isolated from infected humans, eleven out of twelve PB2 sequences either contained the D701N mutation, or the more studied host-specific PB2 mutation E627K (de Jong et al. 2006), suggesting that the presence of either mutation can overcome the avian-mammalian host range restriction. Residue 627 is located between the cap-binding region and NLS domain and a humanizing mutation at this position results in a basic surface patch (Tarendeau et al. 2008), possibly modulating

interplay between PB2 and host factors. Indeed, humanizing 627K was also associated with increased recruitment of importin α (Hudjetz and Gabriel 2012). However none of these studies exclude the role of host factors other than importin α in host adaptation, and research is ongoing on this subject.

1.5.3 Basic polymerase protein 1

PB1 is encoded on RNA segment 2 and forms the core of the polymerase complex, where it is found between PB2 and PA (He et al. 2008, Obayashi et al. 2008). Its main function is the elongation of RNA (Braam, Ulmanen and Krug 1983) using the SDD amino acid sequence located in a conserved motif (motif III) of PB1 (Biswas and Nayak 1994).

1.5.4 Acidic polymerase protein

PA is the third subunit of the influenza polymerase and is encoded on RNA segment 3. The exact role of PA remains unclear, but the protein plays an essential role in endonuclease cleavage of host mRNA caps necessary for mRNA synthesis initiation (Fodor et al. 2002, Hara et al. 2006, Dias et al. 2009), vRNA synthesis (Kawaguchi, Naito and Nagata 2005) and cRNA synthesis (Huarte et al. 2003), packaging of RNA segments (Liang et al. 2012, Regan, Liang and Parslow 2006) as well as promoter binding (Kashiwagi et al. 2009). Single mutations in this protein can promote the production of defective interfering RNAs, possibly linked to destabilisation of the RNA-polymerase complex (Fodor et al. 2003). PA also has proteolytic

activity, of which the function in the influenza life cycle is not yet fully understood (Hara et al. 2001, Perales et al. 2000, Rodriguez, Perez-Gonzalez and Nieto 2007).

1.5.5 Nucleoprotein

NP is encoded on RNA segment 5 and is the most abundant protein in the RNPs, where it encapsidates vRNA by binding along the entire length of the RNA strand at a 24 nucleotide interval (Ortega et al. 2000) and interacts with both PB1 and PB2 (Biswas, Boutz and Nayak 1998). It is further implicated in intracellular trafficking of the viral genome (Digard et al. 1999, O'Neill et al. 1995, Wang, Palese and O'Neill 1997), viral RNA replication (reviewed in (Portela and Digard 2002)) and the viral switch from transcription to replication by stabilizing cRNA (Vreede, Jung and Brownlee 2004, Beaton and Krug 1986, Perez et al. 2010), although the polymerase complex might play a role as well (Vreede and Brownlee 2007).

1.5.6 Neuraminidase

NA is encoded on RNA segment 6 and acts as the receptor-destroying protein, in contrast to receptor-binding protein HA. The protein is expressed as a homotetramer on the surface of the viral particle and is regarded as the second major surface antigen. Nine different subtypes have been identified thus far, which are not serologically cross-reactive (Webster et al. 1992). NA's main function is cleavage of the α -ketosidic linkage between terminal sialic acid and an adjacent sugar residue (*Figure 1.4a*), using the catalytic site located in the head of the protein. This

cleavage activity helps to remove sialic acids linkages between the host cell and HA protein on the virus surface as well as virus:virus interactions. This function was elegantly demonstrated by infection of cells with neuraminidase-deficient virus (Liu et al. 1995), or using neuraminidase inhibitors (Palese and Compans 1976). In both studies, the virus was found to accumulate in clusters and chains on the cell surface.

NA is further thought to be important in the initial infection of the human host. Sialic acids displayed on glycosylated mucins present in the mucosal layer in the respiratory tract are the first line of defence against influenza virus infection; these sugars capture the virus which is then transported out of the body using the mucociliary escalator. NA is thought to cleave these sialic acids, thereby creating a pathway through the mucosal layer towards the target respiratory cells (Matrosovich et al. 2004b).

Because HA and NA have such contrasting roles in the viral life cycle but target the same host cell molecule, sialic acid, the ratio of expression on the viral surface and activity of the proteins needs to be well-balanced (reviewed in (Wagner, Matrosovich and Klenk 2002)).

The NA enzyme active site is the target of the neuraminidase inhibitor (NAI) antiviral drugs of which oseltamivir and zanamivir are currently licensed (Sugrue et al. 2008). Oseltamivir resistance linked to the H275Y mutation in NA was found widely in H1N1 viruses isolated in the 2007-2008 season, increasing to 56% of detected H1N1 influenza viruses in Europe by week 19 of 2008 (Meijer et al. 2009).

1.5.7 Matrix protein

M1 is one of two proteins encoded on RNA segment 7 and is the most abundant protein in the virus particle, produced in the late stages of infection. In the viral particle, M1 forms a coat underneath the viral envelope, providing rigidity to the membrane (Lamb and Krug 1995, Shishkov et al. 1999) and interacting with both viral RNPs and the cytoplasmic tails of HA and NA (Ali et al. 2000, Bui, Whittaker and Helenius 1996, Bui et al. 2000, Baudin et al. 2001, Martin and Helenius 1991a). Furthermore, M1 is crucial in viral budding and morphogenesis, as shown by infection of cells with influenza virus encoding a nuclear-retained M1 protein resulting in reduced particle formation (Whittaker, Kemler and Helenius 1995, Rey and Nayak 1992), influenza virus with low expression of M1 and M2 proteins which delays budding (Bourmakina and Garcia-Sastre 2005) and influenza virus with mutated M1 protein which significantly changes morphology (Burleigh et al. 2005, Elleman and Barclay 2004).

1.5.8 Ion channel protein

M2 is the second protein encoded on RNA segment 7, specifically derived by alternative splicing of mRNA (Lamb, Lai and Choppin 1981), and is the target of antiviral drug amantadine (Sugrue et al. 2008). M2 spans the entire viral membrane surface once. Its ion channel function was suggested based on its homotetrameric amphiphilic transmembrane region forming an alpha helix (Sugrue and Hay 1991) and confirmed by electrophysiological experiments using oocytes of *Xenopus laevis* expressing M2 protein (Pinto, Holsinger and Lamb 1992). Finally, the

M2 protein has recently been found to play a crucial role in the pinching off of virions using its amphipathic helix (Rossman et al. 2010)

1.5.9 Non-structural protein 1 (NS1)

The non-structural protein 1 (NS1) is encoded on RNA segment 8 and plays an important role in the regulation of the immune response as well as cellular host functions. It does this via several different mechanisms; NS1 binds dsRNA, thereby preventing detection by pattern recognition sensors such as retinoic acid inducible gene I (RIG-I) within the host cell and subsequent activation of the dsRNA-dependent response which includes type I interferon production (Wang et al. 1999, Min and Krug 2006), NS1 suppresses the RNA silencing-based antiviral response (Li et al. 2004), NS1 blocks Protein Kinase R (PKR) activation, a component of the cellular antiviral system activated by binding to dsRNA (Li et al. 2006a), NS1 blocks nuclear export of poly(A)-containing mRNAs, most likely to allow cap-snatching by PB2 (Qiu and Krug 1994), NS1 inhibits pre-mRNA splicing of host mRNAs, but not viral NS1 mRNA (Lu, Qian and Krug 1994) and finally NS1 activates phosphatidylinositol-3-kinase (PI3K), resulting in enhanced viral replication via an as yet unknown mechanism (Hale et al. 2006).

1.5.10 Nuclear Export Protein

NEP (or NS2) is the second protein encoded on RNA segment 8 and is derived from the alternative splicing of mRNA. As its name implies, this protein is of importance in the export of viral RNPs from the nucleus to the cytoplasm by interaction with cellular nucleoporins (O'Neill, Talon and Palese 1998). NEP is also involved in viral assembly through its interaction with the M1 protein (O'Neill et al. 1998) and has been implicated in maintaining the balance between transcription and replication products of the viral genome independent of its export function (Robb et al. 2009). Lastly, NEP has been associated with adaptation of H5 to the human host by increasing polymerase activity (Manz et al. 2012).

1.5.11 PB1-F2

PB1-F2 is a non-essential protein encoded by an alternative open reading frame (+1) on RNA segment 2, which was identified serendipitously while searching for CD8⁺ T-cell reactive epitopes (Chen et al. 2001). PB1-F2 is found in the mitochondria, nucleus and cytoplasm and is thought to induce apoptosis (Chen et al. 2001, Gibbs et al. 2003, Zamarin et al. 2005). Furthermore, this protein has been linked to enhancement of secondary bacterial infection, resulting in increased pathogenesis in a mouse model (McAuley et al. 2007) as well as IFN suppression (Conenello et al. 2011). However, the importance of PB1-F2 remains controversial as not all influenza strains express full-length PB1-F2 (Zell et al. 2007). Furthermore, restoration of full-length PB1-F2 in A(H1N1)pdm09 virus does not influence pathogenicity in a ferret or

mouse model (Hai et al. 2010) and only moderately enhances pathogenicity in pigs (Pena et al. 2012). The introduction of 1918 H1N1 PB1-F2 in previous seasonal H1N1 influenza viruses delayed proinflammatory responses, but did not cause a difference in pathogenicity in ferrets compared to previous wild type seasonal H1N1 (Meunier and von Messling 2012).

1.5.12 PB1-N40

PB1-N40 is a recently discovered protein expressed during influenza infection, and is encoded on RNA segment 2 using start codon 5 in the same open reading frame as PB1, resulting in an identical protein except for the lack of the first 39 amino acids (Wise et al. 2009). This truncation results in a loss of the primary interaction site with PA, and PB1-N40 is insufficient for influenza virus replication (Wise et al. 2009). Due to the recent discovery of PB1-N40, its function remains unclear.

1.5.13 PA-X

The PA-X protein discovery was published in June 2012. PA-X is encoded on RNA segment 3 and is produced via ribosomal frameshifting at codon 190 due to the presence of motif UCC UUU CGU C. This protein contains the N-terminal endonuclease domain of the PA protein with a short C-terminal encoded by the +1 ORF. Although limited information is available on the function of this protein, a role in the modulation of host-gene expression and host-cell shutoff is implicated. PA-X is not required for viral replication (Oh et al. 2009).

1.6 Infection and disease in the human host

Influenza is a serious public health problem affecting 5-15% of the population annually. Epidemics are documented practically every year, although severity and extent may vary widely. Human-to-human transmission of influenza occurs via the respiratory route. Patients infected with influenza virus display a variety of symptoms, often including fever, a sore throat and aching muscles but may also be asymptomatic. Annually, influenza virus infections cause approximately 3-5 million cases of severe illness and 250,000-500,000 deaths worldwide, particularly in higher risk populations including the very young (<5 years), elderly (>65 years) and chronically ill patients (WHO Accessed 26-01-2012). Increased severity of disease is often associated with pneumonia; either primary viral pneumonia or secondary bacterial pneumonia. Secondary bacterial infection is hypothesized to be caused by a change in the indigenous microbiota in the upper respiratory tract (URT) as well as an altered immune state during or after infection (Snelgrove, Godlee and Huseell 2011, Goulding et al. 2011, Huseell and Cavanagh 2009, Ballinger and Standiford 2010, McAuley et al. 2007). Furthermore, NA could potentially expose receptors for pathogenic bacterial invaders whilst stripping the lungs of sialic acid (McCullers and Bartmess 2003).

Influenza virus infections impose a substantial economic burden in the form of health care costs and lost productivity, estimated to be \$87.1 billion yearly in the USA (Molinari et al. 2007). Besides annual epidemics, major antigenic changes in the virus can result in periodic, global pandemics due to a lack of herd immunity. Estimates put the direct medical costs of a pandemic

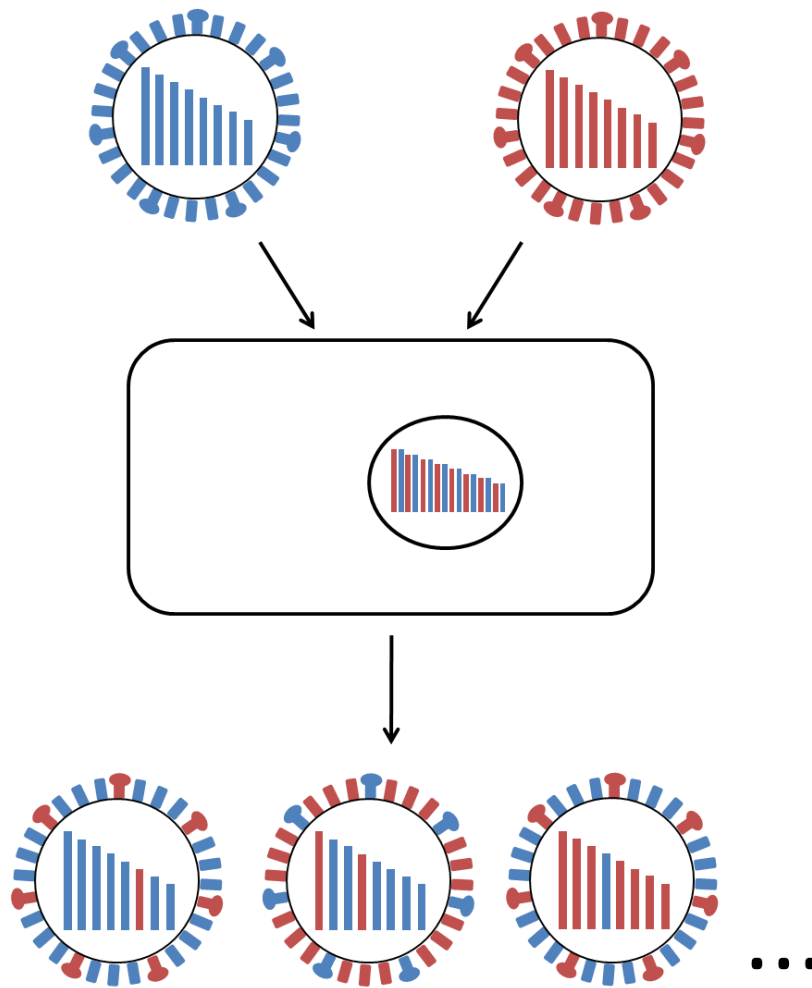


Figure 1.5 Reassortment of influenza virus

When two influenza viruses infect one cell, reassortment of the genetic information can occur due to the segmented genome of the virus. This phenomenon is called antigenic shift. Image adapted from 'Fields Virology', Chapter 48 'Orthomyxoviruses'.

with an attack rate of 30% at €843 million in the Netherlands alone (Hak, Meijboom and Buskens 2006).

Due to the prominent position of HA and NA on the virion surface (80% and 20% of the viral surface on average depending on virus strain (Lamb and Krug 1995)) these proteins constitute the major surface antigens of influenza virus. Accumulation of mutations in the viral genome is driven by the lack of proofreading of the RdRp which results in the generation of approximately one mutation per novel virion (Drake 1993). Mutations in the antigenic sites of HA and NA causing minor antigenic changes give significant advantage to viruses evading the host response. This process is called antigenic drift and elucidates the ability of influenza to re-infect the human host.

Due to its segmented genome, influenza viruses are capable of a second form of evasion of the immune system via major antigenic changes; antigenic shift. This occurs when two different influenza viruses infect one cell and during replication exchange segments, thereby creating a novel genomic arrangement. For example, an avian HA protein not formerly encountered by the immune system of the human host combined with human-adapted internal proteins could form a virus capable of replication within the human host which is completely overlooked by the immune system. Such viruses are thought to be particularly important in the emergence of influenza pandemics (*Figure 1.5*).

Two examples of such reassortant viruses causing pandemics occurred in 1957 and 1968, when H2N2 and H3N2 respectively caused influenza pandemics. The influenza pandemic caused by H2N2 in 1957 is known as the 'Asian flu' and this virus resulted from a reassortment

event between circulating human H1N1 and avian H2N2 viruses. The pandemic virus obtained HA, NA and PB1 gene segments from the avian H2N2 virus, while the remaining five segments were of human H1N1 origin (Lindstrom, Cox and Klimov 2004, Kawaoka, Krauss and Webster 1989). The so-called 'Hong Kong flu', was caused by H3N2 in 1968. As with H2N2, this virus arose after reassortment of a circulating human H2N2 virus with an avian H3N2 virus, obtaining both HA and PB1 from the avian virus (Lindstrom et al. 2004, Fang et al. 1981, Kawaoka et al. 1989). The new pandemic virus H3N2 replaced H2N2 as the circulating human seasonal influenza strain.

Not all pandemic viruses are generated via antigenic shift. The most severe influenza pandemic in recent documented human history is known as 'Spanish flu' and claimed the lives of approximately 20 to 50 million people in 1918. The general consensus is that this virus, 1918 H1N1, was entirely of avian origin and changed its receptor binding abilities in order to infect the human host and cause human-to-human transmission (Taubenberger et al. 2005, Reid, Taubenberger and Fanning 2004). However, recent research has suggested that 1918 H1N1 was not directly introduced in the human host, but was in fact also a reassortment virus (Smith et al. 2009a). If this is indeed the case, then all influenza pandemics in the 20th and 21st century were preceded by reassortment of influenza virus.

1918 H1N1 circulated in the human population until 1957, when it was displaced by H2N2. Interestingly, H1N1 was re-introduced into the human population in 1977. This 'novel' virus was genetically identical to the H1N1 virus circulating in 1950 (Nakajima, Desselberger and Palese 1978). Three possible places for this virus to hide have been suggested; the animal host, the

human genome or a frozen source. As no antigenic drift was present in the virus compared to isolates from 1950 (Nakajima et al. 1978), and no documented cases of integration of the influenza genome in the human genome exist, this reintroduction presumably originates from a frozen source. Due to existing immunity in the older population, disease was mainly found in younger subjects (Zimmer and Burke 2009). This virus co-circulated with H3N2 until recently, when it was replaced by A(H1N1)pdm09.

Based on the ability of influenza viruses to reassort and the constant detection of influenza virus in the avian host, the emergence of another pandemic influenza virus has never been in question for most virologists. As such, the more pressing questions were *when* and *where* the next pandemic virus would arise. In preparation for such an event, the World Health Organization (WHO) established the Global Influenza Surveillance and Response System (GISRS) in 1952, which is responsible for worldwide surveillance of the human population for influenza viruses. Currently, there are 136 National Influenza Centres (NICs) members of GISRS located in 107 WHO Member States and these process >1,000,000 samples per year (WHO 2012). Another influenza surveillance initiative, overseen by the European Centre for Disease Prevention and Control (ECDC), gathers influenza surveillance information from European Union (EU) countries. The foundation of these global and regional surveillance initiatives are the national influenza surveillance programs which exist all over the world. For example, in the UK a virological surveillance scheme is in place that collects and tests samples from patients presenting with influenza-like illness (ILI) at one of 100 participating GP Surgeries (Fleming et al. 1995). This scheme allows for the rapid detection and monitoring of influenza

activity within the community as well as providing both genetic and antigenic information on circulating strains within the UK population.

Surveillance of influenza in the human host resulted in early detection of the first pandemic of the 21st century which was caused by an H1N1 virus, A(H1N1)pdm09. On April 21, 2009 the Centre for Disease Control and Prevention (CDC) reported two epidemiologically unrelated cases of swine-origin influenza infection in California following reports of increased influenza-like illness in Mexico (CDC 2009). In the following weeks this virus spread globally, causing the WHO to declare a pandemic on June 11, 2009. By the time the pandemic had passed in August 2010, two pandemic waves had been documented in the Northern hemisphere and the virus had spread to more than 214 countries, causing over 18,000 confirmed influenza-related deaths (WHO 2010). A(H1N1)pdm09 vaccines were not available until October 2009, six months after the first cases were reported, and none of the methods undertaken to try and quarantine the virus were successful; A(H1N1)pdm09 reached the UK on April 27 2009, only six days after the first case report by CDC.

A(H1N1)pdm09 originates from a swine source and contains a unique gene constellation; the NA and M genes originate from Eurasian swine influenza, all other genes originate from triple reassortant swine influenza, a virus circulating in the American swine population since 1998 and itself containing PB2 and PA from an avian influenza virus, PB1 from a human influenza virus and HA, NP, NA, M and NS from classical swine influenza (Vincent et al. 2006, Zhou et al. 2000). A(H1N1)pdm2009 closest relatives were isolated on average 10 years ago, indicating that the virus or a precursor of it had been circulating within the swine population undetected for

a prolonged time (Garten et al. 2009). This finding highlights the necessity of surveillance not only in the human host but also in the avian and swine population, which has been undertaken by the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization (FAO). Unfortunately, surveillance in the swine host lags behind surveillance levels in the avian host; only 902 full-length HA sequences were deposited in the NCBI Influenza Virus Resource Database before April 27, 2009 of which only 742 sequences were unique. However, such surveillance is predicted to be an immense undertaking.

Currently, A(H1N1)pdm09 is co-circulating with H3N2 in the human population; the 2010-2011 influenza season in the UK was dominated by A(H1N1)pdm09 but during the 2011-2012 influenza season (with notably low influenza activity) H3N2 has been the dominant virus. The majority of the human population has most likely been infected with and produced antibodies against A(H1N1)pdm09 (Miller et al. 2010). This brings us back to the central question; *when* and *where* will the next influenza pandemic arise?

1.7 The human pandemic threat from zoonotic influenza viruses

As explained in section 1.6, previous pandemics were caused by influenza viruses with genes originating from avian (1918 H1N1, H2N2 and H3N2) or swine hosts (A(H1N1)pdm09), either directly or via reassortment events.

A large number and variety of different influenza viruses can be found in the avian host (Asplin 1970, Easterday et al. 1968, Dasen and Laver 1970, Winkler, Trainer and Easterday 1972, Downie and Laver 1973, Slemons et al. 1974, Stallknecht and Shane 1988) with aquatic birds (the Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and waders)) regarded as the primary natural reservoir of influenza virus (Krauss and Webster 2010, Gorman et al. 1991, Gorman et al. 1990a, Gorman et al. 1990b, Okazaki, Kawaoka and Webster 1989, Schafer et al. 1993, Kawaoka et al. 1989). Infection and replication of influenza virus takes place in the intestinal tract of the avian host and is usually asymptomatic. Transmission of influenza virus between birds most likely occurs via water; the virus remains infective in lakes for days to weeks depending on water temperature, salinity, pH and viral strain (Stallknecht et al. 1990a, Stallknecht et al. 1990b). Migrational behaviour of aquatic birds inadvertently leads to extensive spread of influenza virus, which is for example observed in a specific clade of H5N1, that has travelled to Europe from Asia via migrational routes (Kilpatrick et al. 2006).

The first influenza virus isolate was obtained from a swine host in 1931 by Richard Shope, who made the pioneering observation that pigs suffered very similar symptoms to humans during the Spanish flu in 1918 (Shope 1931). Indeed, 1918 H1N1 was widespread in swine herds then and pigs have been known to be infected by both human and avian influenza (reviewed in (Van

Reeth 2007)). Due to this ability, the swine host has traditionally been regarded as a potential mixing vessel for the generation of novel reassortant influenza viruses.

History teaches us that both swine and avian hosts have the potential to produce a pandemic influenza virus. However, overcoming the species barrier is not straight forward and evolving to become a pandemic influenza virus involves much more than just jumping from one host to another. Several different factors can play a fundamental role; the virus will need to be able to infect appropriate cells in the URT; the initial infection must be followed by replication within the cell; replication within the host must lead to a high viral load; virus must be released from the host cell; released virus must be found as single particles, and not aggregates; and a high amount of virus must be released (Sorrell et al. 2011). This is not an exhaustive list of pandemic viral determinants, and several other unknown factors may have a significant role as well, which only underlines the complexity of the formation of a new pandemic influenza virus. Nonetheless, influenza pandemics occur every few decades and research on these different factors is paramount to provide a better understand and possibly prevention of influenza pandemics.

1.7.1 Cell tropism

A noted difference between avian and human influenza viruses is the receptor binding preference, which has been discussed in section 1.4.; avian influenza virus have a higher affinity for α 2,3-linked sialic acid, which is found abundantly in the avian gut, whereas human influenza viruses preferentially bind to α 2,6-linked sialic acid which is found in the human URT.

α 2,3-linked sialic acid is also found in the human lower respiratory tract (LRT) and consequently avian viruses can infect the human lungs, but infection of the URT is limited and therefore sustained human-to-human transmission does not occur (CDC 1997, Fouchier et al. 2004). It is generally assumed that avian influenza virus needs to change its receptor binding preference to α 2,6-linked sialic acid and decrease its binding to α 2,3-linked sialic acid before it can infect the appropriate cells in the human URT and obtain pandemic potential. Most likely, this receptor binding preference change is more complex than just switching from α 2,3-linked sialic acid to α 2,6-linked sialic acid but involves binding to specific α 2,6-linked sialic acids such as longer branched and umbrella-like structures, which have been found to be selectively bound by human influenza virus (Chandrasekaran et al. 2008).

Specific mutations in the HA protein have been linked to a change in receptor binding preference in earlier pandemic viruses, as discussed in section 1.4. In H2 and H3 influenza virus, residues 226 and 228 play an important role, whereas in H1 influenza viruses, residues 190 and 225 are particularly important. These and other mutations, whether found in natural isolates or engineered, in the HA of H5, H7 and H9 avian influenza viruses as researched previously, have been linked with a change in receptor binding and thus cell tropism (Ayora-Talavera et al. 2009, Stevens et al. 2008, Auewarakul et al. 2007, Watanabe et al. 2011, Yang et al. 2010, Wan and Perez 2007). However, these changes on their own have not yet led to aerosol transmission in the ferret model, a necessary feature of a pandemic influenza virus (Chen et al. 2012, Belser et al. 2008, Wan et al. 2008). Therefore these mutations are probably necessary, but not sufficient for the establishment of a new pandemic virus. Recent research by

Imai *et al.* conferring aerosol transmission to a reassortant virus containing the HA of H5N1 and all other genes of A(H1N1)pdm09 suggests that in addition to receptor binding preference changes, the stability of the HA protein in response to pH and heat via mutations in the stalk region is also important (Imai *et al.* 2012).

1.7.2 Replication leading to high viral yields

Following viral entry into cells of the human URT, replication of the viral genome needs to result in viral yields high enough to cause transmission between humans. Adaption-mediating mutations in the PB2 gene have been particularly well studied, and have been discussed to some extent in section 1.5.2. For example, humanizing mutation D701N in H5N1 was found to be a prerequisite of direct contact transmission within a guinea pig model (Gao *et al.* 2009) and avianising mutation K627E was associated with a decrease in transmission efficiency of H5N1 and H3N2 in a direct contact model of guinea pigs (Steel *et al.* 2009). These mutations are thought to increase the transmission potential of an influenza virus by increasing polymerase activity at 32-33°C, the temperature of the human URT (Massin, van der Werf and Naffakh 2001), ensuring large numbers of released progeny virus are present in aerosols excreted from the nose and mouth upon breathing, sneezing and coughing.

1.7.3 Release of single particles, not aggregates from the host cell

It has long been recognized that the balance between HA and NA in respect to receptor binding and release is important in viral fitness. A good balance allows viral release from the host cell by cleavage of sialic acid by NA (Palese and Compans 1976, Liu et al. 1995), but is also very important in the release of single virus particles. HA does not differentiate between sialic acids on the host cell and sialic acids on the glycoproteins of influenza virus. Therefore, upon release of the virus from the host cell, HA binds to other virions and viral aggregates are formed. Cleavage by NA disrupts these interactions and single viral particles instead of viral aggregates are released (Rudneva et al. 1993). One study showed that replacing the NA of swine H1N1 with the NA of a recent A(H1N1)pdm09 with increased relative activity led to the production of single particles in MDCK cells, and consequently this virus showed an increase in respiratory droplet (RD) transmission in the ferret model (3/3) compared to the wild type (1/3) (Yen et al. 2011b). Another study showed that the increased activity of NA in A(H1N1)pdm09 was correlated with a consistent release of influenza particles in the air – a factor that will undoubtedly affect aerosol transmission (Lakdawala et al. 2011).

1.7.4 Release of high viral titres

Finally, high titres of virus should be released, increasing the chance some of the virions will reach the next susceptible host. Evidence for a potential role for the M gene in the release of high viral titres can be found in some recent publication studying A(H1N1)pdm09. Chou *et al.*

investigated transmission of influenza in guinea pigs after infection with reassortant viruses containing the NA and M gene of A(H1N1)pdm09 and internal genes of either a swine origin H1 virus or PR8. It was consistently found that inclusion of the NA gene, but predominantly the M gene resulted in an increased viral titre in nasal wash samples obtained from inoculated guinea pigs, and consequently an increase in infected sentinels (Chou et al. 2011). Likewise, Yen *et al.* found an increased viral titre in human airway epithelium culture infected with influenza viruses containing the Eurasian M gene like A(H1N1)pdm09, compared to those infected with swine origin influenza virus without the Eurasian M gene (Yen et al. 2011b). This research suggests a role for the M gene in release of virus into the mucus.

1.7.5 Concluding remarks and aims

The adaptations discussed above may not be the only changes sufficient to cause the next pandemic, and changes in any of the other proteins could potentially play an important role as well. Other factors such as induction of sneezing and coughing, length of viral shedding and the level of immune response could significantly change the outcome. For example, recent research correlates the timing of expression of pro-inflammatory molecules in the URT to the point in infection when transmission and peak virus shedding occur (Maines et al. 2012). To better understand the factors important for transmission, further research is necessary and currently in progress, potentially using improved models. Influenza research is currently performed on several different models; in vivo, ex vivo and in vitro models are in use. However, no model is

perfect and as such there is a constant search for better models. An intriguing example is the high-technical solution of using the 'lung-on-a-chip' model, which has the advantages of using highly differentiated cells seeded on an air-liquid interface in a system that mimics the complex mechanical and biochemical behaviours of a human lung (Huh et al. 2010). One chapter in this thesis will look at the possibility of using a whole porcine lung, which would also have the advantage of highly differentiated cells, mechanical and biochemical behaviour of the porcine lung, but on top of that the complex 3D structure of the respiratory tract system.

Here, we try to address some of the questions posed above using pandemic influenza viruses and viruses with pandemic potential, tested in different models of infection and transmission;

- What are the molecular changes in the HA of A(H1N1)pdm09 that explain why this virus transmits between humans, whereas earlier swine-origin human isolates do not?
- Are there any molecular differences between a human and an avian isolate of H7, and do differences exist between the human isolates obtained from different anatomical sites (the eye and throat of a single patient), which explain the ability to jump species and the different infection sites respectively?
- Can we infect whole porcine lungs with influenza virus and use this as a model of influenza virus infection?

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

Name	Source	Comments
293T	GlaxoSmithKline	Human Embryonic Kidney cells expressing simian virus 40 (SV40) large T antigen
HAE	Raymond Pickles, University of North Carolina or Epithelix Srl	Primary differentiated human airway epithelium cultured at air-liquid interface
IOBA-NHC	Virginia Calder, University College London	Normal human conjunctival cell line
MDCK	WHO-Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, Mill Hill (Health Protection Agency Microbiology Services (HPA MS)) or GlaxoSmithKline (Imperial College (IC))	Madin Darby Canine Kidney cell line
MDCK-SIAT1	Mikhail Matrosovich, Philips University Marburg	MDCK cells expressing increased levels of α 2,6-linked sialic acid (Matrosovich et al. 2003)
SF9	Invitrogen	<i>Spodoptera frugiperda</i> (Fall armyworm) cells

Table 2.1 Cell lines used in this project

2.1.2 Virus strains

Name	Abbreviation	Source	Subtype	RG*	Comments
A/England/195/09	E195	IC	A(H1N1)pdm09	Yes	First fully sequenced UK A(H1N1)pdm09 virus
A/England/195/09	E195c	HPA MS	A(H1N1)pdm09	No	Clinical isolate
A/England/195/09 E227A	E195 E227A	This project	A(H1N1)pdm09	Yes	Mutation in HA protein at position 227
A/England/195/09 D225G	E195 D225G	This project	A(H1N1)pdm09	Yes	Mutation in HA protein at position 225
A/England/331/10	E331	HPA MS	A(H1N1)pdm09	No	Isolated in December 2010 (third wave of A(H1N1)pdm09 pandemic)
A/England/480/06	E480	HPA MS	H7N3	No	Isolated from throat patient
A/England/481/06	E481	HPA MS	H7N3	No	Isolated from eye patient
A/Mallard/Sweden/105/02	M105	HPA MS	H7N7	No	Isolated from Mallard
A/Ohio/01/07	Ohio01	This project	Swine-origin H1N1	Yes	HA gene: Ohio01 All other genes: E195
A/Ohio/01/07 A227E	Ohio01 A227E	This project	Swine-origin H1N1	Yes	Mutation in HA gene at position 227 HA gene: Ohio01 All other genes: E195

Table 2.2 Viruses used in this project

*RG: Rescued by reverse genetics using 12-plasmid transfection

2.1.3 Recombinant proteins

Name	Gene	Origin	Tags	Source	Expression model	Comments
E195	HA	A/England/195/09	His Human Fc	This project	Baculovirus expression in SF9 insect cells	
E195 E227A	HA	A/England/195/09	His Human Fc	This project	Baculovirus expression in SF9	Mutated at position 227
E480	HA	A/England/480/06	His Human Fc	This project	Baculovirus expression in SF9	
M105	HA	A/Mallard/Sweden/105/02	His Human Fc	This project	Baculovirus expression in SF9	
Ohio01	HA	A/Ohio/01/07	His Human Fc	This project	Baculovirus expression in SF9	
Ohio01 A227E	HA	A/Ohio/01/07	His Human Fc	This project	Baculovirus expression in SF9	Mutated at position 227

Table 2.3 Recombinant proteins used in this project

2.1.4 Oligonucleotides

Name	Sequence	Purpose	Designer
ND-H1swHA-BAC1-F	GCG AAT GGC CAT TAT GGC CCC GAC ACA TTA TGT A	Amplification of H1 HA for baculovirus expression	This project
ND-H1swHA-BAC1-R	GGA ATA GGC CGA GGC GGC CGT AAA TCC TTG TT	Amplification of H1 HA for baculovirus expression	This project
A227EOhio_F	GGT GAG GGA CCA AGA AGG GAG AAT GAA C	Mutagenesis A227E in Ohio01	This project
A227EOhio_R	GTT CAT TCT CCC TTC TTG GTC CCT CAC C	Mutagenesis A227E in Ohio01	This project
E227AE195_F	CCC AAA GTG AGG GAT CAA GCA GGG AGA ATG AAC TAT TAC	Mutagenesis E227A in EE195	This project
E227AE195_R	GTA ATA GTT CAT TCT CCC TGC TTG ATC CCT CAC TTT GGG	Mutagenesis E227A in E195	This project
480COseq_F1	ATT ATG GCC CCG ACA AGA T	Sequencing codon optimised E480	This project
480COseq_F2	CTG GAA TTC TCC GCT GAC C	Sequencing codon optimised E480	This project
480COseq_F3	CAC CAT CAC CTT CAG CTT CA	Sequencing codon optimised E480	This project
480COseq_F4	ATC AAC TGG ACC CGT GAC TC	Sequencing codon optimised E480	This project
480COseq_R1	GTC TCG GTA GCG TTG ACC AC	Sequencing codon optimised E480	This project
480COseq_R2	TGG TGG ATA CCC CAG ATG AT	Sequencing codon optimised E480	This project
480COseq_R3	TCT GGT GAC GGA AAC CGT A	Sequencing codon optimised E480	This project
480COseq_R4	GTC CTT GTA ACC GGA GGA CA	Sequencing codon optimised E480	This project
ND_seqHAH7_F1	CAG GGG ATA CAA AAT GAA CAC	Sequencing full-length HA of H7	This project
ND_seqHAH7_F3_rep	AGT AAC TTG CCC TTT CAG A	Sequencing full-length HA of H7	This project
ND_seqHAH7_R4	GTG TTT ACT TTA GTC CCG TTT G	Sequencing full-length HA of H7	This project
Seq.Ohio_E195F1	GCC TGC AGA GAG GAT CTC G	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E195F2	CGC AAA TGC MGA CAC ATT AT	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E195F3	GAA RGA AGT TCT CGT GCT ATG G	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E195F4	GGC CAT TGC TGG YTT YAT T	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E195F5	GAT GGG GTA AAG YTG GAA TCA A	Sequencing s-o H1N1 and A(H1N1)pdm09	This project

Seq.Ohio_E1 95R1	GTG TCC TTG GGT TGA CCA GA	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E1 95R2	TTY TTC ACA TTT GAG TCG TGG	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E1 95R3	TAG CAC CMT TGG GTG TCT G	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E1 95R4	ACA CTG AGC TCA AYT GCT CTC	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Seq.Ohio_E1 95R5	GCT AAG GGA GAG CTC GAT CC	Sequencing s-o H1N1 and A(H1N1)pdm09	This project
Bm-PB2-1	TAT TGG TCT CAG GGA GCR AAA GCA GGT C	WGS H7 gene segment 1, PB2	Hoffmann(Hoffmann et al. 2001)
HPB2R421	GGC CAA AGG TTC CAT GTT TTA ACC	WGS H7 gene segment 1, PB2	RD(Baillie et al. 2012)
HPB2H7F72 4	GGG ACC TGC TGG GAA CAA ATG	WGS H7 gene segment 1, PB2	This project
HPB2R960	GCA TAT RTC CAC AGC TTG TTC TTC	WGS H7 gene segment 1, PB2	RD(Baillie et al. 2012)
HPB2R1459	TCC TCT CAT TGA CAT CTC	WGS H7 gene segment 1, PB2	RD(Baillie et al. 2012)
PB2-1105F	TAY GAR GAR TT CAC AAT GGT	WGS H7 gene segment 1, PB2	RD(Baillie et al. 2012)
HPB2F1441	GAG ATG TCA ATG AGA GGA	WGS H7 gene segment 1, PB2	RD(Baillie et al. 2012)
HPB2H7F19 07	CTT GTG AGA GGC AAC TCC CC	WGS H7 gene segment 1, PB2	This project
Bm-PB2- 2341R	ATA TGG TCT CGT ATT AGT AGA AAC AAG GTC GTT T	WGS H7 gene segment 1, PB2	Hoffmann(Hoffmann et al. 2001)
Bm-PB1-1	TAT TGG TCT CAG GGA GCR AAA GCA GGC A	WGS H7 gene segment 2, PB1	Hoffmann(Hoffmann et al. 2001)
HPB1F370	CAA ACA AGG GTG GAC AAA CT	WGS H7 gene segment 2, PB1	RD(Baillie et al. 2012)
HPB1R389	AGT TTG TCC ACC CTT GTT TG	WGS H7 gene segment 2, PB1	RD(Baillie et al. 2012)
PB1-1123F	CAR ATA CCN GCA GAR ATG CT	WGS H7 gene segment 2, PB1	RD(Baillie et al. 2012)
PB1-1261R	TTR AAC ATG CCC ATC ATC AT	WGS H7 gene segment 2, PB1	RD(Baillie et al. 2012)
HPB1H7R16 98	CGG TAC GTG TAC CTA TAA TCC	WGS H7 gene segment 2, PB1	This project
HPB1F1711	AGA GGW GAC ACA CAA ATW CA	WGS H7 gene segment 2, PB1	RD(Baillie et al. 2012)
Bm-PB1- 2341R	ATA TGG TCT CGT ATT AGT AGA AAC AAG GCA TTT	WGS H7 gene segment 2, PB1	Hoffmann(Hoffmann et al. 2001)
Bm-PA-1	TAT TGG TCT CAG GGA GCR AAA GCA GGT AC	WGS H7 gene segment 3, PA	Hoffmann(Hoffmann et al. 2001)
HPAR620	TCT TCG CCT CTT TCG GAC TGA CGA	WGS H7 gene segment 3, PA	RD(Baillie et al. 2012)

PA-745F	TRC ATT GAG GGC AAG CTK TC	WGS H7 gene segment 3, PA	RD(Baillie et al. 2012)
HPAH7R984	CGA TGT TGG GTT CTT TCC AAC C	WGS H7 gene segment 3, PA	This project
HPAF1435	AAT GCA TCC TGT GCA GCA ATG GA	WGS H7 gene segment 3, PA	RD(Baillie et al. 2012)
PA-1498R	TNG TYC TRC AYT TGC TTA TCA T	WGS H7 gene segment 3, PA	RD(Baillie et al. 2012)
HPAR1824	GGA CTC RGC TTC AAT CAT GCT CTC	WGS H7 gene segment 3, PA	RD(Baillie et al. 2012)
Bm-PA- 2233R	ATA TGG TCT CGT ATT AGT AGA AAC AAG GTA CTT	WGS H7 gene segment 3, PA	Hoffmann(Hoffmann et al. 2001)
Bm-HA-1 or HAFUc	TAT TCG TCT CAG GGA GCA AAA GCA GGG G	WGS H7 gene segment 4, HA	Hoffmann(Hoffmann et al. 2001)
ND_seqHAH 7_F2	CTA TGC AGA GAT GAA ATG G	WGS H7 gene segment 4, HA	This project
ND_seqHAH 7_F4	GCT GAR GAA GAT GGC ACT G	WGS H7 gene segment 4, HA	This project
ND_seqHAH 7_R1	GAT GCC CCG AAG CTA AAC CA	WGS H7 gene segment 4, HA	This project
ND_seqHAH 7_R2	GRG TGC TTT TGT AAT CTG C	WGS H7 gene segment 4, HA	This project
ND_seqHAH 7_R3_rep	CAC TCC CGT ATA GCT TGG TCT G	WGS H7 gene segment 4, HA	This project
Bm-NS-890R or HARUc or NSRUc	ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TT	WGS H7 gene segment 4, HA and 8, NS	Hoffmann(Hoffmann et al. 2001)
NPFUc	TAT TCG TCT CAG GGA GCA AAA GCA GGG TWR ATA ATC	WGS H7 gene segment 5, NP	RD(Baillie et al. 2012)
HNP7R594	CCA TCA CCA TTG TCC CGA CTC	WGS H7 gene segment 5, NP	This project
NPUR664-83	CGY CCR TTY TCR CCY CTC CA	WGS H7 gene segment 5, NP	RD(Baillie et al. 2012)
HNP7F101 7	GGT ATG GAT GGC ATG CCA TTC	WGS H7 gene segment 5, NP	This project
HNP7R110 9	GCA ATT TGA ACT CCT CTG GTG G	WGS H7 gene segment 5, NP	This project
NPRUc	ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT AWT TTT	WGS H7 gene segment 5, NP	RD(Baillie et al. 2012)
Ba-NA-1 or NAFUc	TAT TGG TCT CAG GGA GCA AAA GCA GGA GT	WGS H7 gene segment 6, NA	Hoffmann(Hoffmann et al. 2001)
H7NAF615_s eq	CCA TTA AAT CCA TGG AGA AAG G	WGS H7 gene segment 6, N3	This project
H7NAR664_ seq	GGG TAC CAT CAA TGC ATT GAC	WGS H7 gene segment 6, N3	This project
H7NAF1105 _seq	GTC ACA GAG GGG TGG ATT AAC	WGS H7 gene segment 6, N3	This project
H7NAR1145 _seq	CAC TAA TGT TTG TGT AAC TG	WGS H7 gene segment 6, N3	This project

Ba-NA1413R or NARUc	ATA TGG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT	WGS H7 gene segment 6, NA	Hoffmann(Hoffmann et al. 2001)
MFUc	TAT TCG TCT CAG GGA GCA AAA GCA GGT AGA	WGS H7 gene segment 7, M	Hoffmann(Hoffmann et al. 2001)
HMH7R366	CGA CCT CCT TAG CTC CAT GG	WGS H7 gene segment 7, M	This project
HMF581	GCT AAG GCT ATG GAG CAA ATG GCT	WGS H7 gene segment 7, M	RD(Baillie et al. 2012)
Bm-M-1027R or MRUc	ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT	WGS H7 gene segment 7, M	Hoffmann(Hoffmann et al. 2001)
Bm-NS-1	TAT TCG TCT CAG GGA GCA AAA GCA GGG TG	WGS H7 gene segment 8, NS	Hoffmann(Hoffmann et al. 2001)
HNSH7F355	GCC TTA TGG TGA AAA TGG ACC	WGS H7 gene segment 8, NS	This project
HNSH7R474	TAT TTC GGC CAC AAT AGC ACC	WGS H7 gene segment 8, NS	This project

Table 2.4 Oligonucleotides used in this project

WGS = Whole genome sequencing, s-o = swine-origin

Wobbles used: R (A+G); W (A+T); M (A+C); Y (C+T); K (G+T); N (A+C+G+T)

2.1.5 Plasmid vectors

Name	Comments	Source
pAc3CFc His Sfil	Empty baculoviral expression vector expressing His-tag and human Fc-tag	Ian Jones, University of Reading
pAc3CFc His Sfil GFP	Baculoviral expression vector containing GFP gene	Ian Jones, University of Reading
pAc3CFc His Sfil E195 HA	Baculoviral expression vector containing E195 HA gene	This project
pAc3CFc His Sfil E195 E227A HA	Baculoviral expression vector containing E195 E227A HA gene	This project
pAc3CFc His Sfil E480 HA	Baculoviral expression vector containing E480 HA gene, HA is codon-optimised for use in insect cells	This project
pAc3CFc His Sfil M105 HA	Baculoviral expression vector containing M105 HA gene	This project
pAc3CFc His Sfil Ohio01 A227E HA	Baculoviral expression vector containing Ohio01 A227E HA gene	This project
pAc3CFc His Sfil Ohio01 HA	Baculoviral expression vector containing Ohio01 HA gene	This project
pCMV-VictoriaPB2	Helper plasmid for reverse genetics containing segment 1 of A/Victoria/3/75	Dr. T Zurcher, GlaxoSmithKline
pCMV-VictoriaPB1	Helper plasmid for reverse genetics containing segment 2 of A/Victoria/3/75	Dr. T Zurcher, GlaxoSmithKline
pCMV-VictoriaPA	Helper plasmid for reverse genetics containing segment 3 of A/Victoria/3/75	Dr. T Zurcher, GlaxoSmithKline
pCMV-VictoriaNP	Helper plasmid for reverse genetics containing segment 4 of A/Victoria/3/75	Dr. T Zurcher, GlaxoSmithKline
pPoll	Reverse genetics plasmid with the human Poll promoter site and sequences from the hepatitis delta virus ribozyme	Dr. T Zurcher, GlaxoSmithKline
pPoll-E195PB2	pPoll vector containing segment 1, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195PB1	pPoll vector containing segment 2, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195PA	pPoll vector containing segment 3, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195HA	pPoll vector containing segment 4, cloned from E195	Dr. Lorian Hartgroves, Imperial College and this project

pPoll-E195NP	pPoll vector containing segment 5, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195NA	pPoll vector containing segment 6, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195M	pPoll vector containing segment 7, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195NS	pPoll vector containing segment 8, cloned from E195	Dr. Lorian Hartgroves, Imperial College
pPoll-E195 E227A HA	pPoll vector containing segment 4 cloned from E195 mutated at position 722 (A→C, H1 numbering)	This project
pPoll-Ohio01 A227E HA	pPoll vector containing segment 4 cloned from Ohio01 mutated at position 722 (C→A, H1 numbering)	This project
pPoll-Ohio01 HA	pPoll vector containing segment 4 cloned from Ohio01	This project

Table 2.5 Plasmids used in this project

2.1.6 Antibodies and Lectins

Name	Purpose	Source
<i>Primary antibodies</i>		
Anti-HIS tag HRP	Western Blot of recombinant HA	Invitrogen Ltd
Goat anti-human IgG Fc-specific	Immunohistochemistry with recombinant HA on HAE slides	Sigma Aldrich Ltd
Goat anti-human IgG Fc-specific HRP	Western Blot of recombinant HA	Sigma Aldrich Ltd
Mouse anti-influenza A NP	Immunohistochemistry against NP protein	AbD Serotec
Mouse anti-influenza A NP	Immunohistochemistry against NP protein	Dhan Samuel, HPA MS
Rabbit anti-alpha tubulin (polyclonal)	Immunohistochemistry against tubulin	AMS Biotechnology
Rabbit anti-alpha tubulin (polyclonal)	Immunohistochemistry against tubulin	Abcam
<i>Secondary antibodies</i>		
Donkey anti-goat Alexa 488	Immunohistochemistry on HAE (HA protein)	Invitrogen Ltd
Donkey anti-rabbit Alexa 647	Immunohistochemistry on HAE (HA protein and infection)	Invitrogen Ltd
Goat anti-mouse Alexa 488	Immunohistochemistry on HAE (infection)	AbD Serotec
Streptavidin Alexa 488	Immunohistochemistry with lectins	Invitrogen Ltd
Streptavidin Alexa 594	Immunohistochemistry with lectins	Invitrogen Ltd
Streptavidin HRP	Immunohistochemistry with lectins	Life Technologies Inc.
<i>Lectins</i>		
MAA biotin	Detection of α 2,3-linked sialic acid	TCS Bioscience
SNA biotin	Detection of α 2,6-linked sialic acid	Vector Laboratories Ltd

Table 2.6 Antibodies used in this project

2.1.7 Bacterial and cell culture

2.1.7.1 Competent cells

The competent cells (*Escherichia coli* (*E.coli*)) used in this study were TOP10 Oneshot TM cells (Invitrogen) and XL10 Gold ultracompetent cells (Stratagene).

2.1.7.2 Luria Broth (LB) medium

1% Oxoid tryptone; 0.5% Oxoid yeast extract; 100 mM NaCl; 5.5 mM glucose; 100 µg/ml ampicillin.

2.1.7.3 SOC recovery medium

2% Oxoid tryptone; 0.5% Oxoid yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄·7H₂O; 20 mM glucose.

2.1.7.4 NZY+ broth

1% NZ amine (casein hydrolysate); 0.5% Oxoid yeast extract; 100 mM NaCl; 12.5 mM MgCl₂; 12.5 mM MgSO₄; 20 mM glucose.

2.1.7.5 MDCK cell medium

Imperial College: Dulbecco's Modified Eagles Media (DMEM, Invitrogen) supplemented with 10% foetal calf serum (FBS, Biosera); 1% Non-essential amino acids (NEAA, Gibco) 50 U/ml Penicillin (Invitrogen); 50 µg/ml Streptomycin (Invitrogen).

Health Protection Agency: DMEM (Gibco) supplemented with 10% FBS (Invitrogen); 50 µg/ml gentamycin (Invitrogen).

2.1.7.6 SIAT cell medium

DMEM supplemented with 10% FBS; 50 µg/ml gentamycin and 0.4 mg/ml geneticin (Gibco).

2.1.7.7 Spodoptera frugiperda cell medium

SF900 II medium (Gibco) supplemented with 2% FBS; 2.5 µg/ml Fungizone (Invitrogen); 100 U/ml Penicillin (Invitrogen); 100 µg/ml Streptomycin (Invitrogen).

2.1.7.8 IOBA-NHC cell medium

DMEM/F12 (Invitrogen) supplemented with 10% FBS; 50 U/ml Penicillin; 50 µg/ml Streptomycin; 2.5 µg/ml Fungizone; 2 ng/ml human epidermal growth factor (Sigma); 1 µg/ml bovine pancreas insulin (Sigma); 0.1 µg/ml cholera toxin (Sigma); 5 µg/ml hydrocortisone (Sigma).

2.1.7.9 293T cell medium

DMEM supplemented with 10% FBS (Biosera); 1% NEAA; 50 U/ml Penicillin; 50 µg/ml Streptomycin.

2.1.7.10 Plaque assay overlay

1x Earle's minimal essential medium (Invitrogen) supplemented with 0.2% w/v Bovine Serum Albumin (BSA, Invitrogen); 1 % v/v L-glutamine (Invitrogen); 0.15% v/v NaHCO₃ (Invitrogen); 10mM HEPES (Invitrogen); 0.005% w/v filtered DEAE Dextran (Sigma); 50 U/ml Penicillin; 50 µg/ml Streptomycin; 0.6% number 3 agarose (Oxoid); 1.6 µg/ml trypsin (Worthingtons).

2.2 Methods

2.2.1 Cell lines

2.2.1.1 MDCK cells

MDCK cells were cultured at 37°C in 5% CO₂ in DMEM supplemented with 10% FBS, 1% NEAA and 50 µg/ml gentamycin or DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin.

2.2.1.2 SIAT cells

SIAT cells were cultured at 37°C in 5% CO₂ in DMEM medium supplemented with 10% FBS, 1% NEAA, 50 µg/ml gentamycin and 0.4 mg/ml geneticin to maintain stable expression of the α 2,6-sialyltransferase gene (Matrosovich et al. 2003).

2.2.1.3 *Spodoptera frugiperda* cells

Spodoptera frugiperda (SF9) insect cells (Invitrogen) were cultured in suspension at 27°C in SF900 II medium supplemented with 2% FBS, 2.5 µg/ml Fungizone, 100 U/ml penicillin and 100 µg/ml streptomycin at a maximum volume of 30 ml in a 250 ml Corning Erlenmeyer flask (Sigma). SF9 cells were diluted every 3-4 days, ensuring cell density did not exceed 10⁷ cells/ml.

2.2.1.4 IOBA-NHC cells

IOBA-normal human conjunctival tissue (IOBA-NHC) cells, a kind gift from Dr. Virginia Calder (UCL), were cultured at 37°C in 5% CO₂ in DMEM/F12 medium supplemented with 1 µg/ml bovine pancreas insulin, 2 ng/ml human EGF, 0.1 µg/ml cholera toxin, 5 µg/ml hydrocortisone, 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml Fungizone.

2.2.1.5 293T cells

293T cells were cultured at 37°C in 5% CO₂ in DMEM supplemented with 10% FBS (Biosera), 1% NEAA, 50 U/ml Penicillin and 50 µg/ml Streptomycin.

2.2.1.6 HAE cultures

HAE cultures from Epithelix Sàrl were delivered in medium containing agar. Upon arrival, inserts were placed in 24-well plates containing 700 µl of pre-warmed (37°C) MucilAir™ medium (Epithelix Sàrl). Only the eight wells in the middle of the plate were used, all other wells were filled with 1 ml nuclease free water (Severn Biotech) to maintain a high humidity. HAE cultures were incubated at 37°C in 5% CO₂. Medium was replaced every 2-3 days.

HAE cultures from Cystic Fibrosis/Pulmonary Research and Treatment Center, Department of Microbiology and Immunology, UNC at Chapel Hill were grown in custom made media with provision of an air-liquid interface for 4 to 6 weeks to form differentiated, polarized cultures that resemble in vivo pseudostratified mucociliary epithelium (Fulcher et al. 2005).

2.2.2 Viral methods

2.2.2.1 *Viral cell passage*

A confluent layer of MDCK or SIAT cells in a T25 flask (Nunc) was washed twice with sterile PBS (Gibco) after which 5 µl of virus in 1 ml serum-free DMEM was added. The flasks were rocked regularly while incubating at 37°C in 5% CO₂ for 1 hr. After removal of the inoculum 5 ml of serum-free MDCK or SIAT medium (See section 2.1.7) containing 1 µg/ml trypsin (Worthington) was added. The cells were incubated until cytopathic effect (CPE) was observed, after which the medium was removed and centrifuged at 5000 rpm for 5 mins to remove cell debris. Before storage at -80°C, viral titre was determined using HA assay and/or plaque assay.

2.2.2.2 *Viral egg passage*

10 days-old embryonated eggs were inoculated allantoically with 100 µl of serially diluted influenza virus (10^{-4} to 10^{-7}) and incubated at 37°C for 48 hrs. Eggs were then placed at 4°C for 4 hrs. Allantoic fluid was collected and centrifuged at 2000 rpm at 4°C for 10 mins to remove debris. Before storage at -80°C, viral titre was determined by HA assay and/or plaque assay.

2.2.2.3 *Haemagglutination (HA) assay*

80 µl PBS was added to the first column of a 96-well plate, 50 µl PBS was added to the wells in columns 2-12. 20 µl of virus-containing fluid was added to the first column in duplicate, and

serially diluted 1:1 up to column 12, leaving 50 µl in every well. PBS was used as a negative control. 50 µl 0.5% red blood cells (RBC) was added, using guinea pig, chicken or turkey RBC dependent upon the virus subtype. After incubation at 4°C for 1 hr, HA titre was determined as the highest dilution that gave full agglutination.

2.2.2.4 Plaque assay

Preparation of lung tissue for plaque assay was done by weighing the tissue and homogenising 10% w/v in serum-free DMEM medium in a 50 ml falcon tube. Cell homogenate was centrifuged at 500 rpm for 5 min to remove cell debris. Six or 12-well plates with confluent layers of either MDCK or SIAT cells were washed twice with sterile PBS, after which six dilutions of 100 µl of serially diluted virus or cell supernatant (between 10^{-1} – 10^{-8}) in virus transport medium (VTM) or serum-free medium in duplicate were added. The plates were rocked regularly while incubating at 37°C for 1 hr. After removal of the inoculum, 3 ml DMEM containing 1 µg/ml trypsin and 1% agarose (MP Biomedicals) (HPA) or plaque overlay medium (IC, section 2.1.7.10) was added. After agarose was set, plates were incubated at 37°C in 5% CO₂ for three days. Cells were fixed with 2 ml/well 5% gluteraldehyde solution (Sigma) for at least 2 hrs, followed by staining with 2 ml of 5% carbol fuchsin (Fluka) for 30 mins (HPA) or stained with crystal violet (IC, Sigma). To determine viral titre, the following formula was used:

$$P \times \frac{1000}{V} \times \frac{1}{D}$$

Equation 2.1 Formula to determine viral titre from plaque assay

P = plaque forming units per well

V = volume of virus added per well in µl (e.g. 100)

D = Dilution of virus (e.g. 10⁻⁶)

2.2.2.5 Determining the 50% Tissue Culture Infectious Dose (TCID₅₀)

MDCK cells were seeded in 96-well plate (90% confluent) and washed with sterile PBS. One plate per virus was used. 100 µl serum-free medium was added to column 2-12. 145.4 µl diluted virus (dilution of 2½ log) was added to column 1, of which 45.4 µl was added to column 2, up to column 11 (serial dilution of ½ log). 45.4 µl was removed from column 11. Column 12 was used as negative control. After 1 hr of incubation at 37°C in 5% CO₂, cells were washed with PBS. 150 µl of serum-free medium containing 1 µg/ml trypsin was added to each well and plates were incubated at 37°C in 5% CO₂ for four days. After aspiration of media, the cells were stained with crystal violet and TCID₅₀ was calculated using the Spearman-Kärber formula as explained in

Figure 2.1:

$$Xp + d(0.5 - 0.5 \left(\frac{r}{n}\right))$$

Equation 2.2 Spearman-Kärber formula to calculate TCID₅₀

Xp = Highest dilution with CPE in all samples

d = Spacing between dilutions (e.g. 0.5)

r = Wells with CPE between Xp and lowest dilution with no CPE in all samples

n = number of wells per dilution (e.g. 8)

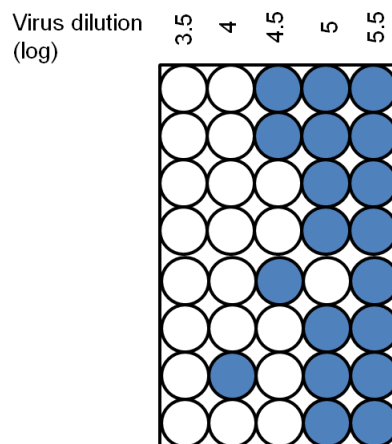


Figure 2.1 Calculation of TCID₅₀ value

Value for Xp = -3.5; value for d = 0.5; value for r = 21; value for n = 8. TCID₅₀ value is therefore

$$-3.5 + 0.5(0.5 - 0.5 \left(\frac{21}{8}\right)) = -3.09375$$

2.2.2.6 Rescue of recombinant influenza virus by 12 plasmid transfection

293T cells were seeded in 6-well plates overnight to achieve up to 70-80% confluency. 2 hrs before transfection, media was replaced with 0.5 ml DMEM with 3% FCS. Twelve reverse genetics plasmids (eight pPolI plasmids containing the required viral gene segments, and four helper pCMV expression plasmids encoding the polymerase and nucleoprotein of A/Victoria/3/75) were aliquoted into eppendorf tubes, each at 0.5 µg apart from pCMV-NP which was aliquoted at 1 µg. 200 µl serum-free DMEM was combined with 20 µl Fugene (Roche) and incubated at RT for 5 min. DNA was added to the medium/Fugene mixture and incubated for 15 min at RT. DNA/medium/Fugene mixture was then added drop wise to the 293T cells. Transfected 293T cells were incubated overnight at 37°C in 5% CO₂. The next day, one confluent T75 flask of MDCK cells was resuspended in 25 ml DMEM with 10% FCS. One ml of MDCK cell suspension was added to each well of transfected 293T cells and gently pipetted up and down. The cell mixture was transferred to a T25 flask together with a further 4 ml of MDCK cell suspension. The co-culture was incubated at 37°C in 5% CO₂ until the cells were attached (6-8 hrs), washed and incubated with 5 ml of fresh serum-free MDCK medium containing 2 µg/ml trypsin, 50 U/ml penicillin and 50 µg/ml streptomycin. When CPE was observed, the medium was harvested and tested for presence of virus by standard plaque assay on a confluent monolayer of MDCK cells (see section 2.2.2.4).

2.2.2.7 Purification of viral stocks by plaque picking

Using a sterile aspirator tip, isolated plaques were extracted from plaque plates and incubated in sterile PBS containing 0.35% BSA, 50 U/ml penicillin and 50 µg/ml streptomycin at 4°C overnight. T75 flasks were seeded with MDCK cells (90% confluent) and washed with sterile PBS. Three ml of serum-free medium was mixed with 100 µl of virus diluent, added to MDCK cells and incubated for 1 hr at 37°C in 5% CO₂. Inoculum was then replaced with 12 ml serum-free MDCK cell medium with 1 µg/ml trypsin and incubated at 37°C in 5% CO₂ until CPE was observed. When CPE was observed, the medium was harvested and tested for presence of virus by standard plaque assay on a confluent monolayer of MDCK cells (see section 2.2.2.4).

2.2.2.8 Infection and study of viral replication in MDCK and SIAT cells

MDCK or SIAT cells were seeded on 6-well plates or 24-well plates with a coverslip (90-100% confluent) and washed with sterile PBS. Virus inoculum was applied to cells. After 1 hr incubation at 37°C in 5% CO₂, cells were washed with PBS. Serum-free cell-specific medium with 1 µg/ml trypsin was added to each well and plates were incubated at 37°C in 5% CO₂ for the duration of the experiment. Viral growth kinetics were determined by taking and replacing 500 µl of serum-free medium. Samples were stored at -80°C prior to analysis.

2.2.2.9 Infection and study of viral replication in HAE cultures

HAE were washed with sterile PBS or MucilAir medium to remove apical secretions. Virus inoculum was applied to the apical surface of HAE. After 1 hr of incubation at 33°C or 37°C in 5% CO₂, inoculum was removed and HAE cultures were washed with PBS or MucilAir medium and incubated at 33°C or 37°C in 5% CO₂ for the duration of the experiment. Viral growth kinetics were determined by performing apical washes with 300 µl of serum-free DMEM for 30 min at 33°C or 37°C in 5% CO₂. Washes were then harvested and stored at -80°C prior to analysis.

2.2.2.10 Infection and study of viral replication in IOBA-NHC cells

IOBA-NHC cells were seeded on 6-well plates (90-100% confluent) and washed with sterile PBS. Virus inoculum was applied to IOBA-NHC. After 1 hr of incubation at 37°C in 5% CO₂, cells were washed with PBS. Three ml of serum-free IOBA-NHC medium with 1 µg/ml trypsin was added to each well and plates were incubated at 37°C in 5% CO₂ for the duration of the experiment. Viral growth kinetics were determined by taking and replacing 500 µl of serum-free medium. Samples were stored at -80°C prior to analysis.

2.2.2.11 Microneutralisation assay for influenza A virus on MDCK cells

MDCK cells were seeded in 96-well plate (90% confluent) and washed with sterile PBS. Four rows of wells per virus were used. Viruses were diluted to 100TCID₅₀ (see section 2.2.2.5). 60 µl of sterile PBS was added to columns 2-12 of an empty 96-well plate. Serum raised against E195 in ferrets was diluted 0-320x and 120 µl was added to all wells in column 1. 60 µl of serum from column 1 and serially diluted 1:1 up to column 12, leaving 60 µl in every well. Then 60 µl of diluted virus was added to each well and plates were incubated at 37°C in 5% CO₂ for 1 hr. Hereafter, 100 µl of virus-serum mixture was added to plates containing MDCK cells which were incubated at 37°C in 5% CO₂ for 1 hr. Inoculum was replaced with 100 µl of serum-free medium containing 1 µg/ml trypsin. Plates were incubated at 37°C in 5% CO₂ for four days and stained with crystal-violet. Wells containing PBS instead of serum were used as positive controls; wells containing no virus were used as negative controls. MN value is the highest serum dilution with no CPE in any wells (*Figure 2.2*).

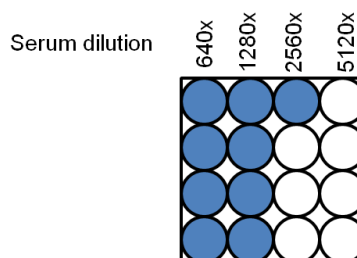


Figure 2.2 Calculation of MN value

The highest serum dilution with no CPE is 1280x (all wells still contain cells), one well with serum diluted 2560x has no CPE. The final MN value is 1280x.

2.2.3 Molecular techniques

2.2.3.1 *DNA synthesis by GeneArt*

As cloning of the HA of A/England/480/06 (and A/England/481/06) was not successful, this gene was codon-optimised for insect cells by GeneArt (Invitrogen). Hereby, the protein sequence of the gene stays the same, but the nucleotide sequence is optimised by for example eliminating AT- and GC-rich sequences and using codons that are most common in insect cells. Furthermore, as the virus A/Ohio/01/2007 was not available, this HA gene was also synthesised (but not codon-optimised) by GeneArt.

2.2.3.2 *Viral RNA extraction from cell supernatant*

RNA was extracted from 130 µl of cell supernatant using Qiagen Viral RNA mini kit (Qiagen) according to manufacturer's instructions except for lysis buffer, which was purchased from Roche (MagnaPure LC total nucleic acid kit).

2.2.3.3 *Viral RNA extraction from porcine lung tissue*

Porcine lung tissue was weighted and RNA was extracted using Qiagen RNeasy mini kit (Qiagen) according to manufacturer's instructions.

2.2.3.4 cDNA synthesis

10 µl of extracted viral RNA was incubated at 65°C for 1 min with 1 µl of 10 pmol forward primer (*Table 2.4*) and 1 µl of 25 mM dNTPs (Invitrogen). Then, 4 µl 5x first strand buffer (Invitrogen), 2 µl 0.1 M DMSO (Invitrogen) and 1 µl RNaseOUT (Invitrogen) were added, followed by incubation for 2 min at 42°C. Finally, 1 µl of Superscript reverse transcriptase (Invitrogen) was added and the mixture was incubated at 42°C for 50 min followed by 15 min at 72°C to inactivate the enzyme.

2.2.3.5 Polymerase Chain Reaction

All PCR reactions were optimised for MgCl₂ concentration and melting temperature. 2-10 µl of cDNA was added to 5 µl 10x buffer for KOD DNA polymerase (Novagen), 4 µl forward primer, 4 µl reverse primer (*Table 2.4*), 5 µl 2 mM dNTPs (Novagen), 1-5 µl of MgCl₂ (Novagen) and 0.4 µl KOD DNA polymerase (Novagen). Water was added to a final volume of 50 µl. Depending on the primer set, reaction times and temperatures varied.

2.2.3.6 Agarose gel electrophoresis

For visualisation of DNA, 2 µl of DNA was mixed with 2 µl BlueJuice (Invitrogen) and 18 µl nuclease free water. For purification of DNA, the entire PCR reaction volume was mixed 9:1 with BlueJuice. DNA fragments were separated on agarose gels in 1x TBE (Gibco). Concentration of agarose gels, running voltage and running time were dependent on the

expected size of the DNA band. DNA was visualised by incubating gels in an ethidium bromide-containing (Invitrogen) bath after electrophoresis and photographed using the Gel Doc 2000 system (BioRad).

2.2.3.7 DNA purification

DNA bands of interest were excised from agarose gels and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. DNA was measured using the Nanodrop (Thermo).

2.2.3.8 Restriction enzyme digestion

In general, 1 µg of DNA was digested with 1 U of restriction enzyme (New England Biolabs) in 1x recommended NEB buffer (New England Biolabs) in a final volume of 20 µl. If necessary, BSA (New England Biolabs) was added to a final concentration of 100 µg/ml.

2.2.3.9 Ligation into non-commercial plasmids

Insert and plasmid of interest were mixed in different concentrations (1:1, 1:3 and 1:10) and added to 1 µl of T4 ligase (Promega) and 1x T4 buffer (Promega). Nuclease free water was added to a final volume of 10 µl and ligation was performed overnight at 16°C. As a negative control, plasmid without insert was included.

2.2.3.10 Transformation into competent cells

TOP10 OneShot TM competent cells (Invitrogen) were thawed on ice after which 2 µl of DNA was added. Incubation on ice for 10 mins was followed by a heat shock for 30 mins at 42°C. 250 µl of SOC medium (Invitrogen) was added and the cell mixture was incubated at 37°C for 1 hr at 200 rpm. 10-100 µl was plated out using appropriate antibiotics (ampicillin (Sigma) 10 µg/µl, kanamycin (Sigma) 50 µg/µl or chloramphenicol (Sigma) 68 µg/µl) and incubated overnight at 37°C.

2.2.3.11 Plasmid isolation

Bacterial cultures were started from a glycerol stock or a single colony from a plate into LB medium containing selective antibiotics (ampicillin 10 µg/µl, kanamycin 50 µg/µl or chloramphenicol 68 µg/µl) and grown overnight at 37°C. Three ml of culture media was pelleted at 13,000 rpm for 5 min and plasmids were isolated using the Qiaprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. DNA was dissolved in nuclease free water and the concentration was measured using Nanodrop (Thermo).

2.2.3.12 Site-directed mutagenesis

Specific mutagenesis primers were designed using primerX (www.bioinformatics.org/primerx) and plasmids were mutated using QuickChange site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions.

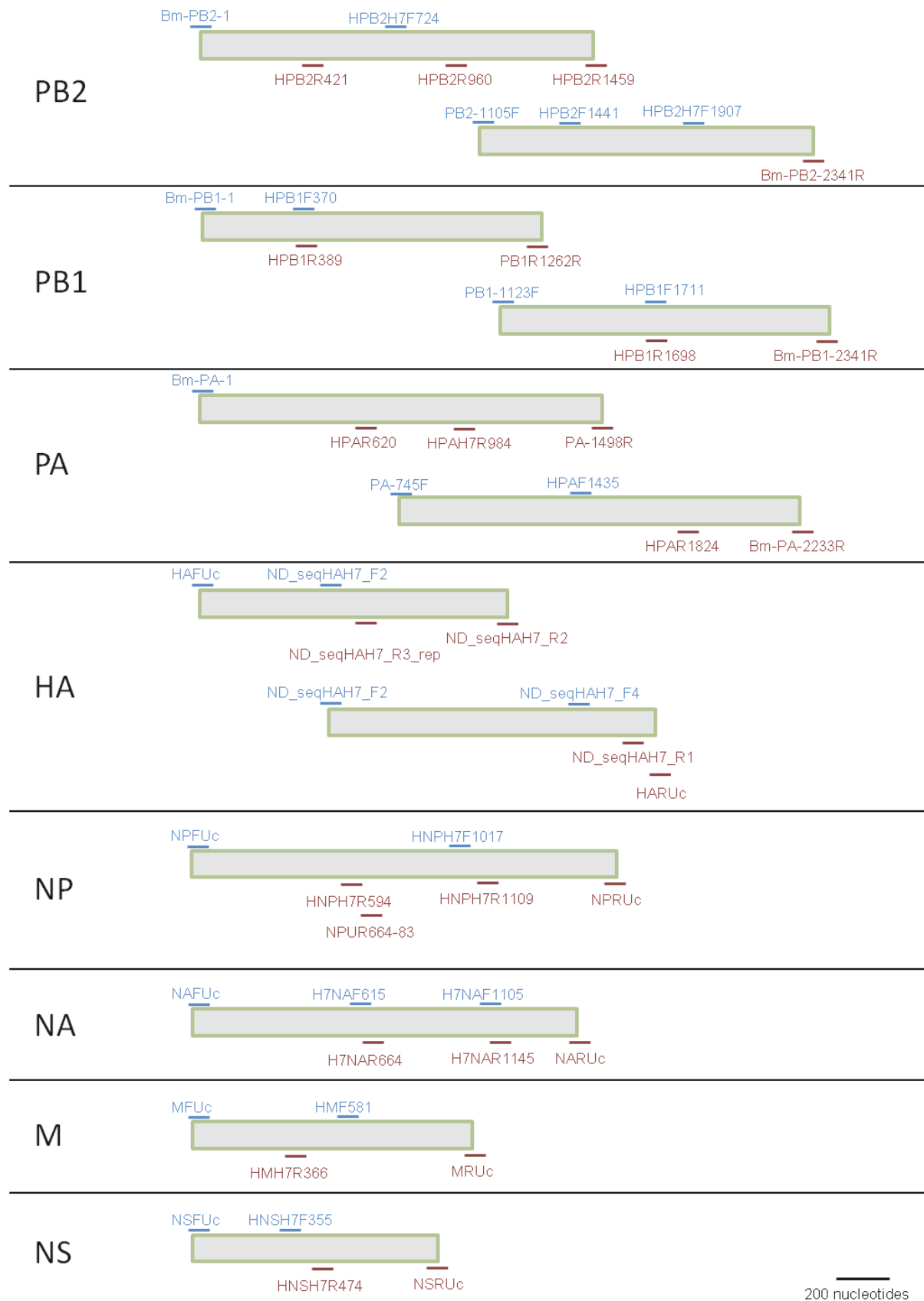


Figure 2.3 Schema of sequencing strategy of H7 influenza viruses

Every influenza virus RNA segment was amplified by polymerase chain reaction to produce one or two fragments depending on gene segment length. Hereafter, every fragment was sequenced using the primers illustrated in this figure. Scale of segments: 1 cm represents 200 nucleotides. Forward primers are in blue, reverse primers in red.

2.2.3.13 Quantitative real-time PCR

RNA was isolated as described in section 2.2.3.2. Concentration was measured by Nanodrop and standardized to 1 µg per reaction. Using the SuperScript III Reverse Transcriptase kit (Invitrogen), cDNA was amplified: 1 µl random hexamers, 1 µl 10mM dNTPs, 2.5 µl nuclease-free water and 8.5 µl RNA was heated at 65°C for 5 min, then incubated on ice for 1 min. To the RNA mixture 4 µl 5x First-strand buffer, 1 µl 0.1M DTT, 1 µl RNase OUT and 1 µl Superscript III was added and incubated at 50°C for 1 hr followed by enzyme deactivation at 70°C for 15 min. TaqMan Fast Universal PCR master mix was used for qRT-PCR on the ABIqPCR system using standard curve program. Two µl of cDNA was mixed with 0.5 µl primer MFUc (10 pmol), 0.5 µl primer MRUc (10 pmol), 2 µl M Probe, 6.8 µl nuclease-free water and 10 µl TaqMan 2x master mix. The standard curve was made using 10^9 - 10^0 copy numbers of pPoll-E195M plasmid.

2.2.3.14 Whole genome sequencing of H7 viruses

RNA was isolated as described in section 2.2.3.2. cDNA was produced according to section 0 and PCR was performed according to section 2.2.3.5 using primers specified in *Table 2.4*. PCR products were purified using in-house facilities (HPA) and DNA concentration was measured by Nanodrop. DNA was diluted to 30 ng/µl and primers to 10 pmol/µl and sent to GATC Biotech (<http://www.gatc-biotech.com>) for sequencing. Chromatographs were assembled using Seqman 9.0.5 software by DNASTAR Lasergene. Please see *Figure 2.3* for a schematic explanation of the alignment of sequence primers.

2.2.4 Statistics and Bioinformatics

2.2.4.1 *Statistical analyses*

All statistical analyses were performed using Microsoft Excel 2007 or GraphPad Prism 5. Student's T-test was performed on unpaired samples; One-way Anova was performed on multiple unpaired samples followed by Tukey post test to provide relevant p-values between samples. Samples were marked significantly different if the p-value was 0.05 or lower. Area under the curve was calculated using GraphPad Prism 5.

2.2.4.2 *Multiple protein alignments*

Amino acid sequences were compared using standard settings of Geneious 5.3.6 Blosum62 (Biomatters Limited) (Drummond AJ 2011).

2.2.4.3 *Phylogenetic analysis*

Phylogenetic trees were created using multiple alignment files of Geneious 5.3.6 using standard bootstrap settings.

2.2.4.4 *Modelling of HA protein of E195 and mutants*

The crystal structure of A(H1N1)pdm09 HA protein A/California/04/2009 (Protein Data Bank ID code 3LZG) was used as a template for homology modeling of HA of E195 using SwissProt (Bairoch et al. 2004) (http://web.expasy.org/docs/swiss-prot_guideline.html). Yasara force field

minimization server (Krieger et al. 2009) (<http://www.yasara.org/minimizationserver.htm>) was used to calculate energy minimization values. Molecular surfaces images were created using Autodock (Park, Lee and Lee 2006) (<http://autodock.scripps.edu/>) and coloured per amino acid.

2.2.5 Recombinant baculovirus techniques and protein production

2.2.5.1 *Bacmid preparation*

E.coli containing bacmid (a kind gift from Professor Ian Jones, University of Reading) was cultured overnight at 37°C in LB medium containing 50 µg/ml kanamycin and 68 µg/ml chloramphenicol. Bacmid DNA was isolated using the BacMAX Kit (Cambio Ltd) according to manufacturer's instructions. Bacmid DNA concentration was measured using the Nanodrop. To linearise bacmid DNA, 1 µg of bacmid was digested with 3 U of Bsu361 (New England Biolabs) in 1x NEBuffer 3 (New England Biolabs) and 100 ng/µl BSA (New England Biolabs) at 37°C for 2 hrs. An extra 3 U of Bsu361 was added followed by a further incubation at 37°C for 2 hrs. Bsu361 was heat inactivated by incubation at 80°C for 20 min. Linearised bacmid DNA was stored at -20°C.

2.2.5.2 *Construction of P₀ generation virus*

418 ng linearised bacmid was added to 418 ng transfer vector in a total volume of 12 µl. Lipofectin (Invitrogen) was mixed 2:1 with nuclease free water. Bacmid and lipofectin mix were mixed 1:1 and incubated at RT for 30 min. 5×10^5 SF9 cells per well were seeded in a 6-well plate (50% confluent). After 30 min, cells were washed twice with serum-free media. 1 ml serum-free media containing the lipid/DNA complexes (24 µl) was added to the cells and the sealed plate was incubated overnight at 27°C. Serum-free media was replaced with 2 ml normal

insect media (See section 2.1.7.7) and the sealed plate was incubated at 27°C for 3-7 days. As a positive control, transfer vector containing a gene construct for GFP was transfected simultaneously. Protein production was inspected microscopically; when >40% of the cells were GFP positive, cells were scraped from the well and centrifuged at 13,000 rpm for 2 min. Supernatant was aspirated and stored in aliquots at 4°C in the dark.

2.2.5.3 Construction of P_1 generation virus

15×10^6 SF9 cells were seeded in a T150 flat tissue culture flask. After 30 min, media was removed leaving 2 ml medium remaining. 0.5 ml P_0 generation virus was added. After incubation at 27°C for 1 hr inoculum was replaced with 10 ml insect media and cells were incubated for a further 3-5 days at 27°C. When the cells showed CPE, they were harvested and after centrifugation at 13,000 rpm for 2 min supernatant was stored at 4°C in the dark.

2.2.5.4 Small scale protein production

Whilst constructing P_1 generation virus, HA production by SF9 cells was tested on a small scale. 5×10^5 SF9 cells per well were seeded in a 6-well plate (50% confluent). After 30 min, cells were washed twice with SFM and 0.5 ml of P_0 generation virus was added. After 1 hr incubation at 27°C media containing virus was replaced with 2 ml normal insect media. The sealed plate was incubated at 27°C for 3-4 days, after which cells were harvested and centrifuged 2 min at 13,000 rpm. Supernatant was analysed by Western Blot (WB) (see section 2.2.5.6).

2.2.5.5 Large scale protein production

In a 500 ml Corning Erlenmeyer flask, 500 µl P₁ generation baculovirus was added to 6 x 10⁷ SF9 cells in a total volume of 5 ml. After 2 hrs of incubation at 27°C while shaking at 225 rpm, 45 ml insect media was added. The cells were incubated for 6-7 days at 27°C in suspension and pelleted by centrifugation at 4000 rpm for 10 min at 4°C. Supernatant was further concentrated by centrifuging at 3500 rpm for 1.5 hrs at 4°C using vivaspin columns (Sartorius).

2.2.5.6 Western Blot

10.2 µl protein was added to 1.6 µl reducing agent (Invitrogen) plus 4 µl loading buffer (Invitrogen) and incubated at 100°C in a water bath for 5 min. 15 µl of sample was added to a 10% Bis/Tris gel (Invitrogen) next to 5 µl NuPage Magic Marker (Invitrogen). Gels were run at 125 V for 80 min and transferred onto a protein membrane with the iBlot™ Gel Transfer system (Invitrogen). Using Ponceau S (Fluka), proteins on the membrane were localised. After blocking the membrane for 1 hr at RT with 10% milk (Marvel) in PBS containing 0.05% Tween, the first antibody at an appropriate dilution in 10% milk/PBST was added (*Table 2.6*) and incubated for 1 hr at RT. The membrane was then washed 5x, 3x for 10 sec and 2x for 5 min. Secondary antibody at an appropriate dilution in 10% milk/PBST (*Table 2.6*) was added and incubated for 1 hr at RT. After a washing step as above, the blot was developed using western blotting detection reagents (Amersham) according to the manufacturer's instructions.

2.2.6 Immunohistochemistry

2.2.6.1 *Lectin staining*

Cells (MDCK, SIAT or IOBA-NHC) were seeded on coverslips in a 24-well plate and left overnight at 37°C to adhere. After blocking the cells for 1 hr at RT with 3% BSA in PBS containing 0.05% Tween (PBST), the biotin-conjugated lectins SNA (Sambucus Nigra lectin, binds to α -2,6 sialic acid) (Vector Laboratories) or MAA (Maackia amurensis, binds to α -2,3 sialic acid) (TCS Bioscience) diluted 1:200 in PBST with 1% BSA were incubated with the cells for 1 hr at RT. Cells were washed 3x 5 min with PBST and incubated with streptavidin conjugated with Alexa 594 (Invitrogen) 1:1000 in PBST with 1% BSA for 1 hr at RT. After repeating the wash step, the coverslips were mounted in 5 μ l mowiol (Invitrogen). Images were taken on the Nikon Eclipse Ti.

2.2.6.2 *Detection of infection of mammalian cells with influenza virus using immunofluorescence*

Cells (MDCK, SIAT or IOBA-NHC) were seeded on coverslips in a 24-well plate and left overnight at 37°C to adhere. After washing the cells twice with PBS, cells were infected with a low MOI (between 0.1 and 0.5) by incubating them with inoculum for 1 hr at RT (See section 2.2.2.8 and 2.2.2.10). After replacing the inoculum with serum-free cell-specific medium (See section 2.1.7), cells were incubated at 37°C for 16 hrs followed by fixation with ice-cold methanol:acetone 1:1. Cells were blocked for 1 hr at RT with 3% BSA in PBST, followed by

incubation with anti-NP antibody raised in mice 1:400 in PBST with 1% BSA for 1 hr at RT. To remove excess antibody, cells were washed 3x 5 min with PBST and incubated with anti-mouse antibody raised in goat conjugated with Alexa 488 (AbD Serotec) 1:500 in PBST with 1% BSA for 1 hr at RT. After repeating the wash step, the coverslips were mounted in 5 μ l mowiol. Images were taken on the Nikon Eclipse Ti.

2.2.6.3 Detection of infection of HAE cultures with influenza virus

HAE cultures were infected according to section 2.2.2.9 and fixed with 4% paraformaldehyde with 0.25M HEPES (Invitrogen) for 15 min at RT on both apical and basal surfaces. After a wash step (3x) with PBS containing 0.2% fish gelatin (Sigma), 0.02% sodium azide (Sigma) and 0.02% saponin (Sigma) (PGAS), cells were quenched and permeabilized using 50 mM NH_4Cl and 0.2% Saponin for 10 min at RT. Cells were then washed again 3x with PGAS and blocked for 1 hr at RT with PGAS, followed by incubation with anti-NP antibody raised in mice 1:400 and rabbit anti-tubulin 1:200 in PGAS 1 hr at RT. To remove excess antibody, cells were washed 3x with PGAS and incubated with anti-mouse antibody raised in goat conjugated with Alexa 488 (AbD Serotec) 1:500 and anti-rabbit antibody raised in goat in PGAS for 30 min at RT. After repeating the wash step, HAE cultures were cut out of the culture rings and mounted in 5 μ l mowiol with the apical surface facing upwards. Images were collected on the Zeiss Axiovert 200.

2.2.6.4 *Detection of infection of porcine lung tissue with influenza virus*

Lung tissue samples were fixed in 10% formalin saline for 24 hrs, embedded in wax and cut at 5 μ m. Tissue sections were deparaffinised by immersing the slides 2x5 min in xylene. By immersing the slides in 100% ethanol, 90% ethanol, 70% ethanol and placing under running tap water respectively the tissue sections were rehydrated. Further work was performed in a humidity chamber. After blocking for 1 hr at RT with 3% BSA in PBS, 200 μ l of anti-NP antibody raised in mice 1:400 in 3% BSA in PBS was incubated on the cells for 1 hr at RT. To remove excess antibody, cells were washed 3x with PBS and incubated with anti-mouse antibody raised in goat conjugated with Alexa 488 (AbD Serotec) 1:500. After repeating the wash step, lung tissue was mounted in 5 μ l mowiol. Images were collected on the Zeiss Axiovert.

2.2.6.5 *HA binding assay on HAE culture sections*

The concentrated HA proteins of interest were mixed with PBS to a final volume of 200 μ l (dilution depended on Western Blot result) and pre-complexed with 0.67 μ l anti-human antibody raised in goat (Sigma), 2 mg BSA and 5 μ l rabbit anti-acetylated anti-tubulin (AMS Biotechnology). This was mixed overnight at 4°C on a rotating wheel. Paraffin embedded sections of cultured human airway epithelium or normal human tracheal sections were obtained from Professor Ray Pickles at the University of North Carolina. Tissue sections were deparaffinised by immersing the slides 2x5 min in xylene. By immersing the slides in 100% ethanol, 90% ethanol, 70% ethanol and placing under running tap water respectively the tissue

sections were rehydrated. Further work was performed in a humidity chamber. After blocking for 1 hr at RT with 3% BSA in PBST, 200 µl of the HA mixture was incubated on the cells for 1 hr at RT. To remove excess antibody, slides were washed 3x5 min with PBST, followed by incubation with anti-goat antibody raised in donkey conjugated with Alexa 488 (Invitrogen) 1:1000 and donkey anti-rabbit Alexa 647 (Invitrogen) in PBST with 1% BSA. Cells were washed 3x5 min with PBST and viewed under a confocal microscope. Images were collected on a Zeiss Pascal LSM5 laser scanning microscope.

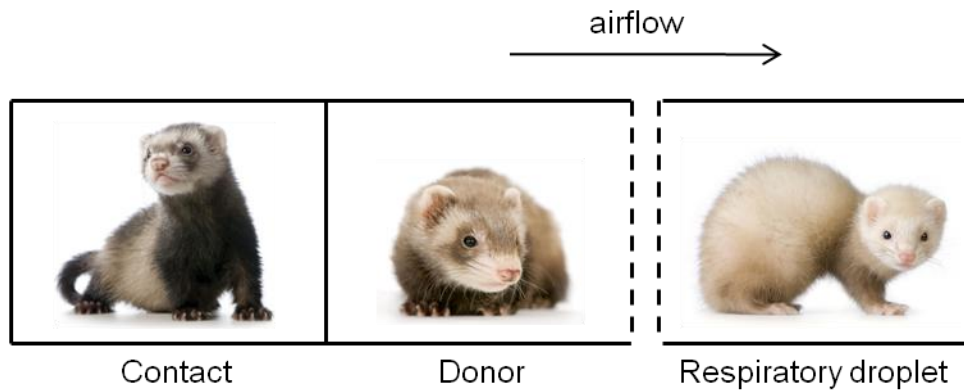


Figure 2.4 Cage setup for transmission experiments with ferrets

Donor animals were infected with influenza A virus. Twenty-four hrs later, sentinels were introduced; the respiratory droplet sentinel was separated from the donor animal by two perforated walls containing 5 mm holes which were 25 mm from each other, whereas the contact sentinel was placed in the same cage as the donor animal. Twenty-four hrs later, the contact sentinel was placed in an adjacent cage to ensure the respiratory droplet sentinel would not receive a double dose of influenza A virus.

2.2.7 Influenza virus transmission in the ferret model

2.2.7.1 Set-up ferret transmission experiments

Twenty to twenty-six week old female ferrets (*Mustela putorius furo*) weighing between 900–1250 g were used. After acclimatisation, sera were obtained and tested by MN assay (see section 0) for antibodies against E195. All ferrets were negative for influenza antibodies at the start of the experiments. Strict procedures were followed to prevent cross-contamination between animals during all procedures. Twenty-four hrs after inoculation of the donor ferrets, sentinels were introduced either as contacts or respiratory sentinels. Contact sentinels were placed in the same cage as donor animals for 24 hrs, and were then transferred to adjacent cages. Respiratory sentinels were placed in adjacent cages with a perforated separation. Airflow was monitored to create optimal conditions for transmission (*Figure 2.4*). When animals were handled or daily husbandry chores were performed, a strict order was used to ensure no cross contamination would occur; aerosol sentinels, then contact sentinels, then donor animals were treated. If influenza infection was suspected in an aerosol or contact sentinel, these animals would be treated last in their respective group (e.g. a suspected infected aerosol sentinel would be the last aerosol sentinel to be treated). Daily nasal washing was performed and donor ferrets were observed for 1 hr per day. At the end of the experiment, terminal blood samples were collected for seroconversion determination.

2.2.7.2 *Inoculation of ferrets with influenza A virus*

For inoculation, ferrets were lightly anaesthetised with ketamine (22 mg/kg) and xylazine (0.9 mg/kg) and inoculated intranasally with virus diluted in PBS, 0.1 ml per nostril.

2.2.7.3 *Nasal washing and weighing of ferrets*

All animals were weighed daily using a veterinary weigh scale and nasal washed consciously, by instilling 2 ml PBS into the nostrils. Expectorate was collected in modified 250 ml centrifuge tubes. BSA (0.3%), antibiotics (1%) and antifungal agent (1%) were added to nasal wash expectorate before viral titre was determined by standard plaque assay on MDCK cells monolayers (see section 2.2.2.4). The limit of virus detection in plaque assays was 10 PFU/ml.

2.2.7.4 *Observation donor animals*

During the first 12 days of the experiment, all donor animals were observed from 10am until 11am. The observer would enter the room at 9.45am and note temperature and humidity. After allowing the ferrets to settle down for 15 min, all coughs, sneezes and sneeze fits were reported during 1 hr. Furthermore, every 15 min the activity of every animal was scored; one point was awarded if the animal was asleep, two points if it was awake but not active and three points if it was active. Behaviour was also noted, e.g. sleeping, washing and eating. Finally, any remarkable observations were noted, e.g. difficulty breathing.

2.2.8 Ex-vivo porcine lung perfusion and infection methods

2.2.8.1 Setup ex-vivo porcine lung perfusion

Both lungs were extracted from a 5-month old pig by Dr. Alastair Proudfoot, Brompton hospital and transported on wet ice, after which the lungs were coupled to the EVLP system developed by the Department of Cardiothoracic Surgery at the University Hospital of Lund, Sweden (Steen et al. 2003). Cannulae were placed in the pulmonary artery and vein after which the lungs were perfused with high oncotic pressure perfusate called Steen solution (Steen et al. 2003) with or without red blood cells from porcine origin, which was circulated via a centrifugal pump. The trachea was attached to a ventilator protected by a filter which ventilates the porcine lungs. This closed system allows for ventilation of the lungs and was expected to maintain them for approximately 12-18 hrs. The lungs were kept in an unsealed dome (Xvivo Perfusion) to provide an environment with a high humidity. Physiological parameters are measured using specialized equipment installed within the circuit.

2.2.8.2 Infection of ex-vivo porcine lungs

Influenza virus was diluted in serum-free medium. Using a tube connected to a fibre-optic bronchoscope, lobes were sought out and (mock-)infected with 10 ml serum-free medium or 10 ml diluted virus per lobe segment. This was repeated for 3-5 lobe segments per lung. One lung

was infected with influenza virus; the other lung was used as a control. After infection, the bronchoscope was removed and cleaned with 70% ethanol.

2.2.8.3 *Sampling ex-vivo porcine lungs*

Tissue samples were taken at different time points from both the uninfected and infected whole porcine lung. The lungs were deflated by inactivating the ventilator. Samples were taken using biopsy equipment, which first staples the lung and then cuts the tissue on the outer side of the staple line. This ensures that the lung stays sealed and consequently no virus escapes. Tissue samples were then divided into different parts and either snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Frozen samples were stored at -80°C. Fixed samples were stored at RT. During the experiment, samples of 0.5 ml were taken of the perfusate via a tap installed in the reservoir. Part of the fixed lung tissue was embedded in wax and cut at 5 µm.

3 Cell tropism and in vitro replication of A(H1N1)pdm09 with altered HA genes

3.1 Introduction

The influenza A (H1N1) virus which caused the first pandemic of the 21st century (A(H1N1)pdm09) is known to originate from a swine source (Garten et al. 2009). The ability of swine influenza virus to transmit to humans was first demonstrated by Wentworth *et al.*, who reported on an accidental transmission of influenza from experimentally infected pigs to two out of four laboratory personnel (Wentworth et al. 1997). To investigate the likelihood of contracting a swine-origin influenza infection in people regularly exposed to swine, such as farmers, in comparison to non-swine exposed individuals, a 2-year prospective study looked at antibody levels against swine influenza in both groups. They reported an odds ratio of 54.9 of swine influenza infections in farmers, showing that farmers are much more likely to contract swine influenza at some point during their working life (Gray et al. 2007). Shinde *et al.* described the clinical features of eleven separate cases that were reported to the CDC between December 2005 and February 2009, in which all patients were infected with swine-origin influenza viruses, mostly after coming into close proximity with pigs (Shinde et al. 2009). The symptoms associated with infection by swine influenza were similar to those reported for human adapted strains including fever, cough and headaches. Similarly, Yassine *et al.* reported an outbreak of swine influenza at an Ohio county fair, where 26 people went on to develop mild respiratory

symptoms (Yassine et al. 2009). These studies show the ability of swine influenza viruses to infect humans, but also emphasize the necessity of close contact between pigs and humans, and suggest a poor ability of these viruses to transmit between humans. Indeed, no or only limited human-to-human transmission of swine influenza virus had been reported (Shinde et al. 2009) and until the recent pandemic with A(H1N1)pdm09 swine were not considered the most likely source of the next influenza pandemic. A recent ferret transmission study of a human isolate from the Ohio county outbreak, A/Ohio/02/2007 (Ohio02), showed that, in line with the observations in the above studies, efficient transmission was detected following direct contact between ferrets, but transmission by RD exposure did not occur (Belser et al. 2011a).

In an attempt to find differences in receptor binding preference which could explain the ability of A(H1N1)pdm09 to transmit between humans, glycan array studies comparing the carbohydrate specificity of the HA of swine-origin viruses involved in dead-end zoonoses with that of A(H1N1)pdm09 HAs were performed by several groups. An obvious ligand difference was not identified by these studies, which may be because these arrays do not completely represent the glycans expressed in the upper respiratory tract (Chen et al. 2011, Bradley et al. 2011, Childs et al. 2009). The exact configuration of the sialic acids bound by human and avian influenza viruses has not yet been fully defined and it is becoming apparent that the composition and configuration of the glycan chain, in addition to the terminal sialic acid linkage, may also be important for receptor specificity. Diverse preferences shown by different influenza viruses for modifications such as fucosylation, sulfation and sialylation linkage of the terminal trisaccharide

have been reported (Stevens et al. 2006a). Mass-spectrometry of the glycome of human bronchial epithelial cells revealed the presence of long α 2,6-linked sialic acids, which formed a so-called 'umbrella-like' glycan topology, in contrast with the 'cone-like' glycan topology formed by α 2,3-linked sialic acid and short α 2,6-linked sialic acid. These findings illustrate the extent of structural diversity amongst cell surface sugars. Both α 2,3-linked sialic acid and short α 2,6-linked sialic acid were bound by avian influenza HA, whereas the ability to bind to long α 2,6-linked sialic acid structures was specific for human influenza HAs (Chandrasekaran et al. 2008). Although these studies bring us closer to identifying the exact sialic acids used for influenza virus receptor binding, until these biological glycans can be studied on glycan arrays, conclusions derived from these experiments must be taken with precaution.

Due to the incomplete surveillance of influenza in swine in the decades before the start of the pandemic, it has not been possible to identify the exact origin of A(H1N1)pdm09 (Smith et al. 2009b, Vijaykrishna et al. 2011) and it is therefore challenging to identify which specific mutations allowed A(H1N1)pdm09 to transmit efficiently enough between humans to start a pandemic. Interestingly the HA of the classical swine H1N1 viruses from which A(H1N1)pdm09 originates already contained the human-like amino acids 190D and 225D (which were associated with the start of the 1918 H1N1 pandemic) but the classical swine H1N1 viruses did not transmit between humans. We hypothesise therefore that other, yet unknown, amino acid changes unique to the HA of the A(H1N1)pdm09 played a crucial role in the ability of the virus to

cause an influenza pandemic. In this chapter we aimed to identify such mutation(s) and their effect on receptor binding and growth within relevant cell cultures.

3.2 Results

3.2.1 Evolution of residue 219 and 227 in the receptor binding protein HA of H1N1 influenza A virus

To investigate the molecular determinants of the ability of A(H1N1)pdm09 to transmit between humans (Maines et al. 2009, Munster et al. 2009, Itoh et al. 2009), compared to earlier swine-origin zoonotic viruses that failed to cause human-to-human transmission (Shinde et al. 2009, Yassine et al. 2009), 173 full-length HA sequences of H1N1 swine influenza viruses isolated before January 2009 in North America were compared to a randomly selected panel of 1397 sequences from human former seasonal H1N1 viruses, isolated between 1918 and 2008, and 1605 A(H1N1)pdm09 isolates, as well as five published HA sequences of H1 swine-origin dead-end zoonotic viruses (Shinde et al. 2009), all selected using the NCBI influenza database (Cypel et al. 2008) (Cypel et al. 2008) (Cypel et al. 2008) (Cypel et al. 2008) (Cypel et al. 2008) (Cypel et al. 2008). The majority of recent human seasonal H1 viruses and A(H1N1)pdm09, both of which transmit efficiently between humans, contain E at residue 227. In contrast, the majority (>95%) of HA sequences of North American classical swine influenza and zoonotic viruses investigated contained A at position 227 (*Table 3.1, Figure 3.1*).

Isolate name		Host	Human-to-human transmission	Residue at	
				219	227
A/SC/1/1918	Pandemic	Human	Y	A	A
A/England/654/07	Seasonal	Human	Y	K	E
A/England/26/08	Seasonal	Human	Y	K	E
A/California/07/09	Pandemic	Human	Y	I	E
A/England/195/09	Pandemic	Human	Y	I	E
A/Ohio/01/07	Dead-end zoonose	Swine/Human	N	T	A
A/Wisconsin/87/05	Dead-end zoonose	Swine/Human	N	T	A
A/Swine/Alberta/56626/03	Classical swine	Swine	N	T	A
A/Swine/Ontario/23866/04	Classical swine	Swine	N	T	A

Table 3.1 Glutamic acid at position 227 is associated with human to human transmission.

Sequence comparison of amino acid 227 and 219 (H3 numbering) of HA protein of H1N1 viruses, showing prototypic examples of previous seasonal H1N1, A(H1N1)pdm09, classical swine influenza and dead-end swine zoonoses.

Residue 227 is found in the RBS of H1 HA (*Figure 3.2*), and substitution A227E at this position may have a potential role in changes in receptor binding specificity. However, the earliest H1N1 viruses, such as the 1918 virus, contained A at position 227 and yet were clearly transmissible between humans. This contradiction may be explained by the additional influence of amino acid 219 which is A in 1918 H1 but lysine (K) in the later seasonal human H1N1 viruses with 227E and is isoleucine (I) in the HA of A(H1N1)pdm09. Cooperation between residue 219 and 227 has been suggested to influence the orientation of residue 186, which forms an interaction network with residues 187 and 189. This network then influences the orientation of residue 190 (Maines et al. 2009), which plays a vital role in receptor binding preference of H1 HA (Glaser et al. 2005, Tumpey et al. 2007). Thus 1918 HA can tolerate the 227A and still bind efficiently to α 2,6-linked sialic acid because it has a compensatory A at position 219, whereas classical swine flu viruses have threonine (T) at this position and require mutation at 227 to accommodate the α 2,6-linked sialic acid.

Most swine H1N1 virus sequences examined contained A at position 227 (>95%). In contrast, three swine influenza viruses isolated in 2005 and 2007 contained E at position 227. However, phylogenetic analysis of the HA sequences of these viruses showed a clustering with swine influenza viruses from the δ -cluster (*Figure 3.3*). This cluster is associated with two separate introductions of human H1N2 and H1N1 into the swine population (Vincent et al. 2009, Karasin, Carman and Olsen 2006). Moreover, these viruses also contained the human-like amino acid lysine (K) at position 219 reinforcing the concept that they were human derived viruses and

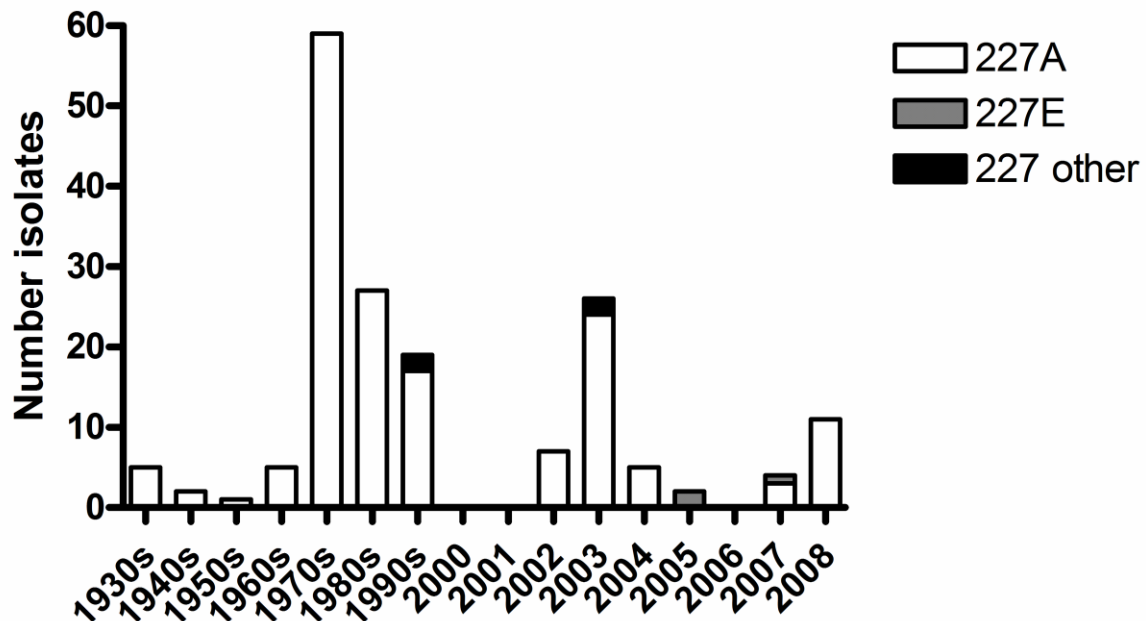


Figure 3.1 Distribution of amino acids at position 227 in North American swine H1 HA

Presence of alanine (A), glutamic acid (E) and other amino acids at position 227 in 173 full-length HA sequences of pre 2009 North-American swine H1 influenza. Sequences were downloaded from the NCBI influenza database on 16th March 2011 using the following search criteria: Type *A*; Host *Swine*; Region *North America*; Subtype *H1N1*; Collection Date *Before 01-01-2009*; Full-length only

not swine-origin influenza virus. This could explain why these viruses contain 227E. One HA derived from a swine-origin dead-end zoonosis, containing E at position 227, also clustered with the δ -cluster and is therefore hypothesized to represent a zoonotic infection (swine to human) of human-origin H1.

Interestingly, the HA of a typical A(H1N1)pdm09 isolate used in this study, A/England/195/2009, clustered with classic swine influenza, just like A/California/04/2009, another A(H1N1)pdm09 isolate, did (Garten et al. 2009), within the γ -cluster (*Figure 3.3*).

Analysis of sequences showed that in the 1980s residue 219 in classical swine H1N1 changed from A to T, potentially changing the interaction network around the receptor binding site and possibly the receptor binding preference (*Figure 3.4*). Interestingly, A(H1N1)pdm09 does not contain T at position 219, but I. This amino acid is only rarely found at this position in either human or swine H1 HA, and is thought to be suboptimal for receptor binding (Maines et al. 2009, de Vries et al. 2011). No human cases of influenza virus infection containing T at position 219 have been reported.

Finally, residue 219 and 227 in H1 influenza virus isolated from Eurasian pigs before 2009 was investigated. After collapsing identical sequences, 343 full-length HA sequences were found in the NCBI influenza database on 12th November 2012 (Type A; Host *Swine*; Region *Asia, Europe*; Subtype *H1N**; Collection Date *Before 01-01-2009*; Full-length only). The majority of sequences contained A at position 227 (261 sequences, 76%), but 58 sequences (17%) contained E at this position. Interestingly, most of the sequences containing 227E were found to

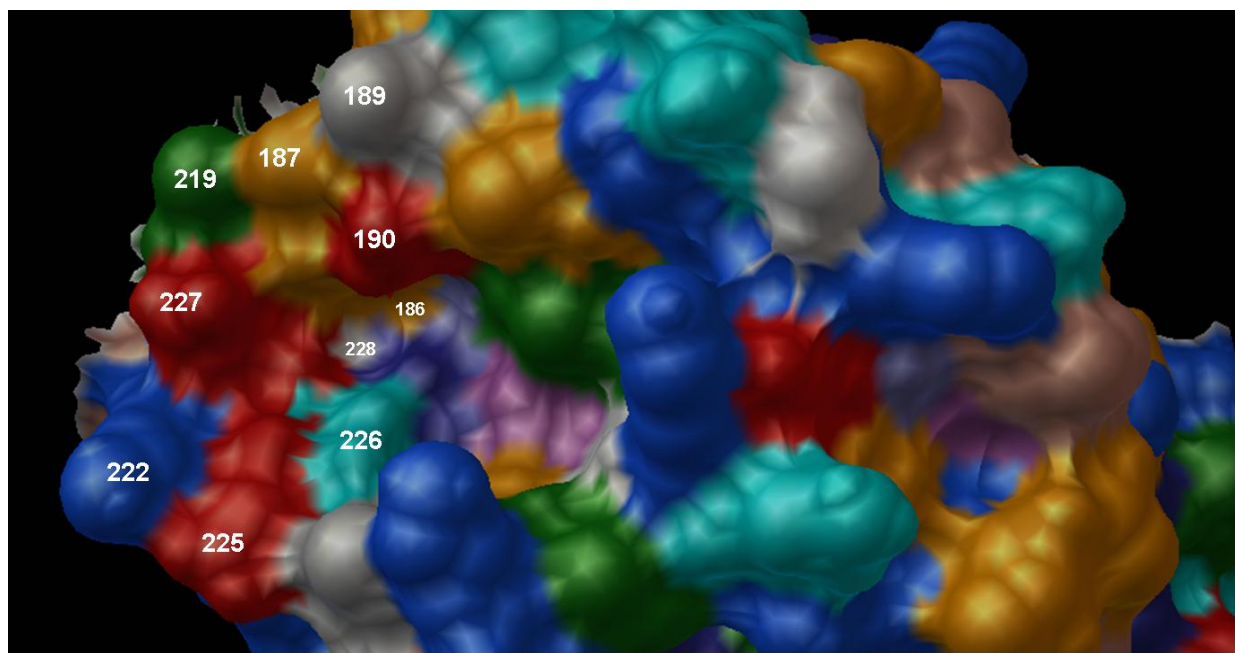


Figure 3.2 Molecular surface of HA protein of wild type E195

The crystal structure of A(H1N1)pdm09 HA protein A/California/04/2009 (Protein Data Bank ID code 3LZG) was used as a template for homology modelling of HA of E195. Yasara (<http://www.yasara.org/minimizationserver.htm>) was used to calculate energy minimization values. Molecular surfaces images were created using Autodock (<http://autodock.scripps.edu/>) and coloured per amino acid.

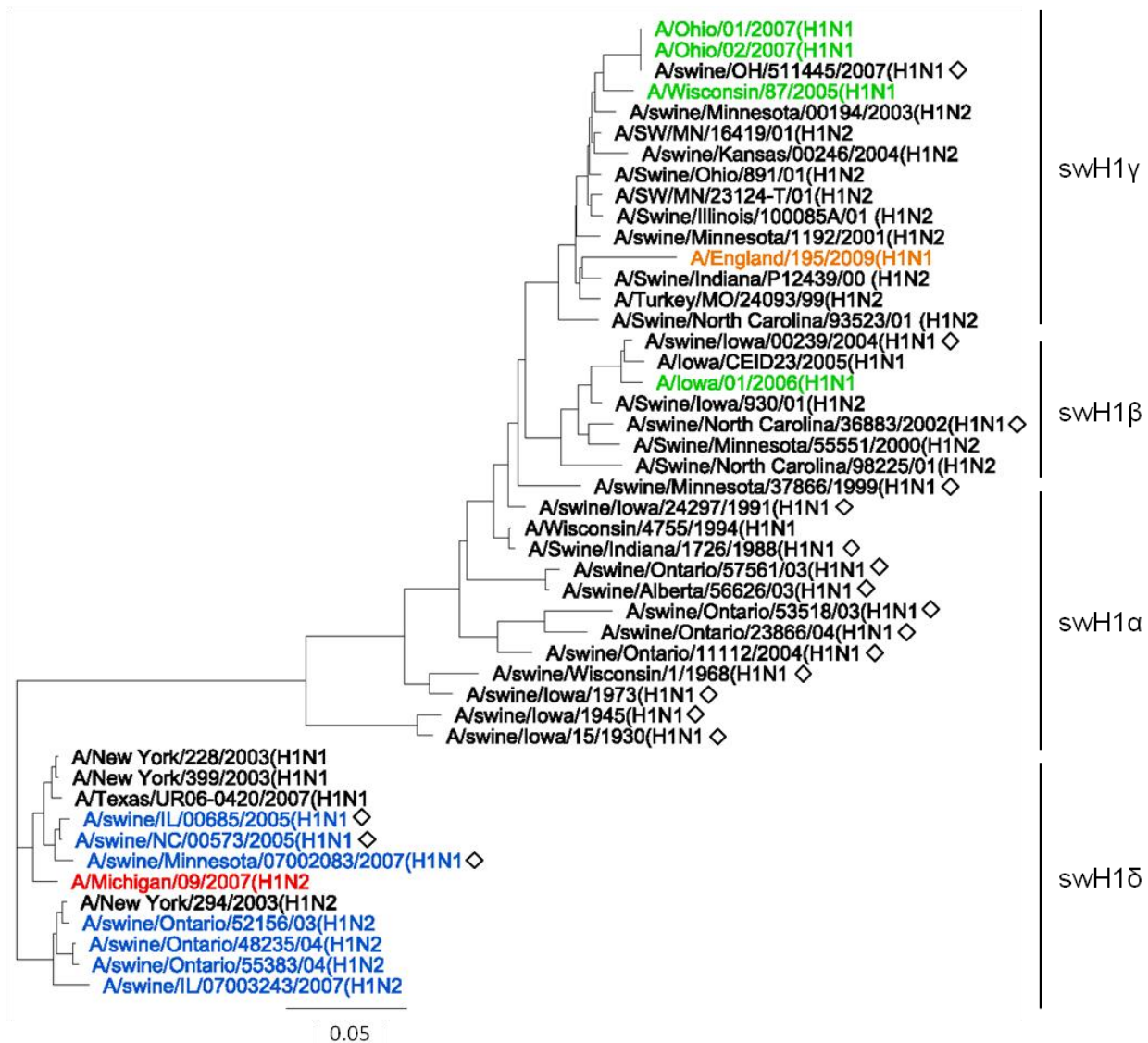


Figure 3.3 Phylogenetic tree of HA sequences based on nucleotide sequences of swine H1 influenza virus

Four clusters of related viruses as suggested by Vincent *et al.* are indicated by bars on the right side of the phylogenetic tree. HA sequences analysed were chosen from phylogenetic trees from Vincent *et al.* (Vincent *et al.* 2009) (black), and supplemented with zoonotic events from swine to human hosts containing amino acid 227A (green) or 227E (red), swine influenza virus containing amino acid 227E (blue) and A(H1N1)pdm09 (orange). Diamonds indicate viruses included in Figure 3.1. Scale indicates number of changes per site. Phylogenetic analysis was conducted in Geneious 5.4.5 (Drummond AJ 2011) and performed using standard settings.

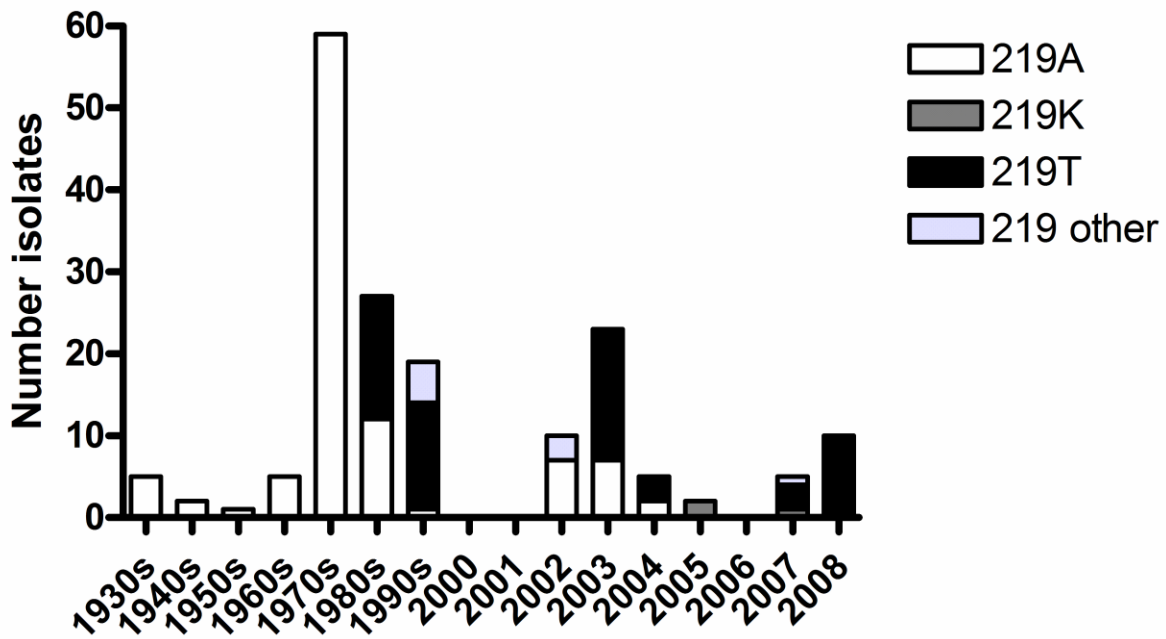


Figure 3.4 Distribution of amino acids at position 219 in North American swine H1 HA

Presence of A, E and other amino acids at position 219 in 173 full-length HA sequences of pre 2009 North-American swine H1 influenza. Sequences were downloaded from the NCBI influenza database on 16th March 2011 using the following search criteria: Type A; Host Swine; Region North America; Subtype H1N1; Collection Date Before 01-01-2009; Full-length only

contain 219K (40 sequences, 69%) and are hypothesised to be introduced into the swine population from a human source. Twenty sequences (6%) contained 227T. Both amino acids A and T were commonly found at position 219 (165 and 104 sequences respectively, 48% and 30% respectively). Amino acid I was not found at position 219.

In summary, these data indicate that the HA of A(H1N1)pdm09 is unique amongst swine influenza H1 HAs, combining a human virus like amino acid at residue 227 (E) with an unique I at residue 219 in the RBS.

Having identified residue 227 as a candidate substitution which may have played a key role in the ability of A(H1N1)pdm09 to transmit between humans, we then looked more closely at the evolution of residue 227 in human influenza H1N1 using both alignments as well as phylogenetic trees based on protein sequences of former seasonal H1N1 isolated in different decades. HA protein sequences containing at least residues 219 and 227 were selected from the NCBI influenza database (Cypel et al. 2008)(Cypel et al. 2008)(Cypel et al. 2008)(Cypel et al. 2008)(Cypel et al. 2008)(Cypel et al. 2008)(Cypel et al. 2008)and stratified per decade. Identical sequences were collapsed. Isolates from the Fort Dix outbreak (Gaydos et al. 1977a, Gaydos et al. 1977b, Hodder et al. 1977, Top and Russell 1977, Gaydos et al. 2006), swine-origin zoonotic infections and laboratory isolates were not included.

A different evolution of residue 227 in human influenza virus is observed compared to swine influenza isolates. Whereas in swine influenza viruses residue 227 is consistently A, human influenza viruses show changes in this residue from A in the period 1918-1970s, to E in the

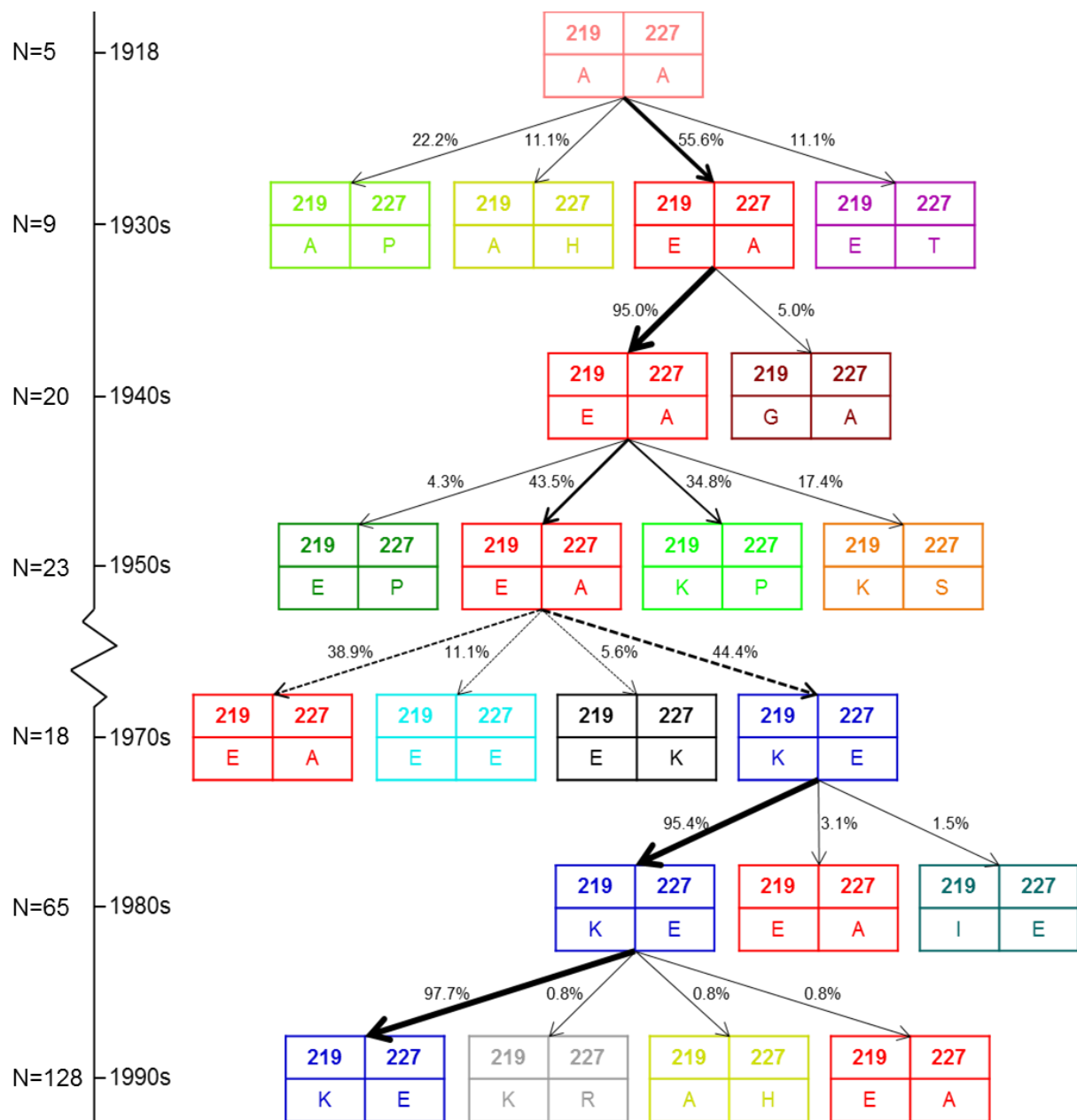


Figure 3.5 Proposal of genetic evolution of human H1N1

All subsets of human H1N1 influenza viruses detailed in *Table 3.2*, divided based on amino acids at position 219 and 227, are represented per decade. The most likely candidate hypothesized to be the progenitor for the next generation of virus is depicted by the start of the arrow. The thickness of the arrows and corresponding percentages show relative distribution of 219-227 amino acid profile. Dashed arrows depict the reintroduction of H1N1 in the 1970s. The N-number on the left-hand side indicates the number of sequences analysed per decade. Boxes are coloured based on amino acid at position 227; Red: 227A, Blue: 227E, Green: 227P, Yellow: 227H, Purple: 227T, Orange: 227S, Black: 227K, Grey: 227R. Sequences were downloaded from the NCBI influenza database on 4th July 2011 using the following search criteria: Type A; Host *Human*; Region *Worldwide*; Subtype *H1N1*; Collection Date *Before 01-01-2000*; Containing amino acids 219 and 227; Collapse identical sequences; Isolates from the Fort Dix outbreak, swine-origin zoonotic infections and laboratory isolates were excluded

period 1970s to date. This change seems to go hand in hand with changes in residue 219; in the 1970s all isolates with the K substitution at residue 219 also had the E substitution at residue 227. Interestingly, the combination of A at both position 219 and 227 is only found in 1918 isolates, and not in any later human isolates, suggesting this amino acid combination is not optimal for infection of the human host. Although other amino acids than A and E are found sporadically at position 227, these strains do not seem to perpetuate (summarized in *Table 3.2*). *Figure 3.5* is a hypothesized proposal of the evolutionary path H1N1 influenza has undertaken in humans since 1918. All isolates are divided by their amino acids at position 219 and 227. This figure shows that early in the 20th century, most human viruses contained 219E and 227A, which was slowly replaced by 219K variants. Influenza A (H1N1) then completely disappeared in 1957, with the emergence of the new H2N2 pandemic virus (reviewed in (Lagace-Wiens, Rubinstein and Gumel 2010)). The first viruses isolated after the reintroduction of H1N1 in 1977 contained 219E and 227A, but these residues rapidly changed to 219K and 227E. No human strains with 219T were found. These findings strengthen the hypothesis that residues 219 and 227 are interconnected, and that the combination of 219A and 227A or 219T and 227A found in swine, is not associated with human-to-human transmission.

Year	219; amino acid (%)	227; amino acid (%)	# of seq
1918	A (100)	A (100)	5
30s	E (66.7), A (33.3)	A (55.6), P (22.2), T (11.1), H (11.1)	9
30s 219A	A (100)	P (66.7), H (33.3)	3
30s 219E	E (100)	A (83.3), T (16.7)	6
40s	E (95), G (5)	A (100)	20
50s	K (52.2), E (47.8)	A (43.5), P (39.1), S (17.4)	23
50s 219E	E (100)	A (90.9), P (9.1)	11
50s 219K	K (100)	P (66.7), S (33.3)	12
70s	E (55.6), K (44.4)	E (55.6), A (38.9), K (5.6)	18
70s 219E	E (100)	A (70.0), E (20.0), K (10.0)	10
70s 219K	K (100)	E (100)	8
80s	K (95.4), E (3.1), I (1.5)	E (96.9), A (3.1)	65
90s	K (98.4), A (0.8), E (0.8)	E (97.7), A (0.8), H (0.8), R (0.8)	128

Table 3.2 Evolution of amino acid 219 and 227 in human H1N1

Summary of findings of multiple alignments of human H1N1, stratified per decade. For isolates from 1930s, 1950s and 1970s a second stratification is used to highlight the separation of amino acids at residue 227 in conjunction with the amino acid at position 219. Sequences were downloaded from the NCBI influenza database on 4th July 2011 using the following search criteria: Type A; Host *Human*; Region *Worldwide*; Subtype *H1N1*; Collection Date *Before 01-01-2000*; Containing amino acids 219 and 227; Collapse identical sequences; Isolates from the Fort Dix outbreak, swine-origin zoonotic infections and lab isolates were excluded.

Recombinant HA protein	Viral Origin	Mutation	Tag
E195	A/England/195/09	-	Human-Fc, His
E195 E227A	A/England/195/09	E227A	Human-Fc, His
Ohio01	A/Ohio/01/07	-	Human-Fc, His
Ohio01 A227E	A/Ohio/01/07	A227E	Human-Fc, His

Table 3.3 Recombinant HA proteins produced as part of this study

Virus	Origin backbone	Origin HA gene	Mutation in HA gene (H3 numbering)
E195	A/England/195/09	A/England/195/09	-
E195 E227A	A/England/195/09	A/England/195/09	E227A
Ohio01	A/England/195/09	A/Ohio/01/07	-
Ohio01 A227E	A/England/195/09	A/Ohio/01/07	A227E

Table 3.4 Recombinant influenza viruses produced by reverse genetics using the genetic backbone based on A/England/195/2009 as part of this study.

3.2.2 Binding of recombinant H1 HA proteins on HAE and human trachea

The initial site of human infection by influenza virus is the respiratory tract. The human respiratory tract is lined with a ciliated epithelium, interspersed with non-ciliated goblet cells that secrete mucus. The combination of these two cell types enables the respiratory tract to create a mucus layer which is able to trap inhaled particles and subsequently excrete them. It is important to consider the different roles of cells in the human airway epithelium during infection to better understand influenza disease. Human airway epithelial (HAE) cell cultures are highly differentiated epithelial cultures grown in an air-liquid interface made from human primary cells which can be sourced from the nasal cavity, trachea or bronchus. HAE cultures are capable of producing mucus and contain beating cilia, tight junctions and several different cells typical of the upper respiratory tract; basal cells, goblet cells and ciliated cells. These cultures are thought to be the best available *in vitro* model of the human respiratory tract, as they represent differentiated cells normally found in the respiratory tract and contain naturally occurring glycans, in contrast to the previously discussed glycan arrays.

Published work using the HAE culture model found that α 2,3-linked sialic acid was found exclusively on ciliated cells and consequently avian viruses infected these cells predominantly. In contrast, human transmissible influenza viruses displayed a preference for non-ciliated cells which expressed α 2,6-linked sialic acid (Ayora-Talavera et al. 2009, Shelton et al. 2011, Thompson et al. 2006, Matrosovich et al. 2007, Matrosovich et al. 2004a). In our laboratory

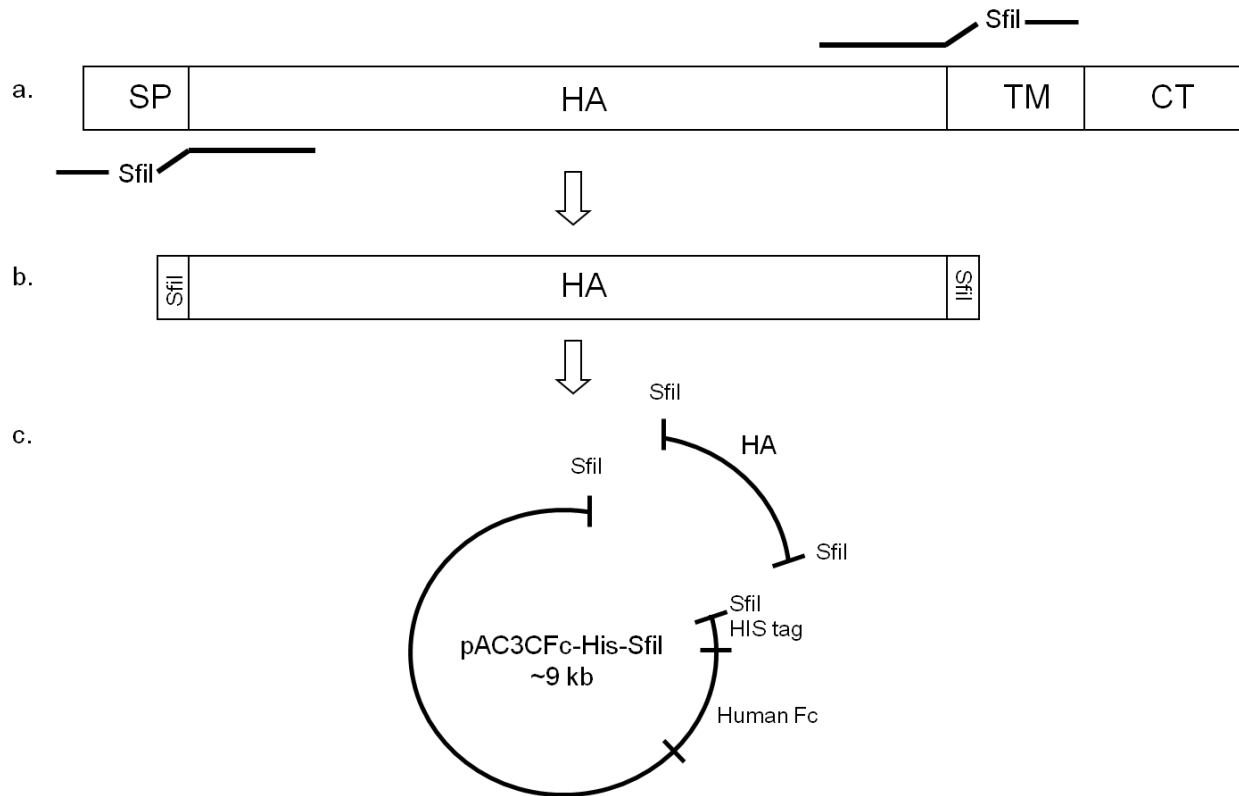


Figure 3.6 Cloning scheme of HA protein for baculoviral expression

Expression of recombinant HA proteins was accomplished by cloning the HA gene excluding the signal peptide (SP), transmembrane domain (TM) and cytoplasmic tail (CT) a), and introducing SfiI restriction sites on either side of the partial HA sequence b). The resulting product was then ligated into the pAc3CFc His SfiI vector c).

Ayora-Talavera *et al.* developed a method to investigate the binding of recombinant HA proteins to HAE sections (as described in Materials and Methods) (Ayora-Talavera *et al.* 2009, Shelton *et al.* 2011). We then compared HA binding to influenza infection, and found the binding of recombinant HA proteins to different cell types in the HAE cultures was representative of the tropism displayed by influenza virus encoding the same HA (Shelton *et al.* 2011).

In order to investigate the effect of the specific amino acid at position 227 in HA on cell tropism of influenza virus, HA proteins of influenza viruses of interest were expressed in SF9 insect cells. First, cDNA of the HA protein sequences were amplified using primers containing the Sfi1 restriction site, excluding the transmembrane domain and cytoplasmic tail. The PCR product was then cloned into the correct reading frame of the pAc3CFc His Sfi1 expression vector using the Sfi1 restriction sites. This vector also contains a human Fc tag for dimerisation of the protein, a 6x His tag for detection and a baculovirus gp67 signal peptide to force secretion of the protein (See *Figure 3.6* for cloning scheme). The E227A mutation at residue 227 could then be introduced using the QuickChange site-directed mutagenesis kit (Stratagene). Co-transfection of this vector with bacmid DNA into SF9 insect cells resulted in expression of soluble recombinant HA protein. Detection of binding was facilitated using the human Fc tag (Shelton *et al.* 2011). A/England/195/2009 (E195, Genbank accession number GQ166661) was chosen as a prototypic A(H1N1)pdm09 virus and a single residue variant, E227A, was created in addition to the parental sequence.

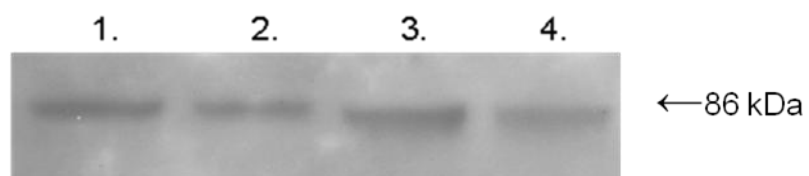


Figure 3.7 Western blot of recombinant HA proteins expressed in SF9 cells

Protein levels of 1) E195, 2) E195 E227A, 3) Ohio01, 4) Ohio01 A227E. Proteins were run on a 10% Bis/Tris gel and stained with anti-human Fc.

The HA of a swine-origin dead-end zoonosis virus A/Ohio/01/2007 (Ohio01, Genbank accession number FJ986620) was expressed similarly. Ohio01 is a swine influenza virus that infected a 36-year old male but did not transmit between humans. Ohio02, which was shown by Belser *et al.* not to transmit via RD in the ferret model (Belser *et al.* 2011a), is a virus isolated from the patient's daughter. It is believed that both of these transmission events originate from the same source and do not represent a case of human-to-human transmission. This is evident by an identical HA sequence as well as the same date of onset of symptoms (Shinde *et al.* 2009). Both Ohio01 and Ohio02 contain internal gene segments from the triple-reassortment internal gene (TRIG) constellation, which predominates in North-American pigs (Olsen 2002, Vincent *et al.* 2008), combined with the H1 HA from classical swine influenza which contains A at position 227 and T at position 219. Again, a single residue variant, Ohio01 A227E, which was a recombinant HA protein mutated to contain the human-like E at position 227 was expressed. A list of all recombinant HA proteins produced can be found in *Table 3.3*. Protein production was confirmed with Western Blot against the human-Fc tag and all four HA proteins were found to be expressed at similar levels and of a similar molecular weight (*Figure 3.7*).

These different recombinant H1 HA proteins were used to investigate whether the cell type in HAE cultures that the HA protein preferentially bound to varied according to the amino acid at position 227, which would give an indication of whether the 227E substitution in A(H1N1)pdm09 was responsible for any change in the cell tropism linked to the outbreak. An equal amount of

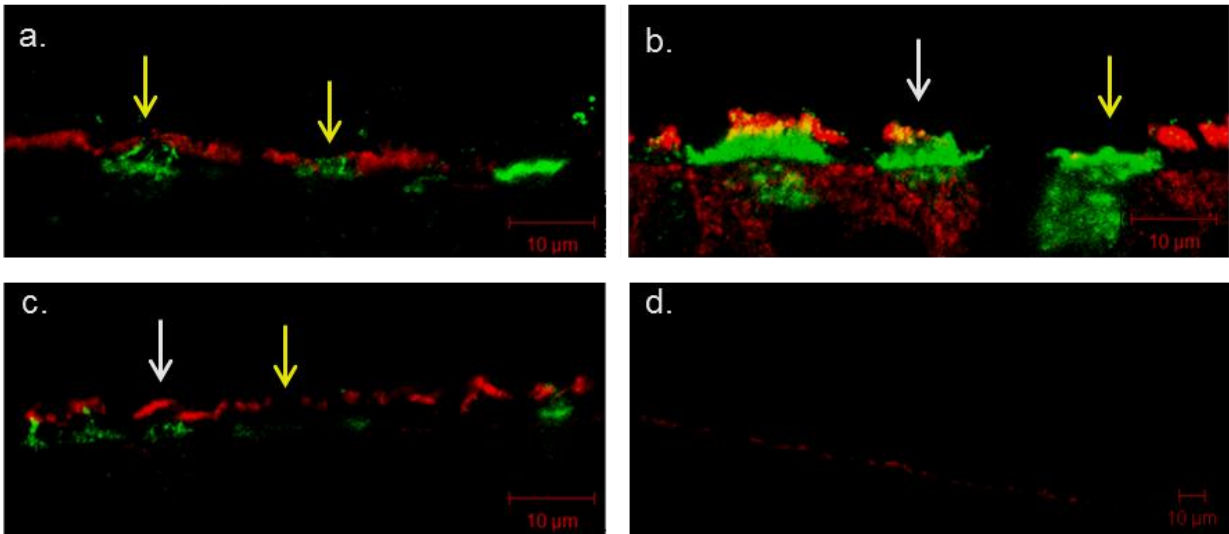


Figure 3.8 Binding of different recombinant H1 HA proteins to HAE sections

HAE culture sections were probed with HA-Fc recombinant proteins from human or swine origin influenza virus. Ciliated cells were identified using anti-acetylated α -tubulin (red) and HA-Fc proteins were identified using anti-human Fc antibody (green). Images are representative of multiple probed sections. Both ciliated (white arrow) and non-ciliated (yellow arrow) cells were found in HAE sections. a) E195; b) E195 E227A; c) Ohio01; d) Ohio01 A227E

HA protein was incubated on fixed sections of HAE cultures for all three HA proteins. Dilutions were based on the strength of WB bands. The HA proteins with lowest concentration (E195 E227A and Ohio A227E) were diluted 2-fold and dilutions of E195 HA and Ohio HA were prepared such that a similar HA protein concentration would be achieved (4-fold dilution). In preliminary experiments, further dilution of HA protein provided no significant difference in HA binding profiles (data not shown). Ciliated cells in the formalin fixed HAE sections were identified using anti-acetylated α -tubulin antibody (red) and HA protein binding to specific cells was detected by anti-human Fc antibody (green) as an Fc-tag was present at the C-terminus of all the HA proteins (*Figure 3.8*). HA protein of wild type E195 showed a preference for non-ciliated cells, 79.7% of the bound cells were non-ciliated (*Figure 3.8a*). In total HA protein of E195 bound to 29.5% of available cells. In comparison to E195 HA, the HA protein of wild type Ohio01 bound to a higher proportion of ciliated cells (61.3% of cells bound were non-ciliated) (*Figure 3.8c*) and a lower percentage of total cells (16.3%). The single mutation at residue 227 in E195 from E to A doubled the percentage of ciliated cells bound, reducing the proportion of non-ciliated cells bound from 79.7% (E195) to 55.0% (E195 E227A) (*Figure 3.8b*) but at the same time increased the percentage of total cells bound to 45.7%. In fact, the number of non-ciliated cells bound by HA protein of E195 and E195 E227A was very similar (47 and 44 cells respectively) but the number of ciliated cells bound by E195 E227A HA protein was tripled compared to the number of ciliated cells bound by E195 HA protein (36 and 12 cells respectively). Surprisingly, mutation of residue 227 in Ohio01 from A to human-like

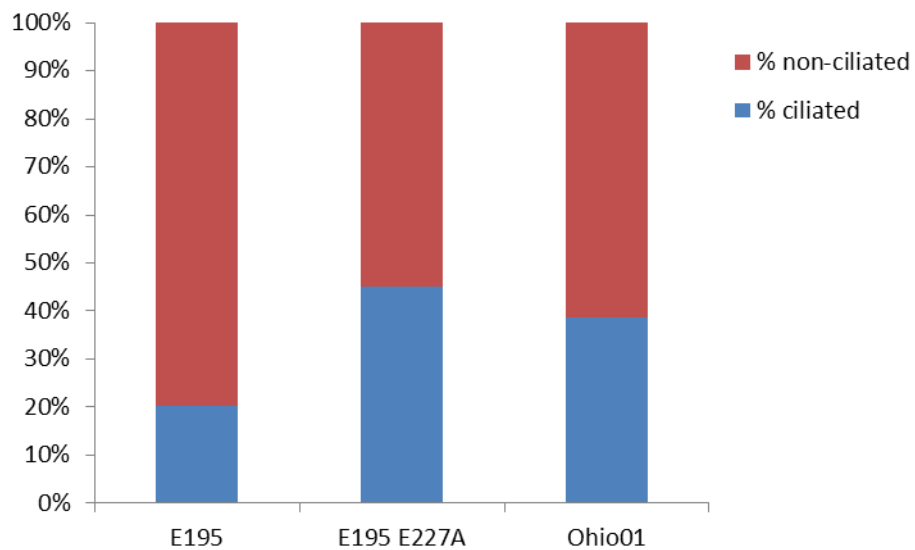


Figure 3.9 Quantification of recombinant HA protein binding to HAE sections

The percentages of non-ciliated and ciliated cells was quantified by blind counting >200 cells per sample.

signature E completely abrogated binding to HAE sections (*Figure 3.8d*) even though the western blot showed that the protein was produced and was stable. Although the sequence of the HA gene in the baculovirus transfer vector was verified it is not possible to exclude mutations within the eventual P₀ or P₁ baculovirus constructs.

Quantification of the binding profiles was obtained by blind counting 5-10 different fields containing around 200 cells in total for each HA (*Figure 3.9*). Using these quantifications, the relative ratio of non-ciliated/ciliated cells bound by the recombinant proteins was calculated, which was higher for E195 HA (3.9) than for E195 E227A HA (1.2) which was similar to Ohio01 HA (1.6) (*Table 3.5*).

Using the same recombinant HA protein binding assay, the receptor binding of all four HA proteins on formalin fixed *ex vivo* sections of human trachea was investigated. Ciliated cells were identified using anti-acetylated α -tubulin antibody (red) and cells bound by HA protein were detected by anti-human Fc antibody (green). Again, HA protein of wild type E195 showed a preference for non-ciliated cells, 79.8% of the bound cells were non-ciliated (*Figure 3.10a*). HA protein of Ohio01 showed more binding to ciliated cells and less to non-ciliated cells (66.2% of cells bound were non-ciliated) (*Figure 3.10c*), although the difference was less pronounced than in HAE cultures (*Figure 3.8c*). The single mutation at residue 227 in E195 from E to A reduced the proportion of non-ciliated cells bound to 71.1% and again increased the total number of cells bound, although not as dramatically as in HAE culture (*Figure 3.10b*). In fact, the percentage of bound cells of all HA proteins was similar on human trachea; E195

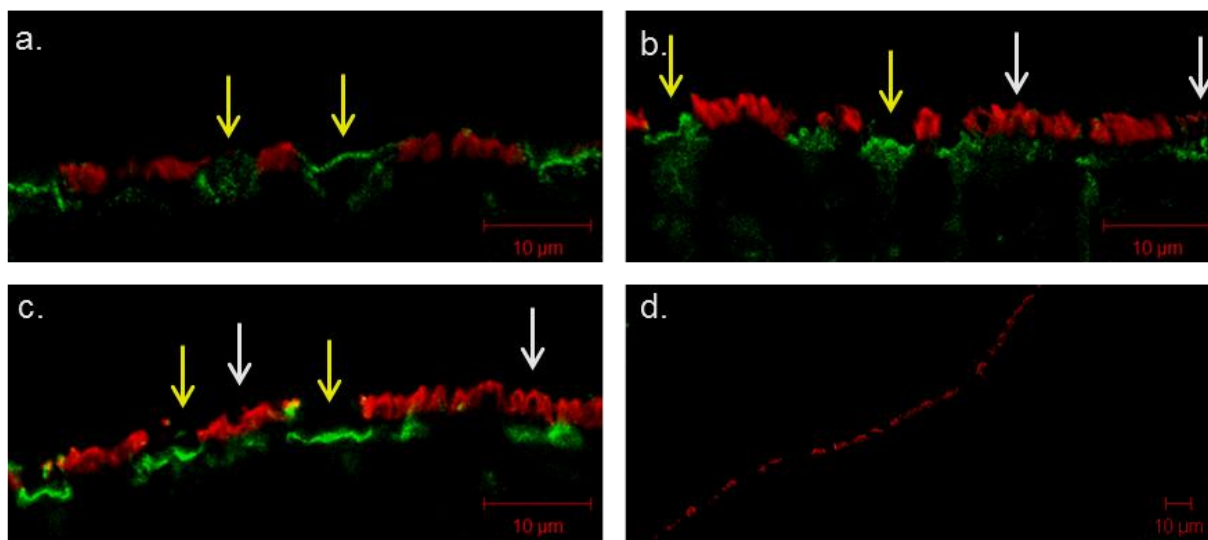


Figure 3.10 Binding of different recombinant H1 HA proteins to human trachea sections

Ex vivo human trachea sections were probed with HA-Fc recombinant proteins from human or swine origin influenza virus. Ciliated cells were identified using anti-acetylated α -tubulin (red) and HA-Fc proteins were identified using anti-human Fc antibody (green). Images are representative of multiple probed sections. Both ciliated (white arrow) and non-ciliated (yellow arrow) cells were found in HAE sections. a) E195; b) E195 E227A; c) Ohio01; d) Ohio01 A227E

bound to 49.4%, Ohio01 bound to 55.1% and E195 E227A bound to 57.5% of total cells. Once more, mutation of residue 227 in Ohio01 from A to human-like signature E completely abrogated binding to the human trachea sections (*Figure 3.10d*). The binding profiles were quantified by blind counting 5-10 different fields containing around 200 cells in total for each HA (*Figure 3.11*). From this quantification, the relative ratio of non-ciliated/ciliated cells bound by the recombinant proteins was calculated, which was higher for E195 (3.9) than E195 E227A (2.5) or Ohio01 (2.0) HA proteins (*Table 3.5*). Although the differences in ratios of ciliated to non-ciliated cells bound were more pronounced for HAE cultures than for ex vivo tracheal sections, the overall trend was maintained as in every experiment the ratio of E195 was higher than that of E195 E227A and Ohio01. This shows that E195 showed a cell tropism more akin to the fully adapted human HAs previously studied in this system, such as H3 HA, than to the avian viral HAs such as H5.

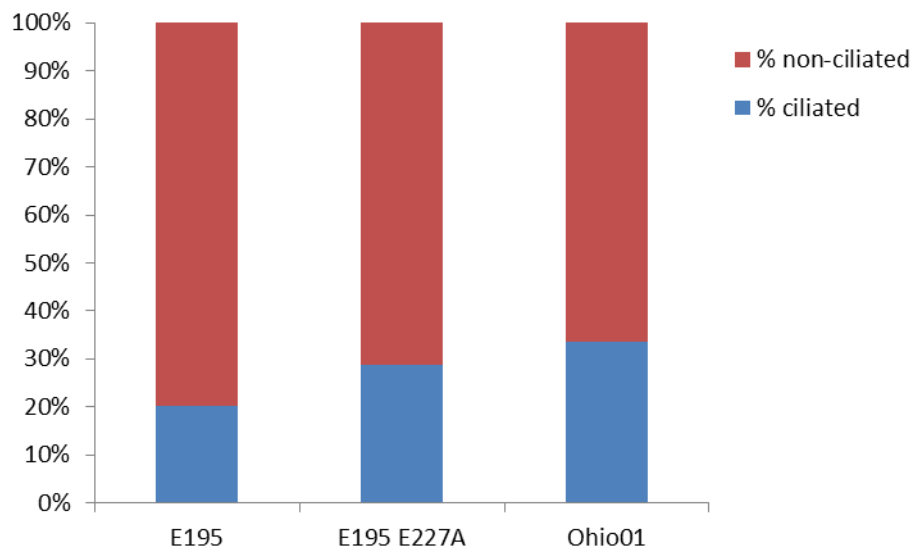


Figure 3.11 Quantification of recombinant HA protein binding to human trachea sections

The percentages of non-ciliated and ciliated cells was quantified by blind counting >200 cells per sample.

3.2.3 Infection of HAE by recombinant influenza viruses with E195, E195 E227A or Ohio01 HA

Work in our laboratory has previously showed that the binding profile of HA proteins to HAE sections mirrored the infection profile observed in HAE cultures for a variety of wild type HA proteins derived from human and avian influenza viruses (Shelton et al. 2011). To confirm the results obtained with HAE sections bound by H1 HA proteins of human and swine origin (Section 3.2.2), reverse genetics was used to create isogenic viruses based on the E195 backbone but containing HA genes of wild-type E195, E195 E227A, Ohio01 or Ohio A227E (Table 3.4) (Elleman and Barclay 2004, Fodor et al. 1999). The viruses were rescued in MDCK cells. Even though Ohio A227E HA protein was unable to bind cells on fixed HAE cultures, this virus did rescue in MDCK cells. Because of doubt about the functionality of the Ohio A227E HA protein it was decided not to use the Ohio A227E virus in the infection assay, as the complementary recombinant protein binding data was not available to confirm a correlation. It is possible that the sialic acid receptors in HAE cultures are more restrictive than sialic acid receptors expressed on MDCK cells, which would then allow for rescue of Ohio A227E in this system but would restrict growth in HAE cultures.

Using a multiplicity of infection (MOI) of 0.1, HAE cultures were infected with virus (E195, E195 E227A or Ohio01) and fixed 16 hrs post infection (h.p.i.). Ciliated cells were identified using anti-acetylated α -tubulin (red) and infected cells were identified using anti-NP antibody (green).

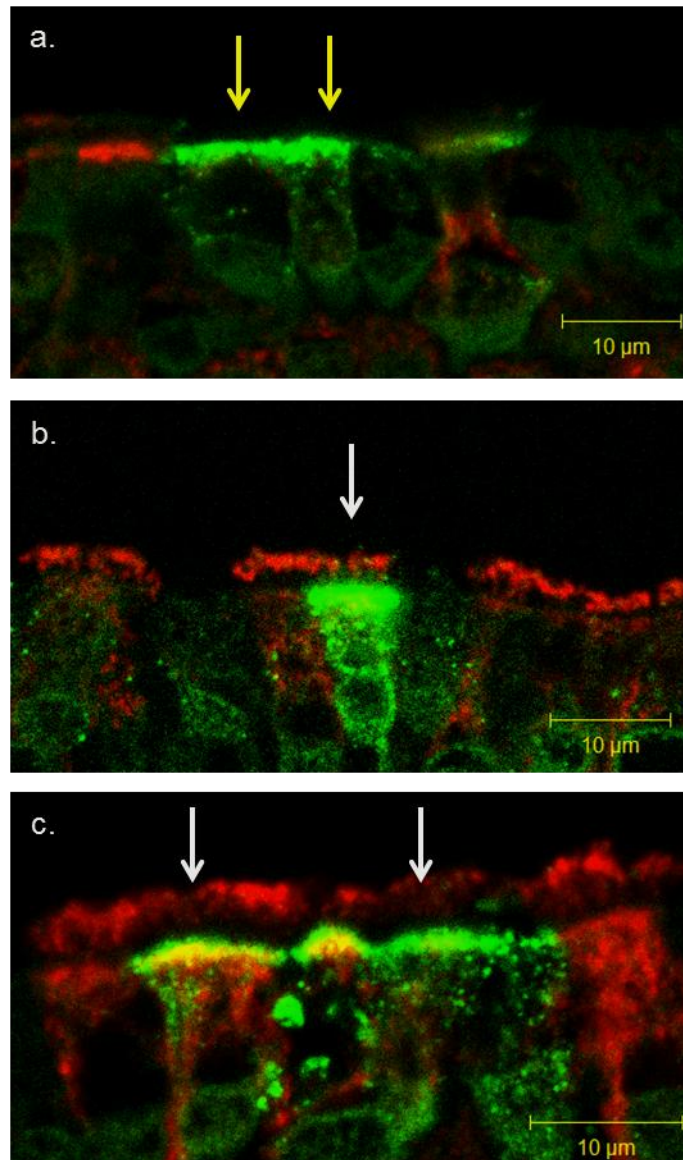


Figure 3.12 Infection of HAE cell cultures with recombinant H1N1 influenza viruses

HAE cultures were infected with isogenic viruses based on the E195 backbone but containing HA genes of wild-type E195, E195 E227A or Ohio01. Ciliated cells were identified using anti-acetylated α -tubulin (red) and virus-infected cells were identified using anti-NP antibody (green). Images are representative of multiple probed sections. Both ciliated (white arrow) and non-ciliated (yellow arrow) cells were found in HAE sections. a) E195; b) E195 E227A; c) Ohio01.

The cell types infected by the recombinant viruses that varied only in their HA genes mirrored the results obtained in the recombinant HA binding assay on HAE sections. E195 preferentially infected non-ciliated cells (60.0% cells infected were non-ciliated) (*Figure 3.12a*), whereas the single HA amino acid substitution at residue 227 from E to A decreased the proportion of non-ciliated cells infected by E195 E227A to 41.4% (*Figure 3.12b*). Virus with Ohio01 HA infected a similar percentage of non-ciliated cells (43.6%) as the E195 E227A mutant virus (*Figure 3.12c*). Both E195 and E195 E227A viruses infected 10% of total cells (10.4% and 10.7% respectively), whereas virus with Ohio01 HA actually infected twice as many cells (20.3%). Cell tropism was quantified by blind counting around 200 cells per HAE section in 8-10 fields for each virus (*Figure 3.13*). Using these quantifications, the relative non-ciliated/ciliated infected cells ratio could be calculated, which was considerably higher for E195 (1.5) than E195 E227A (0.7) or Ohio01 (0.8) viruses (*Table 3.5*).

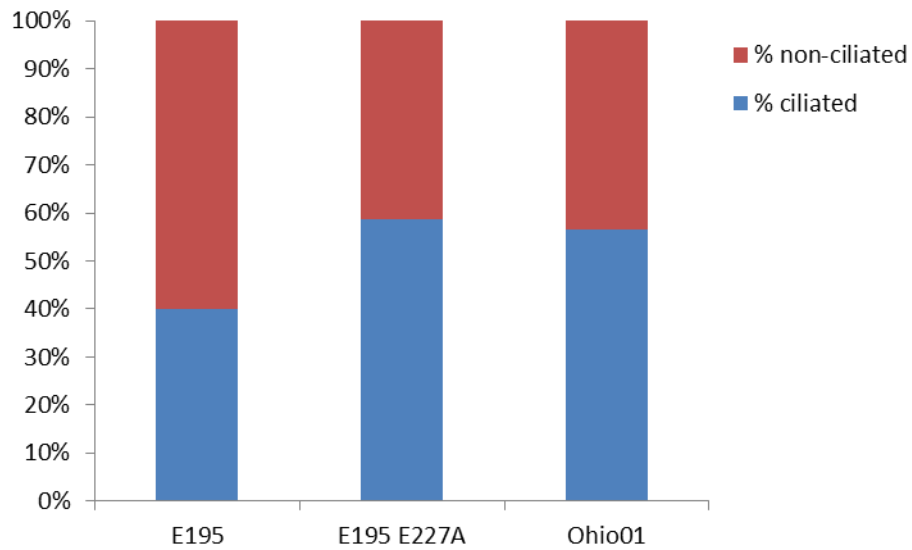


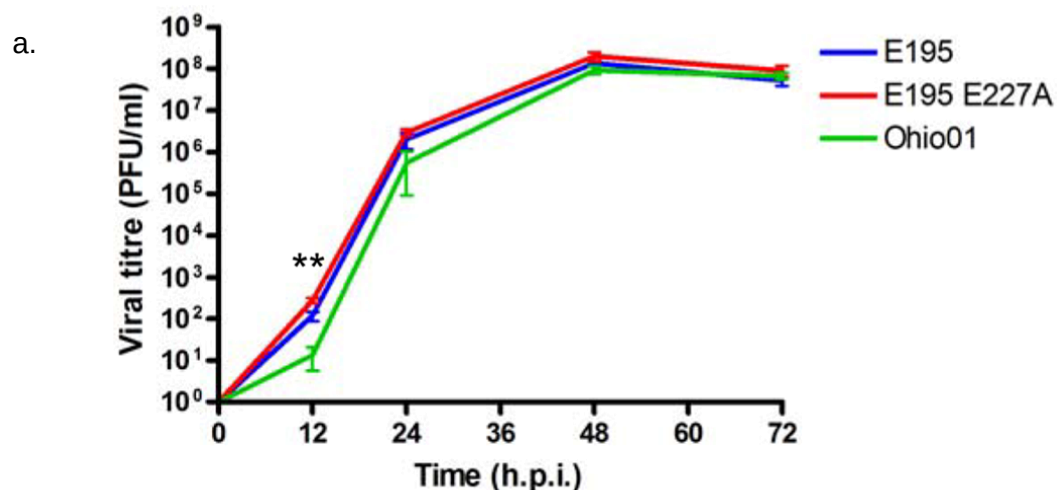
Figure 3.13 Quantification of viral infection of HAE cultures

The percentages of non-ciliated and ciliated cells was quantified by blind counting >200 cells per sample.

Recombinant HA/Virus	Human trachea HA binding	HAE	
		HA binding	Viral infection
E195	3.9	3.9	1.5
E195 E227A	2.5	1.2	0.7
Ohio01	2.0	1.6	0.8

Table 3.5 Ratios of non-ciliated/ciliated cell binding or infection

Ciliated or non-ciliated infected or bound cells were counted on human trachea and HAE (≥ 200 cells/section) and ratios were calculated by dividing the number of non-ciliated cells bound or infected by the number of ciliated cells bound or infected.



b.

	Area Under Curve (Total PFU)		
Time interval	E195	E195 E227A	Ohio01
0-48 h.p.i.	1.636×10^9	2.454×10^9	1.130×10^9
0-72 h.p.i.	3.856×10^9	5.934×10^9	3.050×10^9

Figure 3.14 Multi-cycle growth curves of E195, E195 E227A and Ohio01 recombinant viruses in MDCK cells

Comparison of multi-cycle virus growth in MDCK cells inoculated with isogenic viruses based on the E195 genetic backbone but containing different HA genes; either wild-type E195, E195 E227A or Ohio01 at MOI of 0.001. a) Viral titres were determined by standard plaque assay on MDCK cells. Data shown represent the mean and standard deviation of 4 replicates. Statistical differences between wild type E195 and E195 E227A or Ohio01 were determined using one-way ANOVA. ** = $p < 0.01$. b) Area under the curve was calculated using Graphpad Prism 4 Software and presented for each virus in total PFU released between 0-48 h.p.i. and 0-72 h.p.i. Significant differences could not be calculated with the current experimental setup.

3.2.4 Replication of recombinant influenza viruses with HA genes of E195, E195

E227A and Ohio01 in MDCK cells and HAE cultures

It was previously shown that virus with avian-like sialic acid binding by HA was attenuated for multicycle replication in HAE cultures (Scull et al. 2009). Based on the results obtained from the HA binding assay on HAE sections and recombinant virus infection of HAE cultures, it was hypothesized that the viruses containing swine-like signature amino acid A at residue 227 would also be attenuated compared to wild-type E195 in highly differentiated HAE cultures, but not monolayer MDCK cells which display abundant sialic acids of both linkages and can be readily infected by both swine and human influenza viruses. Confluent monolayers of MDCK cells were infected with each virus at an MOI of 0.001 at 33°C, the proposed temperature of the upper respiratory tract (Scull et al. 2009), and samples of supernatant containing released virus were taken at different time points up to 72 h.p.i. As expected, all viruses grew with similar kinetics. No significant differences were found between viral titres of wild-type E195 and E195 E227A. Recombinant virus with Ohio01 HA did show a small but statistically significant attenuation at 12 h.p.i (*Figure 3.14*). It is possible that this is caused by a mismatch between Ohio HA and E195 NA (Yen et al. 2011b).

HAE cultures were then infected with an MOI of 0.001, apical samples were taken at different time points up to 48 h.p.i. In contrast with the results from MDCK cells, wild-type E195 grew to significantly higher viral titres than E195 E227A at 33 °C at both 12 and 24 h.p.i. (*Figure 3.15*).

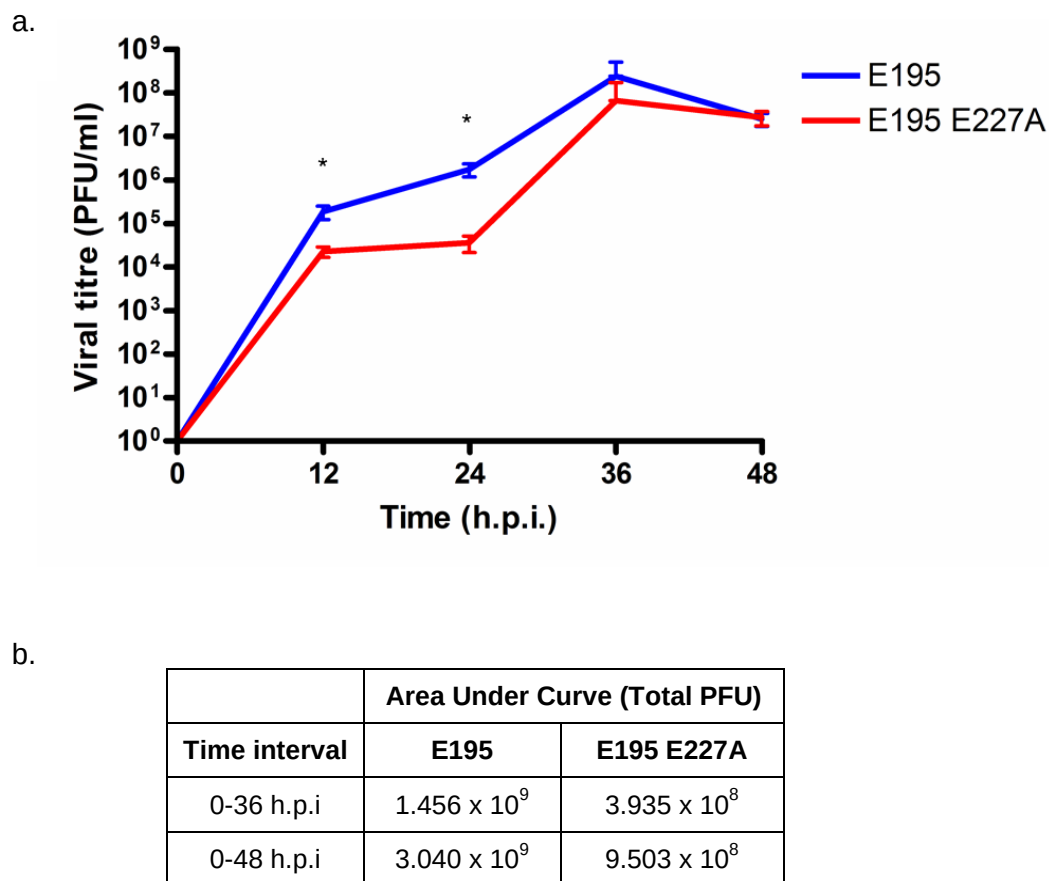


Figure 3.15 Multi-cycle growth curves of E195 and E195 E227A recombinant viruses in HAE cultures

Comparison of multi-cycle virus growth in HAE cultures inoculated with isogenic viruses based on the E195 backbone but containing HA genes of either wild-type E195 or E195 E227A at MOI of 0.001. a) Viral titres were determined by standard viral plaque assay on MDCK cells. Data shown represent the mean and standard deviation of 3 replicate cultures. Statistical differences between wild type E195 and E195 E227A were determined using one-way ANOVA. * = $p < 0.05$

b) Area under the curve was calculated using Graphpad Prism 4 Software and presented for each virus in total PFU released between 0-36 h.p.i. and 0-48 h.p.i. Significant differences could not be calculated with the current experimental setup.

In addition Ohio01 HA virus grew to lower viral titres than E195 at 33°C in HAE at each time point after 36 h.p.i. although the difference was not statistically significant (*Figure 3.16*).

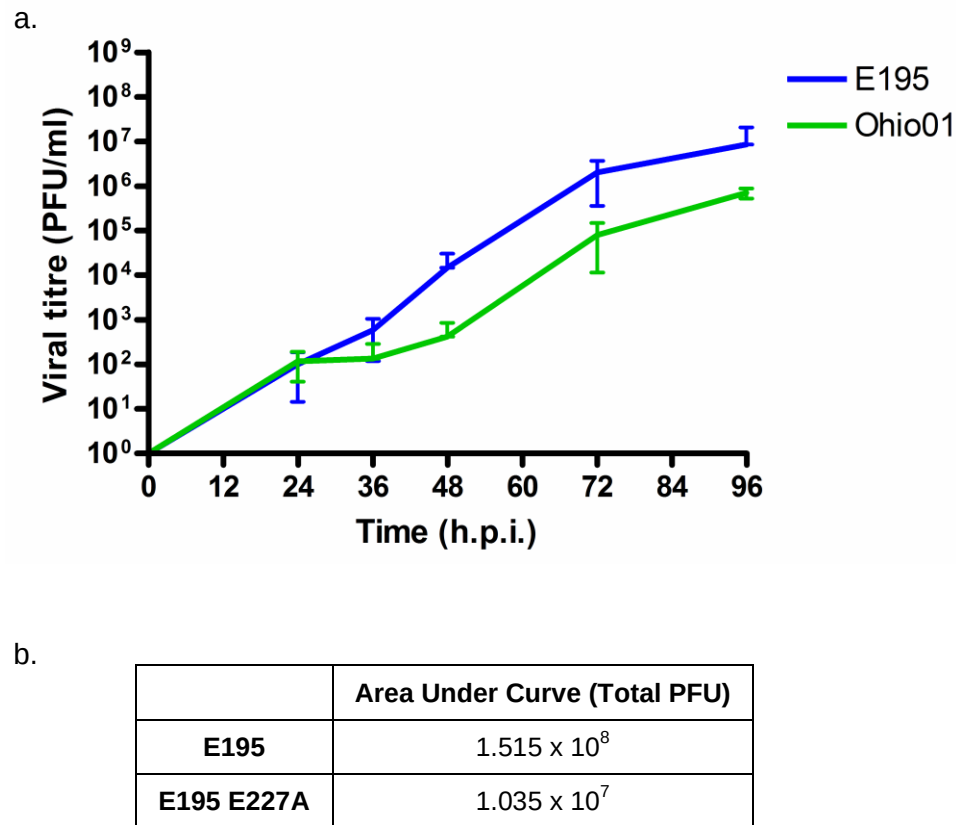


Figure 3.16 Multi-cycle viral growth curves of E195 and Ohio01 recombinant viruses in HAE cultures

Comparison of multi-cycle virus growth in HAE cultures inoculated with isogenic viruses based on the E195 backbone but containing HA genes of either wild-type E195 or Ohio01 at MOI of 0.001. a) Viral titres were determined by standard plaque assay on MDCK cells. Data shown represent the mean and standard deviation of 3 replicate cultures. Statistical differences between wild type E195 and Ohio01 were determined using one-way ANOVA. b) Area under the curve was calculated using Graphpad Prism 4 Software and presented for each virus in total PFU released. Significant differences could not be calculated with the current experimental setup.

3.2.5 Microneutralisation of E195, E195 E227A and Ohio recombinant viruses with E195 antiserum

The efficacy of pre-existing natural immunity or vaccine induced protection for prevention of infection with influenza viruses in humans is mostly monitored by antibodies directed against the highly abundant viral exposed surface protein HA protein (Nayak et al. 2010). The ability of influenza virus to re-infect humans is associated with variations in antigenic sites of the HA protein (Wiley et al. 1981, Caton et al. 1982, Gerhard et al. 1981, Smith et al. 2004), enabling the virus to escape the humoral immune system by antigenic drift (Schmolke and Garcia-Sastre 2010), driven by the selective pressure of neutralizing antibodies in combination with the error-prone RNA-dependent RNA polymerase (Parvin et al. 1986).

Residue 227 is located very close to one antigenic site (Ca2) as identified in A/Puerto Rico/8/34 by Caton *et al.* (Caton et al. 1982). To test whether mutations at residue 227 affected the antigenicity of the viruses Ohio01 HA or Eng195 E227A, their neutralization by antibodies raised against E195 was tested in microneutralisation assays.

The 50% tissue culture infective dose (TCID₅₀) of each recombinant virus; wild-type E195, E195 E227A, Ohio01 and Ohio01 A227E was determined on MDCK cells seeded in 96-well plates. All viruses were then diluted to 100 TCID₅₀ and incubated with serially diluted ferret serum raised against E195, followed by incubation on MDCK cells seeded in 96-well plates. All

Virus	<i>E195</i>	<i>E195 E227A</i>	<i>Ohio01</i>	<i>Ohio01 A227E</i>
MN titre exp. 1	6400	3200	4480	16640
MN titre exp. 2	20480	4480	5760	35840
Average	16640	3840	5120	26240

Table 3.6 Microneutralisation of H1N1 viruses with ferret post-infection antiserum raised against E195.

Viruses were incubated with serial dilutions of anti-E195 antiserum for one hr at 37°C, followed by incubation on MDCK cells for one hr at 37°C. The 96-well plates were stained with crystal-violet after incubation for 4 days at 37°C. Data shown represent the mean of 4 replicate cultures. The average represents the average of both experiments.

viruses were neutralized by ferret serum raised against E195, including Ohio01 and Ohio01 A227E. There were no major differences in microneutralisation titre between any of the viruses examined. Viruses containing A at residue 227 (E195 E227A and Ohio01) showed a small decrease in microneutralisation titre compared to viruses containing E at this position (wild-type E195 and Ohio01 A227E) but variation between assays performed at different times was too large to conclude this was a genuine effect (*Table 3.6*).

Interestingly, serum raised in ferrets against E195 wild type neutralised Ohio01, reaffirming the close relationship of these two viruses.

3.3 Discussion

In this chapter hypothetical changes in the HA sequence were investigated which enabled the virus to transmit between humans. HA multiple sequence alignments found that amino acid 227 in previous seasonal H1N1 viruses is usually E, whereas in swine influenza it is usually A, suggesting that the specific amino acid at this key position in the virus RBS might serve to optimize the interaction between virus and the human host at the point of entry. To investigate this, the swine-like amino acid A was introduced in the HA of E195 (E195 E227A) which was then expressed as recombinant protein or whole virus. E195 virus containing the HA gene of swine influenza virus Ohio01 was rescued. Finally, Ohio01 recombinant protein was produced. In HAE cultures, E195 was shown to bind (HA protein) and infect (reverse genetic virus) more non-ciliated cells than E195 E227A and Ohio01 and multi-cycle viral growth of both E195 E227A and Ohio01 was found to be attenuated in comparison to E195 in HAE cultures.

When H1N1 was reintroduced in 1977 the amino acids 219K and 227E were fixed soon after. However, A(H1N1)pdm09 contained 227E in combination with 219I, an amino acid only found twice in swine isolates and once in human isolates at this position. This dissimilarity, as well as the more prevalent changes of amino acids at residue 219 compared to 227 as shown in *Figure 3.1*, *Figure 3.4* and *Figure 3.5*, led us to focus in this study on residue 227 which seemed to be less tolerant of substitutions and therefore of greater importance in a switch in receptor preference compared to position 219. Nonetheless, amino acid substitution 219K in

A(H1N1)pdm09 influenza virus A/California/04/2009 has been found to result in increased HA binding and RD transmission in a ferret model (Jayaraman et al. 2011).

We first compared the cell tropism of the HA protein of a prototypic A(H1N1)pdm09 strain E195 with the HA mutant E195 E227A and the dead end zoonotic virus Ohio01 in HAE cultures. Both E195 E227A and Ohio01 were found to bind a higher proportion of ciliated cells than wild type E195 virus indicating a shift in receptor preference towards α 2,3-linked sialic acid binding. Published work using the HAE culture model has indicated that α 2,3-linked sialic acid is found exclusively on ciliated cells and consequently avian influenza viruses bind and infect these cells predominantly in contrast to human transmissible viruses that preferentially bind and infect non-ciliated cell types (Thompson et al. 2006, Ayora-Talavera et al. 2009, Shelton et al. 2011, Matrosovich et al. 2007, Matrosovich et al. 2004a). The relevance of these binding profiles has yet to be directly correlated with transmissibility of influenza viruses but the tendency of viruses that are unable to transmit between humans to infect only ciliated cells (Matrosovich et al. 2007, Shelton et al. 2011) suggests an entry preference that could be the foundation of transmission. Therefore by infecting an increased proportion of ciliated cells and a decreased number of non-ciliated cells in the HAE system, the likelihood or efficiency of transmission *in vivo* might decrease.

An interesting example of the interconnectivity of cell tropism and transmission can be found in a set of H3N2 reverse genetic viruses mutated in their RBS. After mutation of the human H3N2

virus A/Hong Kong/1/68 at residues 226 and 228, the infected non-ciliated/ciliated ratio was changed from approximately 3 to <0.05 (Matrosovich et al. 2007).

Our laboratory previously showed that the spread of the human influenza A (H3N2) virus; A/Victoria/3/75, in HAE was profoundly attenuated by the introduction of avian-like amino acid substitutions L226Q and S228G into the HA RBS, (Scull et al. 2009). The same mutations completely abolished transmission of A/Victoria/3/75, in the ferret model (Roberts et al. 2011). Based on these findings, the results of the multi-cycle growth curves performed in HAE in this study suggest that E195 E227A would be less able to transmit in a ferret model.

In generating the opposite ‘humanizing’ mutation in the Ohio 01 HA protein (227E), we were surprised to find that it abrogated HA binding to the HAE in our system (*Figure 3.8d* and *Figure 3.10d*). This might suggest that only a limited number of swine influenza viruses can accommodate the humanizing mutations. Interestingly the same mutation was tolerated in the context of an infectious virus with Ohio01 HA containing the A227E mutation. As can be observed in *Figure 3.8b*, mutating E195 at residue 227 to A greatly increased the HA binding to the HAE section. Similarly, introducing Ohio01 to contain E at 227 could lead to a decrease in sialic acid binding. It may be that the loss of sialic acid affinity linked to E at 227 in Ohio01 results in an HA with undetectable binding to epithelial sialic acid in these conditions. In contrast, the multivalency of Ohio01 A227E recombinant virus increases avidity, allowing the virus to bind sialic acid and to be a viable virus.

For all other HA mutations recombinant viruses were rescued as expected and their tropism reflected that of the HA proteins. This suggests that HA protein binding profiles were not altered by tissue processing or fixation methods. The results with the virus infections were always somewhat less clear cut than with HA. This phenomenon has been observed before (Shelton et al. 2011).

Binding of HA protein to HAE sections also provided a more stringent result than binding to human trachea sections. It is possible that sialic acid distribution on epithelial cells in HAE sections is more discriminative compared to sialic acid distribution in the human trachea. This makes HAE sections a much better tool to determine sialic acid preference than sections of the human trachea.

Maines et al. suggested that residue 227, which is found in the RBS of HA, in combination with residue 219 stabilizes the orientation of amino acid 190 (Maines et al. 2009, Soundararajan et al. 2009) which, in turn, is important in the receptor binding preference of HA (Tumpey et al. 2007). They proposed that the combination of 227E with 219I found in the HA of A(H1N1)pdm09 viruses destabilizes this network and this may explain the lower α 2,6-linked sialic acid binding of A(H1N1)pdm09 HA as compared with that of 1918 HA (Maines et al. 2009). Theoretical modelling of the A(H1N1)pdm09 HA led Soundararajan *et al.* to hypothesize that residue 227 stabilized the orientation of 222K (Soundararajan et al. 2009), itself a component of a “lysine fence” that acts to anchor α 2,6-linked glycans. This interaction and the importance of the lysine fence remain to be proven experimentally.

Jayaraman *et al.* corrected the interaction network between 227 and 219 in A(H1N1)pdm09 by changing residue 219 to the amino acid found in seasonal H1N1 in human isolates (lysine, K), thereby creating a theoretically more stable ionic network. This mutant showed both increased binding to α 2,6-linked sialic acid and increased RD transmission in a ferret model. A second mutant, with the hydrophobic network corrected by changing three residues E227A, S186P and A189T, also showed increased α 2,6-linked sialic acid binding compared to wild type, although no data on its transmissibility in animal models is available (Jayaraman *et al.* 2011). Although these studies suggest mutational pathways by which A(H1N1)pdm09 would increase its binding affinity to human receptors, it is interesting that these substitutions have not yet emerged during the virus transmission through human hosts (Baillie *et al.* 2012). It is likely that yet unknown mutations will be able to increase α 2,6-linked sialic acid affinity. The recent pandemic allows scientists to record mutations in the HA protein and changes in α 2,6-linked sialic acid affinity in real-time, providing the perfect opportunity to gain more insight into the evolution of HA in a new host.

De Vries *et al.* investigated the importance of residue 227 in binding sialic acid by comparing binding of fetuin, which contains α 2,3-linked sialic acid and α 2,6-linked sialic acid in an approximate 2:1 ratio, to different HA proteins. HA proteins of swine influenza showed greater binding to fetuin compared to A(H1N1)pdm09 HA. By creating chimeric proteins with regions of swine influenza HA or A(H1N1)pdm09 HA, as well as by single-residue mutations it was found that introducing only E227A in A(H1N1)pdm09 increased fetuin binding and conversely

introducing A227E in swine HA decreased fetuin binding. This clearly demonstrates that residue 227 alone is capable of changing the receptor binding profile of HA (de Vries et al. 2011). In the present study, an increased binding intensity to HAE was observed for E195 E227A HA (*Figure 3.8b*), which could be due to a correction of the hydrophobic network between 219 and 227 (Maines et al. 2009) similar to the increased binding of the same HA to fetuin reported by de Vries *et al.* (de Vries et al. 2011).

Yewdell has suggested that the neutralization capacity of an antibody depends on the affinity of HA for sialic acid receptors (Hensley et al. 2009). We were therefore interested to see whether we could detect difference in neutralization of viruses that differ only in this receptor binding capacity. The antigenicity of E195, E195 E227A, Ohio and Ohio A227E was investigated using antiserum raised against E195 in ferrets. Although residue 227 is found close to a known antigenic site of HA (Caton et al. 1982), no change in antigenicity was found when comparing E195 to E195 E227A. Further research using monoclonal antibodies would completely exclude a possible role of residue 227 in sensitivity to antibodies. Interestingly, both Ohio and Ohio A227E were sensitive to antibodies against E195, providing further evidence of the close relatedness of E195 and Ohio, which is also observed in the phylogenetic tree (*Figure 3.3*).

We established potential correlates of human to human transmission, by defining the ratios of non-ciliated/ciliated cell binding in the HAE cell model. We and others (Ayora-Talavera et al. 2009, Shelton et al. 2011, Matrosovich et al. 2004a) have observed that human influenza virus preferably bound or infected non-ciliated cells, whereas avian viruses bound or infected ciliated

cells. This ratio (to be found in *Table 3.5*) can potentially predict the ability of a virus to transmit between humans; the virus needs to be able to infect a certain percentage of non-ciliated cells to transmit to another human host.

Future research to establish these correlations and to further determine whether the hypothesized mutation at position 227 in the HA protein of A(H1N1)pdm09 was beneficial in the start of the pandemic should be done using transmission models, e.g. ferrets.

4 Transmission studies of wild type E195 and E195 E227A in ferrets

4.1 Introduction

In Chapter 3 the changes in cell tropism and viral growth resulting from a mutation in residue 227 of HA from E to swine-like A (E227A) in A(H1N1)pdm09 virus (E195 E227A) were discussed. These results, showing a more human-like cell tropism and increased viral titres with E rather than A at position 227, suggested that this residue was important for A(H1N1)pdm09 virus in gaining the ability to transmit from human-to-human, an essential feature of a pandemic virus. To further investigate the importance of residue 227 in transmission, we examined the ability of E195 E227A to transmit within the ferret (*Mustela putorius furo*) model. Several different models are currently available to investigate infection and transmission of influenza virus, which are extensively reviewed by Belser *et al.* (Belser, Katz and Tumpey 2011b, Belser *et al.* 2009b). Traditionally the mouse model has been used to study pathogenesis of influenza virus, due to advantages such as low cost and readily available reagents which allow the study of for example cytokines (Conn *et al.* 1995, Hennet *et al.* 1992). Furthermore, the mouse model permits examination of the role of single genes in influenza infection due to the availability of transgenic mice (Belser *et al.* 2009b). However, due to an absence of α 2,6-linked sialic acid in the respiratory tract of mice (Ibricevic *et al.* 2006) adaptation of human influenza strains is necessary to establish an infection in this model. Moreover, mice do not present with the same clinical signs as humans; fever, nasal secretions or coughing and sneezing are not detected in the mouse model (Belser *et al.* 2011b). Lastly, this model is not suitable to test the transmission

potential of influenza viruses and the organism has to be euthanized to determine viral titres (Belser et al. 2009b).

A relatively new model to study transmission (either via contact or via respiratory droplets) of influenza in small mammals, the guinea pig, has been developed by the Palese laboratory (Lowen et al. 2006). This model does not require prior adaptation of human influenza strains. Like humans, guinea pigs express $\alpha 2,6$ -linked sialic acid in the upper respiratory tract (Sun et al. 2010) which may explain the ability of human influenza virus to transmit between donor and sentinel guinea pigs. However, like mice, guinea pigs lack many clinical signs observed in humans during influenza infection and they are a poor model of pathogenesis; highly virulent or pathogenic influenza strains such as the 1918 and H5N1 viruses which caused high mortality in humans, left guinea pigs almost unaffected (Van Hoeven et al. 2009a).

Non-human primates have been used to study influenza infection, as these mammals are closely related to humans. Features such as the complex immune response to influenza infection can be more relevantly studied in primates than in smaller mammals (Kobasa et al. 2007) and this model has been applied to the study of highly pathogenic influenza strains (Kobasa et al. 2007, Rimmelzwaan et al. 2001). Naturally, experimental procedures using non-human primates are costly and ethical considerations as well as the large size of the animals and the need for specialist facilities have limited the number of experiments performed with this model.

Pigs have been recognised as a host for influenza virus since 1931 (Shope 1931) and therefore form an obvious transmission model. Indeed, replication of avian influenza virus (Hinshaw et al. 1981) as well as human and swine influenza strains (Landolt et al. 2003) can be studied within the swine host without prior adaptation. Furthermore, transmission experiments between pigs have been successful (Brookes et al. 2010). Finally, the pig model can be used to study vaccine efficacy or cytokine production (Van Reeth, Van Gucht and Pensaert 2002). However, pigs are relatively difficult to handle due to their size, and costing is greatly increased when the use of 'high health status herd' pigs (pigs tested against e.g. swine influenza) is required.

A low-cost alternative to the above models is the cotton rat. This model supports replication of influenza virus without prior adaptation and virus can be recovered from nasal washes. However, it is thought that transmission studies cannot be performed in this model due to a lack of the sneeze reflex (Eichelberger 2007), although guinea pigs do not have the sneeze reflex either.

In comparison to all the above models, the ferret model has several obvious advantages for study of influenza pathogenesis and transmission. In ferrets, the anatomical distribution of sialic acid in the respiratory tract is similar to that observed in humans; α 2,6-linked sialic acid is found in abundance in the upper respiratory tract, whereas α 2,3-linked sialic acid is mostly detected in the lungs (Shinya et al. 2006, Kirkeby, Martel and Aasted 2009). A recent study further defines the location of α 2,6-linked sialic acids recognised by humanised influenza viruses to submucosal glands in the lung hilar region in ferrets and compares these tissues to the more

prominent goblet cells in human tissue (Jayaraman et al. 2012). Furthermore, ferrets are naturally susceptible to influenza infection (Swenson et al. 2010) and human influenza viruses are able to replicate efficiently in the respiratory tract without prior adaptation (Maines et al. 2009, Munster et al. 2009, Itoh et al. 2009, Kim et al. 2009). Upon infection, ferrets display clinical symptoms such as fever, nasal secretions, coughing, sneezing, anorexia and lethargy which can all be observed in human patients (Belser et al. 2011b). Furthermore, transmission of influenza virus in the ferret model has been established in both a contact model, where an infected donor animal is co-housed with a naïve sentinel animal, as well as a RD model, where an infected donor animal is housed next to a naïve sentinel animal and direct contact is prohibited (Maines et al. 2006, Herlocher et al. 2001, Yen et al. 2007). The combination of these factors makes the ferret model understandably the best model to study influenza infection.

Ferret transmission experiments can be performed in two different ways. Firstly, the ability of the virus to transmit via contact is investigated by co-housing an influenza virus-inoculated ferret with a sentinel ferret. Secondly, the ability of the virus to transmit via respiratory droplets is investigated by housing an influenza virus-inoculated ferret next to a sentinel ferret. The cages are placed in close proximity (25 mm distance in our set-up) and contain perforated side walls (5 mm holes in our set-up) to allow for air exchange from donor animal towards aerosol sentinel but prevent direct contact between the ferrets. An interesting example of the difference between these two measurements was demonstrated by Belser *et al.* (Belser et al. 2011a). Two different swine-origin triple reassortant viruses isolated from sporadic human cases not associated with

transmission between humans were investigated (A/Ohio/02/2007 and A/Texas/14/2008). Both of these viruses were able to transmit via contact, but not via RD transmission. In comparison, A(H1N1)pdm09 transmitted to all contact sentinel ferrets, and 6 out of 9 RD sentinel ferrets. This suggests that although viruses that are transmitted via contact transmission might be closer to causing a pandemic in the human host, RD transmission is a necessity.

Numerous ferret transmission studies with different influenza virus strains have been performed, and show an excellent predictive value. Human influenza viruses which evidently are able to transmit between humans are found to be transmitted between ferrets (Herlocher et al. 2001, Herlocher et al. 2004, Roberts et al. 2011). In contrast, avian influenza viruses such as H5, H7 and H9 are not transmitted between ferrets, which reflects the inability of avian influenza viruses to cause human-to-human transmission (Wan et al. 2008, Yen et al. 2007, Peiris 2009, Belser et al. 2007). An outbreak of H7N7 in poultry in the Netherlands was linked to 89 human cases with reported human-to-human transmission to three household contacts (Du Ry van Beest Holle et al. 2005). Consequently, contact transmission studies with this virus revealed transmission in 2/3 contact sentinels but no virus was detected in RD sentinels (Belser et al. 2008).

Predictions based on single-point mutations regarding transmissibility of an influenza virus, such as the hypothesis discussed in this chapter, have been investigated previously. Often in the ferret model transmission of viruses that have been generated by reverse genetics has been monitored both via contact and respiratory droplet. The first study of this kind compared the transmission of 1918 virus with transmission of the parental virus mutated at residue 190

(D190E) and 225 (D225G) of the HA protein. These two mutations conferred the virus to a more avian-like receptor binding preference (Glaser et al. 2005, Stevens et al. 2006a). RD transmission of both viruses was assessed by housing a donor animal next to a naïve ferret. Wild type 1918 virus was detected in nasal washes from 3/3 sentinels, but 0/3 sentinels exposed to mutant 1918 D190E D225G had viral titres in nasal washes (Tumpey et al. 2007).

Substitutions in the HA protein of H3 influenza virus at position 226 and 228 have been shown to change receptor binding preference (Connor et al. 1994, Rogers et al. 1983, Vines et al. 1998), as well as cell tropism (Matrosovich et al. 2007). Recent studies showed that mutating an H3 virus to contain the more avian-like amino acid substitutions L226Q and S228G completely abrogated transmission in a ferret model even when the animals were in direct contact with each other during the exposure period (Roberts et al. 2011). The same effect was found in H2N2 viruses isolated during the 1957/58 pandemic; H2N2 with the human residues 226L and 228S transmitted to 3/3 RD sentinels, whereas H2N2 with the avian residues 226Q and 228G transmitted to 1/3 RD sentinels and this transmission event was associated with a Q226L mutation in the HA protein (Pappas et al. 2010).

We hypothesise that the E227A mutation in E195 abrogates or attenuates host-to-host transmission and aim to investigate this hypothesis using the above described ferret transmission model. Due to the increased binding to ciliated cells, it is further hypothesised that E195 E227A-infected ferrets display a more severe disease. This will be investigated by close observation of ferrets inoculated with either wild type E195 or E195 E227A.

4.2 Results

4.2.1 Transmission of E195 and E195 E227A in a ferret model

The ferret model is generally accepted as the most relevant system for investigation of human-to-human transmission of influenza viruses as the distribution of sialic acid in the respiratory tract of humans and ferrets is comparable and ferrets display clinical signs typical of the symptoms of human disease (Shinya et al. 2006, Langlois 2005, Kirkeby et al. 2009, Reuman, Keely and Schiff 1989). An experimental set-up was devised whereby we were able to measure both direct contact transmission, via the co-housing of an inoculated donor ferret with a naive contact sentinel ferret, and RD transmission by placing a sentinel animal in a cage adjacent to the same donor animal, with the RD sentinel separated by a perforated plastic barrier which completely prevented contact (*Figure 4.1*).

In preliminary studies we found that, like many other A(H1N1)pdm09 viruses studied in this manner (Maines et al. 2009, Munster et al. 2009, Itoh et al. 2009), E195 transmitted to all contact ferrets in a co-housed model and to two of three sentinel ferrets housed in adjacent cages by RD transmission (data not shown). In the current study, we confirmed the RD transmission of wild type E195 virus. Three ferrets were inoculated intranasally with 10^3 PFU E195 in 200 μ l under light anaesthetic. This is a lower inoculum than generally used by others in the field in a smaller volume. Twenty-four hrs post inoculation (p.i.) an RD sentinel was introduced to the cage adjacent to the donor animal and infectious viral shedding by both on

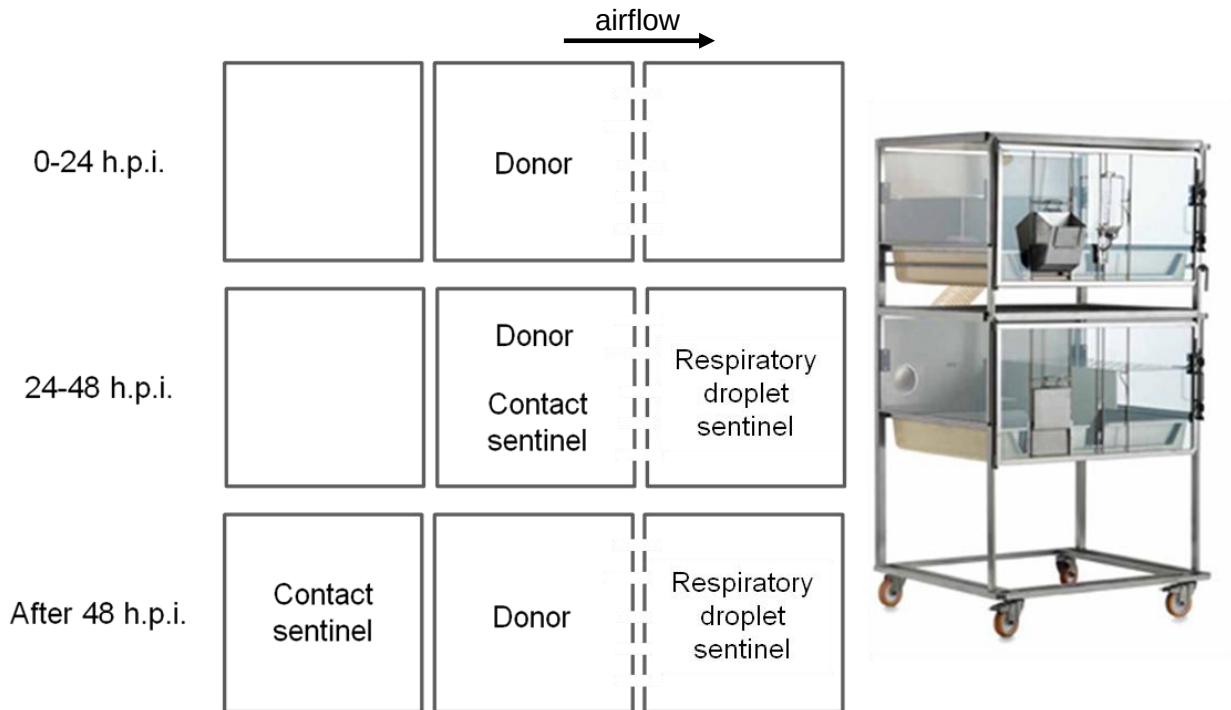


Figure 4.1 Diagram of cage setup transmission experiments

The placement of the different animals in the cages is depicted per timeframe. Every square represents one cage; the dotted line represents the perforated division between two cages. In the first 24 hrs after inoculation, the donor animal was placed in one cage. 24 hrs post-inoculation, a contact sentinel was introduced in the same cage, whereas a respiratory droplet sentinel was introduced in an adjacent cage. The perforated separation walls (5 mm holes) between the cages containing donor and respiratory droplet sentinel animal were 25 mm apart. 48 hrs post-inoculation, the contact sentinel was moved to a separate cage to ensure the respiratory droplet sentinel was only exposed to one infected animal. The photo shows four cages which were occupied by two donor and contact sentinel pairs during the experiment.

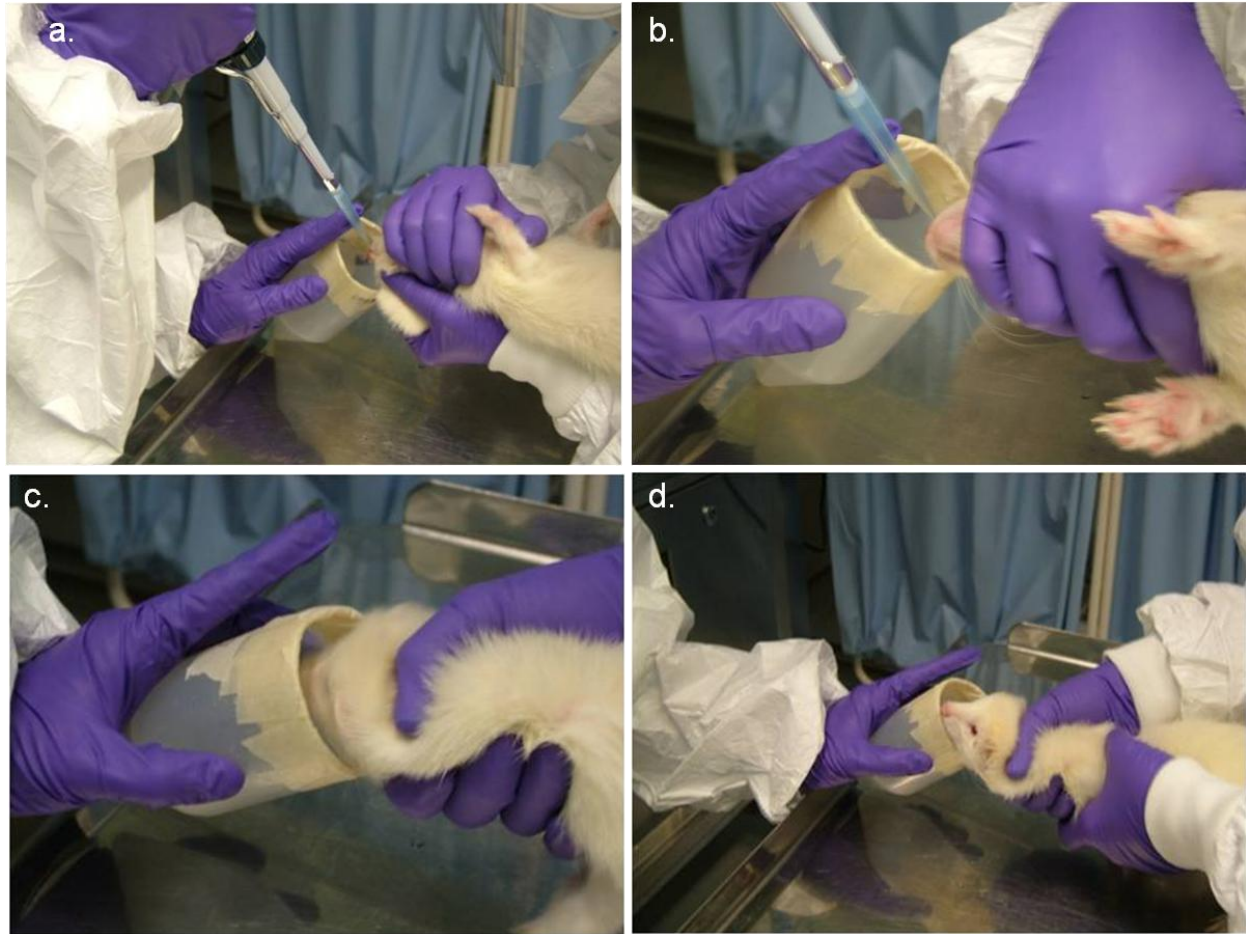


Figure 4.2 Nasal washing ferrets

a/b) Both nostrils of the ferret were washed with a total of 1 ml of sterile PBS. c/d) The expectorate was collected in modified 250 ml centrifuge tubes.

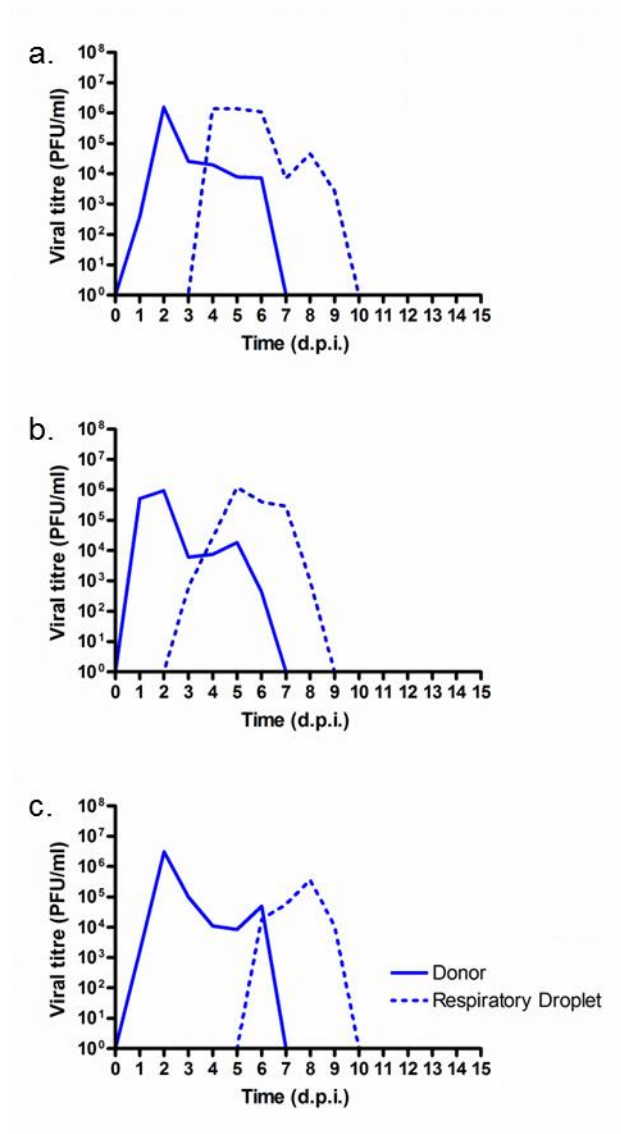


Figure 4.3 Transmission of wild type E195 to respiratory droplet sentinels

Viral titres in nasal wash samples of ferret pairs. Each pair contained one donor ferret (solid lines) and one respiratory droplet sentinel ferret (dotted lines). Donors were inoculated with 10^3 PFU E195 virus. Samples were taken daily and viral titres were determined by standard plaque assay on MDCK cells. Please see *Table 4.1* for exact numbers on days of shedding, peak titre and area under the curve.

donors and respiratory sentinels was monitored by daily nasal washing (*Figure 4.2*) and virus titration MDCK cells. All donors inoculated with E195 became positive for infectious virus in nasal wash on day 1 p.i. Viral shedding was detected in nasal washes from RD sentinels starting on day 3, 4 and 6 p.i.

Peak E195 viral titres reached by RD sentinels (average of 9.7×10^5 PFU/ml) were comparable to peak viral titres reached by donors (average of 1.9×10^6 PFU/ml) and viral shedding was detected for 4 and 6 days (*Figure 4.3*) in line with inoculated donors that all shed infectious virus from the nose for 6 days. Thus it is likely that under these conditions ferrets would be able to support a chain of transmission of E195 by respiratory droplet.

Next we assessed transmission of the E195 virus with the mutation engineered in the HA receptor binding site at position 227 from E to A (E227A). Five donor ferrets were inoculated with 10^4 PFU of E195 E227A via the intranasal route. A higher dose was used for this virus in order to assure robust infection of the donor animals given the modest attenuation of replication in the HAE cultures (see section 3.2.4). Twenty-four hrs p.i. two contact sentinel ferrets were introduced to two out of five inoculated donors to assess contact transmission and placed in an adjacent cage after a further 24 hrs. A RD sentinel was exposed to four out of five inoculated donors in adjacent cages to determine if efficient RD transmission was achieved by this virus (see diagram in *Figure 4.1*).

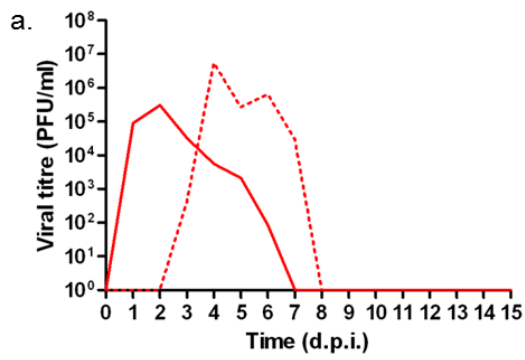
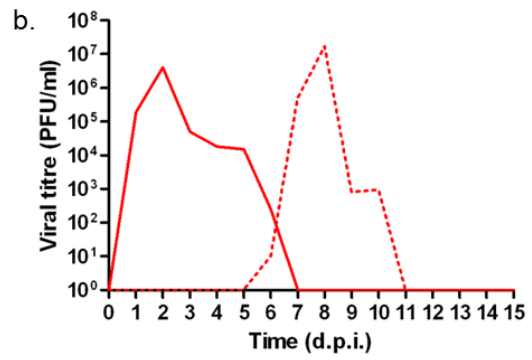
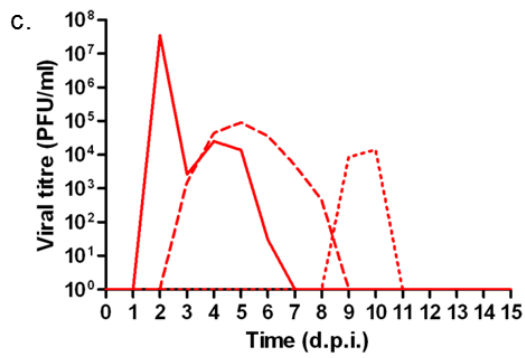
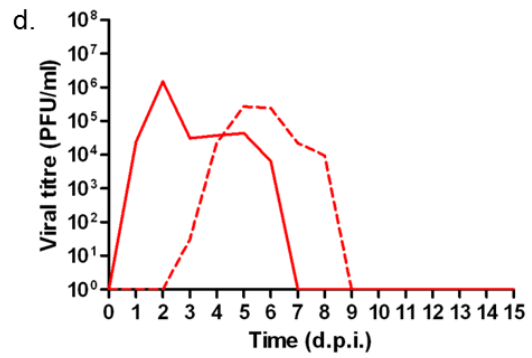
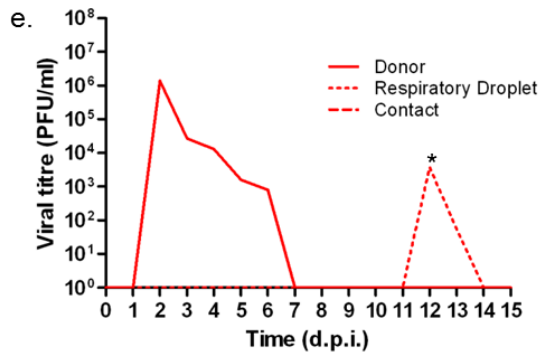


Figure 4.4 Transmission of E195 E227A to contact and respiratory droplet sentinels

Viral titres in nasal wash samples of ferret sets. Each pair contained one donor ferret (solid lines) and one respiratory droplet sentinel ferret (dotted lines) and/or contact sentinel (dashed lines). Donors were inoculated with 10^4 PFU E195 E227A. Samples were taken daily and viral titres were determined by standard plaque assay on MDCK cells. Please see *Table 4.1* for exact numbers on days of shedding, peak titre and area under the curve. Contact sentinels; group c and d; respiratory droplet sentinels; group a, b, c and e; * = Mutated virus E195 A227T obtained from nasal wash

	Duration of shedding (days)		Peak titre (PFU/ml)		Area Under Curve (AUC) (Total PFU shed)	
	<i>E195</i>	<i>E195 E227A</i>	<i>E195</i>	<i>E195 E227A</i>	<i>E195</i>	<i>E195 E227A</i>
Donors	6	6	3.1×10^6	4.0×10^6	3.2×10^6	4.2×10^6
	6	6	1.6×10^6	3.1×10^5	1.6×10^6	4.4×10^5
	6	5	9.5×10^5	1.4×10^6	1.5×10^6	1.4×10^6
		5		3.5×10^7		3.5×10^7
Average	6	5.5	1.9×10^6 ($\pm 1.1 \times 10^6$)	1×10^7 ($\pm 1.7 \times 10^7$)	2.1×10^6 ($\pm 9.8 \times 10^5$)	1×10^7 ($\pm 1.7 \times 10^7$)
Respiratory Droplets	6	5	3.6×10^5	1.7×10^7	4.4×10^5	1.8×10^7
	6	5	1.4×10^6	5.5×10^6	4×10^6	6.5×10^6
	4	2	1.2×10^6	1.4×10^4	1.9×10^6	2.3×10^4
		2		3.8×10^3		3.9×10^3
Average	5.3	3.5	9.8×10^5 ($\pm 5.5 \times 10^5$)	5.6×10^6 ($\pm 8 \times 10^6$)	2.1×10^6 ($\pm 1.8 \times 10^6$)	6×10^6 ($\pm 8 \times 10^6$)

Table 4.1 Summary of results transmission experiments

Duration of shedding, peak titre and AUC were compared between ferrets directly inoculated or infected by respiratory droplet contact with wild type E195 or E195 E227A. Averages are displayed in bold with standard deviation indicated in brackets. None of the groups were significantly different as determined by Student's t-test.

All of the donors inoculated with E195 E227A became positive for infectious virus in nasal wash on day one (3/5) or two (2/5) p.i. (*Figure 4.4*). Viral titres shed were greater than from ferrets infected with wild type E195 virus (mean peak titre of E195 E227A virus shed was 8.2×10^6 PFU/ml). This is potentially caused by the higher inoculum dose used for E195 E227A (10^4 PFU/ferret) compared to E195 (10^3 PFU/ferret). Shedding duration was similar to that of the wild type E195 virus (5-6 days).

Both contact sentinels were robustly infected (average peak shedding of 1.8×10^5 PFU/ml) with no difference in duration of shedding (6 days in both contacts) in comparison to the duration of shedding in inoculated donors of both the wild type E195 and E195 227A viruses.

Infectious virus was detected in at least two consecutive daily nasal washes for all four RD sentinels (4/4). Virus shedding in nasal washes from the RD sentinels exposed to E195 E227A occurred at a range of times after first exposure and to various titres. Two respiratory sentinel animals shed robust peak virus titres (1.7×10^7 and 5.5×10^6 PFU/ml) with duration of 5 days comparable to wild type E195 RD transmission. The other two respiratory sentinel animals showed a reduced duration of shedding (2 days) and considerably lower peak viral titre (1.4×10^4 and 3.8×10^3 PFU/ml) (*Figure 4.4*). However, none of the observed differences were found to be significant.

Table 4.1 shows a summary of the transmission experiments.

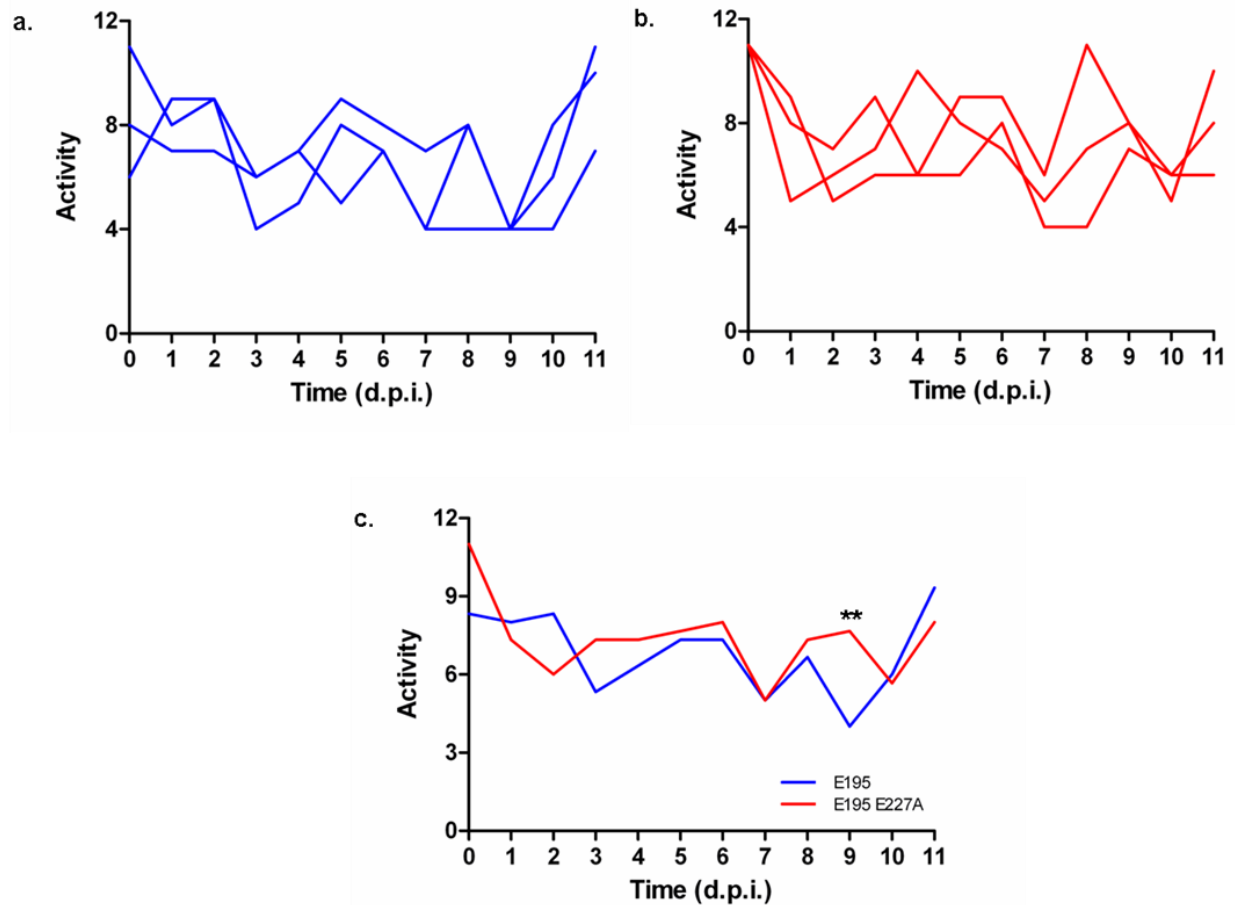


Figure 4.5 Activity chart of E195 and E195 E227A inoculated ferrets

Donor animals were observed daily for one hour before nasal washing during the first 12 days of the experiment. Activity was scored every 15 min, awarding one point if the animal was asleep, two points if it was awake but not active and three points if it was active. a) E195 inoculated animals; b) E195 E227A inoculated animals; c) average of activity per day for E195 inoculated animals (blue) and E195 E227A inoculated animals (red). Statistical differences between wild type E195 and E195 E227A were determined using Student's t-test. ** = $p < 0.01$

4.2.2 Symptoms and behaviour of ferrets during infection with E195 or E195

E227A

As described in Chapter 3, it was observed that recombinant E195 E227A HA bound more intensively to cells in the HAE cultures than E195 HA (*Figure 3.8*). This observation in combination with the increase in ciliated cell binding and infection of ciliated cells by E195 E227A compared to wild type E195 suggests a more avian-like binding profile. It is therefore possible that the virus is capable of penetration deeper into the respiratory tract of the ferret, which in turn could lead to higher pathogenesis. Furthermore, as is hypothesised by Liu *et al.* (Liu *et al.* 2010), the increased infection of ciliated cells could have a marked effect on the mucociliary clearance function of the upper respiratory tract and thereby increase the severity of disease. To investigate the clinical signs associated with infection, three donor animals were observed daily for one hour in the morning for the first 12 days of each experiment.

Activity was scored every 15 min on a 3-point scale; 1 point when the animal was asleep, 2 points if the animal was awake but inactive, 3 points if the animal was awake and active. This allowed for a maximum score of 12 points per day. During the hour, every single sneeze and cough was scored and sneeze fits were scored separately, recording both the number of sneeze fits and the number of sneezes per fit. The body weight of every animal was recorded daily before nasal washes were taken.

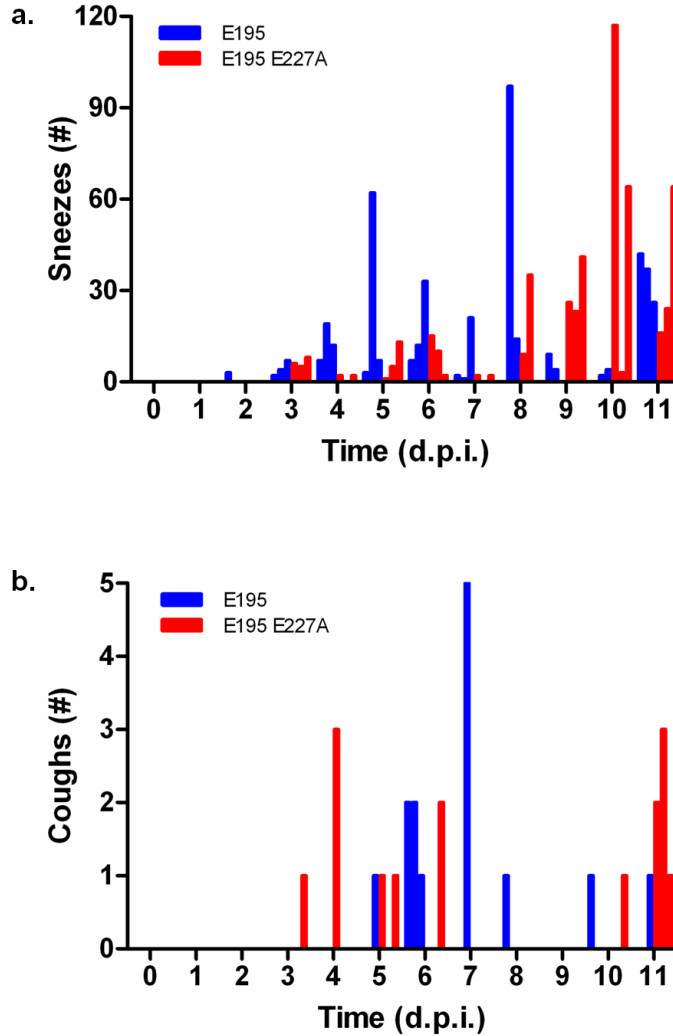


Figure 4.6 Number of sneezes and coughs of ferrets during infection with wild type E195 or E195 E227A

Donor animals were observed daily for one hr before nasal washing during the first 12 days of the experiment; a) total number of sneezes (includes all sneezes in sneezing fits); b) number of coughs; E195 inoculated animals (N=3) (blue) and E195 E227A inoculated animals (N=3) (red).

All ferrets showed decreased activity during infection. Both groups of donor animals were active at the start of the experiment before inoculation at day 0. The first dip in activity for all animals infected with E195 was observed at day 3 (average score; 5.3), with a second dip observed at day 7 (average score; 5) and the last dip at day 9 (average score; 4) (*Figure 4.5a*). Donor animals inoculated with E195 E227A also experienced three dips in activity, their first dip was slightly earlier on day 2 (average score; 6), with a similar dip observed at day 7 as in E195 inoculated animals (average score; 5) and the last dip at day 10 (average score; 7.7) (*Figure 4.5b*). The only significant difference found in activity was at day 9 when ferrets infected with wild type E195 were relatively inactive compared to ferrets inoculated with E195 E227A (*Figure 4.5c*).

No difference was found when comparing the number of coughs of ferrets inoculated with wild type E195 or E195 E227A (*Figure 4.6b*). However, it was observed that animals inoculated with wild type E195 were sneezing more than animals inoculated with E195 E227A at days 4 to 7, whereas the ferrets inoculated with E195 E227A had an increase in the number of sneezes on days 9 to 11 (*Figure 4.6a*). Interestingly, this correlates with the days when the paired RD sentinels became positive; the RD sentinel paired with a donor that had increased number of sneezes from 0 to 42 on day 9 did not show virus in the nasal wash until day 9, whereas the RD sentinel paired with a donor showing an increased number of sneezes from 26 to 117 on day 10 did not show virus in the nasal wash until day 12. It should be noted however that at these later

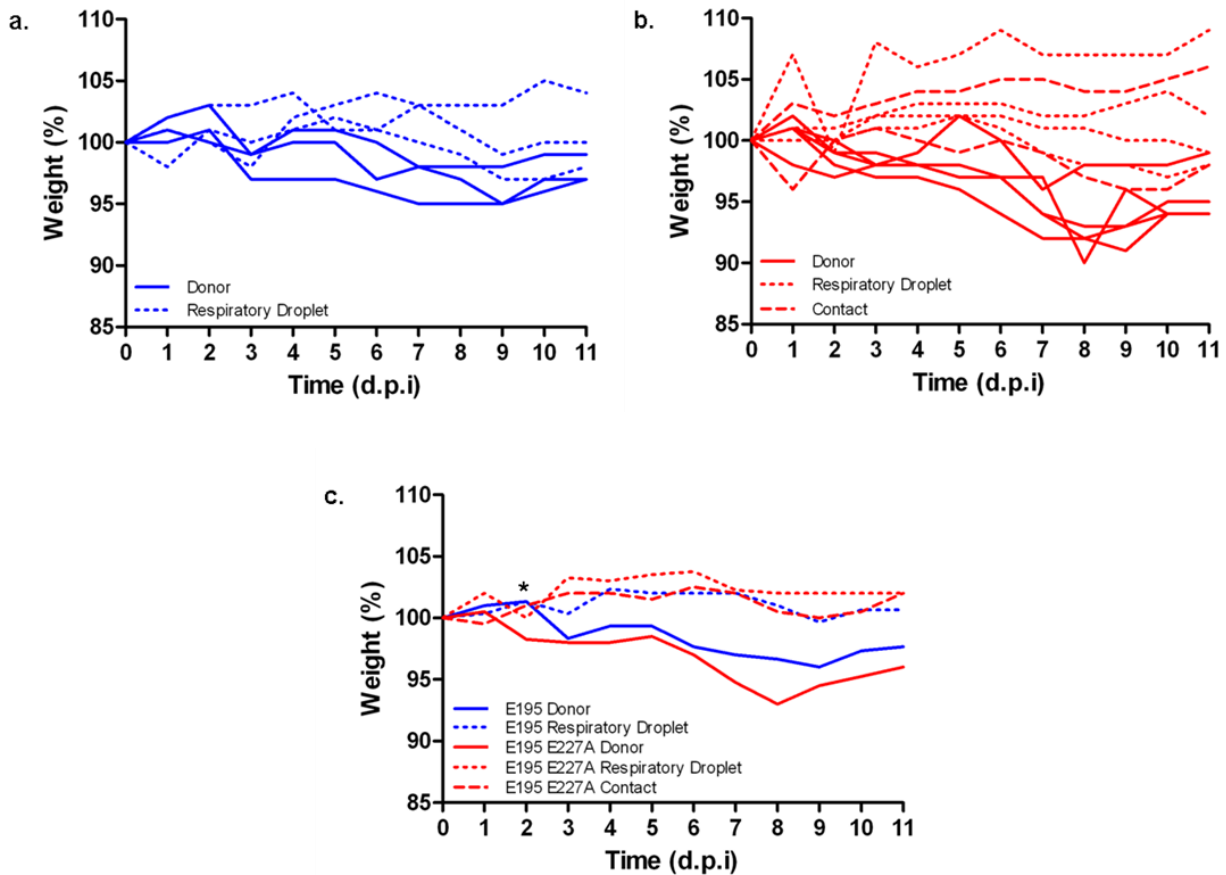


Figure 4.7 Relative body weight of ferrets during infection with either wild type E195 or E195 E227A

The body weight of all animals was recorded daily just before nasal washing; a) Wild type E195; b) E195 E227A; c) Average body weight of donor animal inoculated with wild type E195 (blue solid line) or E195 E227A (red solid line), respiratory droplet sentinels infected with wild type E195 (blue dotted line) and E195 E227A (red dotted line) and contact sentinels infected with E195 E227A (red dashed line). No uninfected animals were monitored. Statistical differences between donors inoculated with wild type E195 or E195 E227A were determined using a Student's t-test. * = $p < 0.05$

time points no virus could be found in the nasal wash of the donors (*Figure 4.4*).

The body weight of every ferret was measured daily to monitor the progression of infection and health of the animal. There was greater variation in body weight of ferrets infected with E195 E227A (*Figure 4.7b*) compared to body weight of ferrets infected with wild type E195 (*Figure 4.7a*). Furthermore, when the average body weight of ferrets were compared by virus (wild type E195 or E195 E227A) and origin (donor, RD or contact) it was observed that donors inoculated with E195 E227A had a lower average body weight than donors inoculated with wild type E195 (*Figure 4.7c*). No significant differences were observed between the RD sentinels infected with wild type E195 or E195 E227A. RD and contact sentinels were less affected by weight loss than donors. This difference was significant between donors and sentinels infected with E195 E227A from 6 days p.i. onwards (*Figure 4.7c*). It should be noted that the donor animals obtained a very different dose of virus than the sentinel animals, which likely explains this difference.

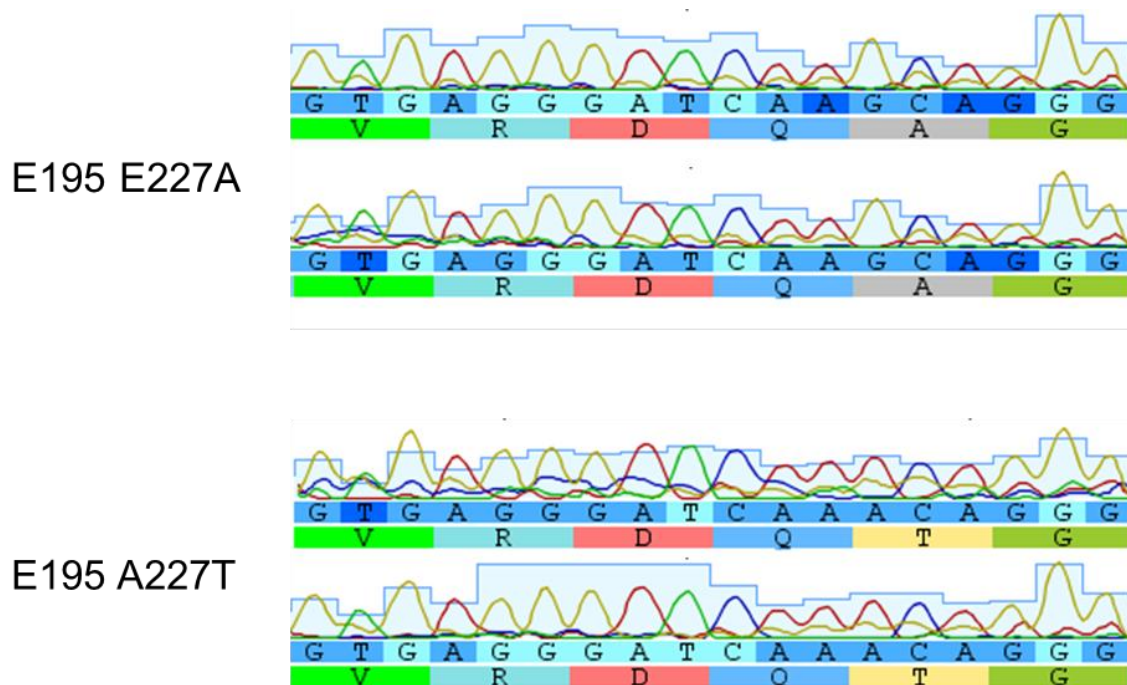


Figure 4.8 Molecular characterization of virus shed by a donor and respiratory droplet pair

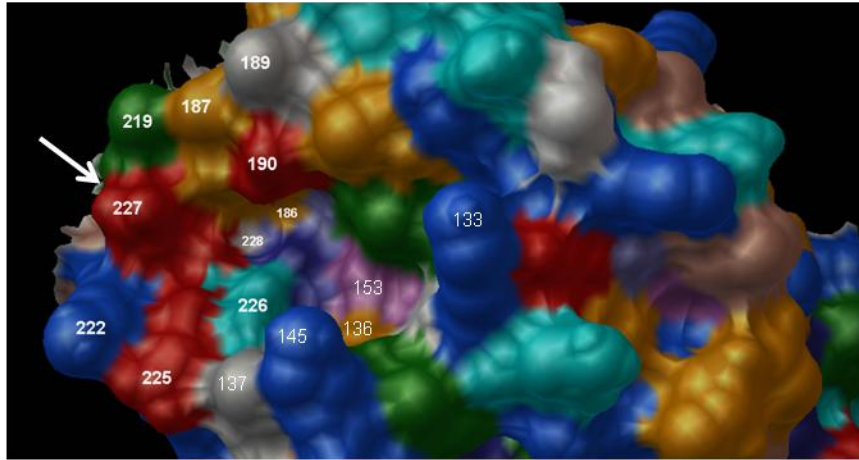
Influenza virus in the nasal wash of ferrets was plaque purified from a MDCK cell monolayer and passaged once in MDCK cells. Viral RNA was extracted from the supernatant using a QIAamp Viral RNA mini kit (Qiagen), and a 1341 nt DNA fragment (HA 42-1382) was amplified by RT-PCR and sequenced using the Sanger method. Nucleotide sequence and corresponding amino acids from HA nucleotides 709 to 726 including residue 227 are shown for two different sequenced plaques of a donor (E195 E227A) and a respiratory droplet sentinel (E195 A227T).

4.2.3 Molecular changes in the HA protein of virus shed by respiratory droplet sentinels

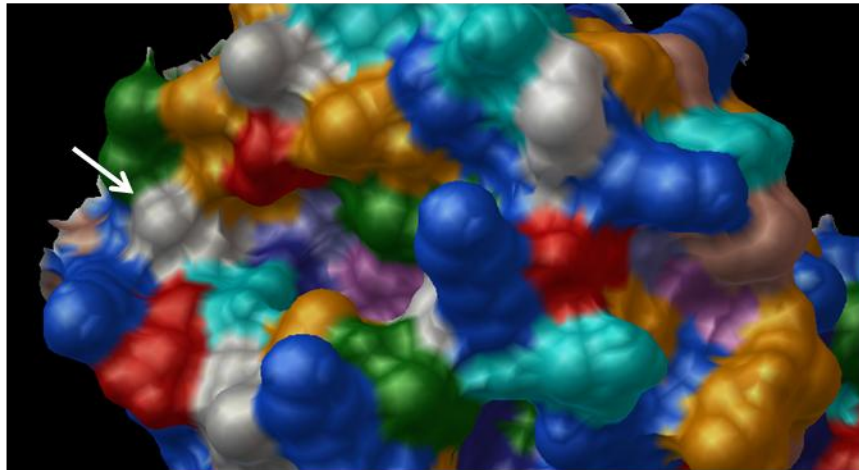
The HA gene of influenza viruses shed by all four donor and RD sentinel pairs in the transmission experiment with E195 E227A were sequenced by plaque-purifying five plaques per ferret, using nasal wash obtained on the first day of positivity from aerosol sentinels and the last day of positivity from donor animals. Although most ferrets (7/8) shed E195 E227A virus identical to the inoculum virus, one RD sentinel was found to have shed a virus containing a mutation at the residue of interest from A to T (A227T) (*Figure 4.4*). The HA gene sequenced from five different plaque picks from the nasal wash of this virus on day 12 consistently contained the A227T mutation. No other mutations were found in the HA protein (*Figure 4.8*). It should be noted that this mutation was not found in influenza virus shed by the donor animal and the viral titre shed by the RD sentinel on day 13 was too low to check for mutational changes.

To investigate the consequence of the A227T amino acid substitution to the HA receptor binding site structure, the molecular surface of all three E195 variants was investigated. The crystal structure of the HA of A/California/04/2009 (Cal04), another prototypic A(H1N1)pdm09 virus, has been solved by Xu *et al.* (PDB 3LZG) (Xu et al. 2010). The protein database file (PDB) was used as a template to mutate all amino acids to the wild type E195 (4 changes), E195 E227A or E195 A227T (5 changes) amino acids in Swiss Prot. The resulting PDF files were then force field minimized using free online software program Yasara, which was expected to reveal a

Wild type E195



E195 E227A



E195 A227T

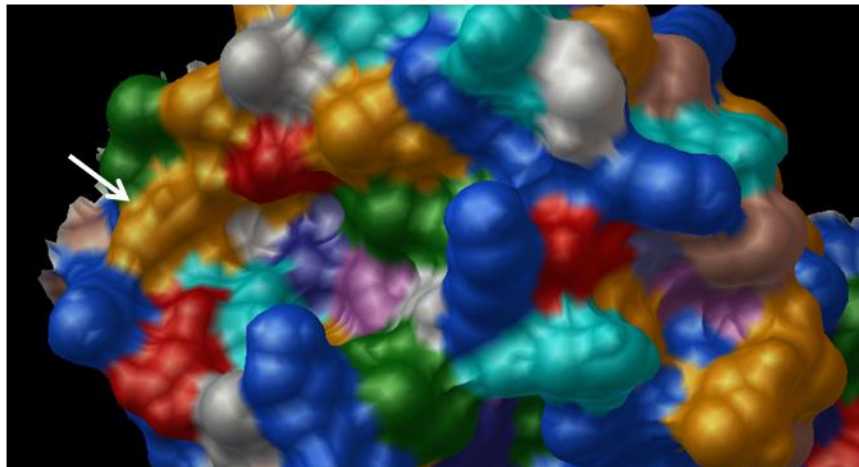


Figure 4.9 Molecular surface of RBS of wild type E195, E195 E227A and E195 A227T

PDB 3LZG (A/California/04/09) was used as a template to mutate all amino acids to the wild type E195 (4 changes), E195 E227A and E195 A227T (5 changes) amino acids in Swiss Prot (<http://www.uniprot.org/>). Yasara was used for force field minimization (<http://www.yasara.org/minimizationserver.htm>). The molecular surface was calculated using Autodock (<http://autodock.scripps.edu/>) and coloured per amino acid in RasMol code. White arrow = residue 227.

more realistic view of the positioning of the different amino acids. The molecular surface was calculated and images were created using Autodock and coloured per amino acid.

The RBS pocket of E195 E227A is more open than the RBS pocket of both wild type E195 and E195 A227T; amino acid 145 is pointing towards the outside of the pocket, whereas it is pointing inwards in both wild type E195 and E195 A227T. Furthermore, amino acid 136 is bulging into the pocket, which is not seen in both wild type E195 and E195 A227T. Overall, E195 E227A looks different from wild type E195 and E195 A227T, which are more similar. This suggests that the shape of the RBS pocket of wild type E195 is preferred for transmissibility in ferrets and the correcting mutation at position 227 from A to T has restored this structural feature (*Figure 4.9*).

4.2.4 Differential plaque phenotype of virus shed by donor animals and respiratory droplet sentinels

After nasal washes were obtained from the ferrets, viral titre was determined on a monolayer of MDCK cells. During these titrations, differences in the plaque phenotypes were observed. To further investigate these differences, nasal washes from two donor ferrets and two RD sentinels infected with E195 as well as the nasal washes from four donor ferrets and four RD sentinels infected with E195 E227A were diluted to 300 PFU/ml and plaque assays were performed using MDCK cells in a 6-well plate. The radii of eleven to fifty-seven plaques per virus were single-blinded measured using a ruler, excluding plaques at the edges of the wells. Values were recorded in 0.5mm increments. The results were first stratified by their origin; one group was formed by all donors, one group was formed by all RD sentinels excluding the mutated E195 A227T virus and the last group consisted of just E195 A227T. All these groups were significantly different; viruses isolated from the nasal washes obtained from RD sentinels had a lower median radius than donor animals, whereas the mutated E195 A227T showed a higher median radius (*Figure 4.10a*). Secondly, the results were further stratified by virus, forming five different groups. The only two groups that were still significantly different from the others were the RD sentinels infected with E195 E227A and E195 A227T (*Figure 4.10b*). Lastly, the results were stratified per ferret. The most interesting observation from this graph is the lack of plaque

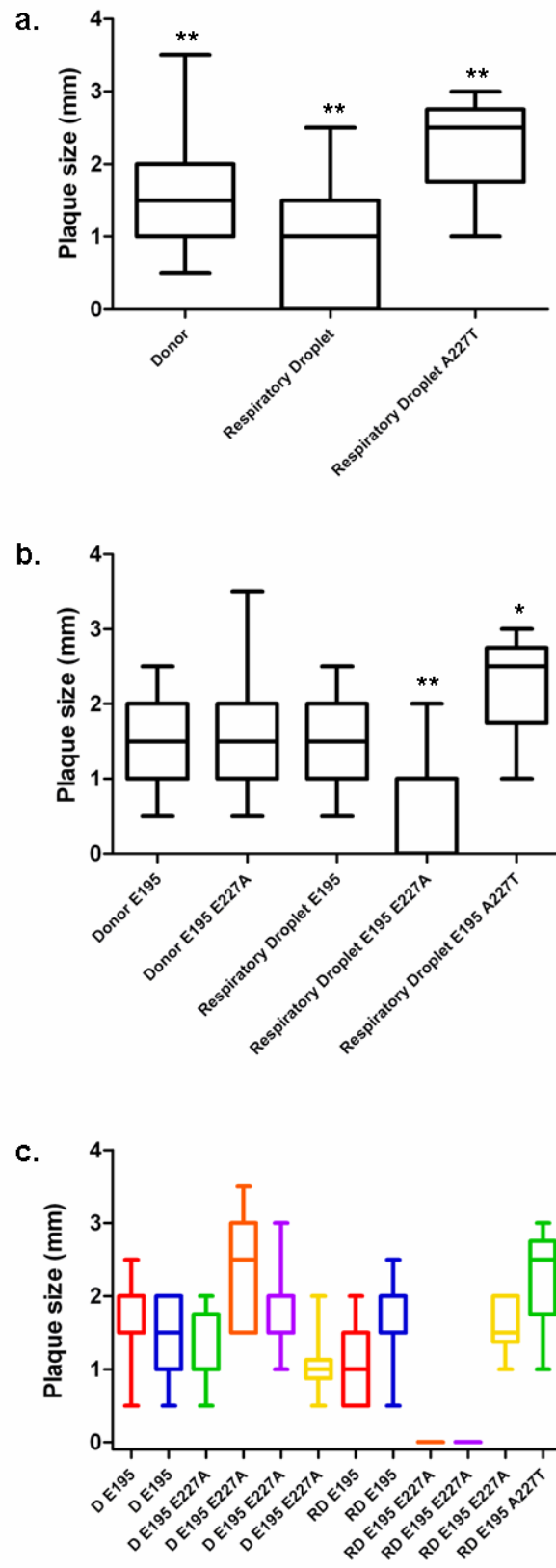


Figure 4.10 Plaque size phenotype of influenza virus isolated from nasal washes obtained from ferrets infected with wild type E195, E195 E227A or E195 A227T

Plaque assays were performed using MDCK cells and the radius of the plaques formed by wild type E195, E195 E227A or E195 A227T viruses were single-blinded measured using a ruler, recording results in 0.5mm increments. Plaque size phenotypes of; a) Results stratified by origin; b) Results further stratified per virus; c) Results per ferret, colour coded per pair. Graphs were produced using Graphpad Prism 5.01 software. Statistical differences between wild type E195 and E195 E227A and E195 A227T were determined using ANOVA one-way test. * = $p < 0.05$, ** = $p < 0.01$. Due to complication of significance of panel c, results can be found in *Table 4.2*.

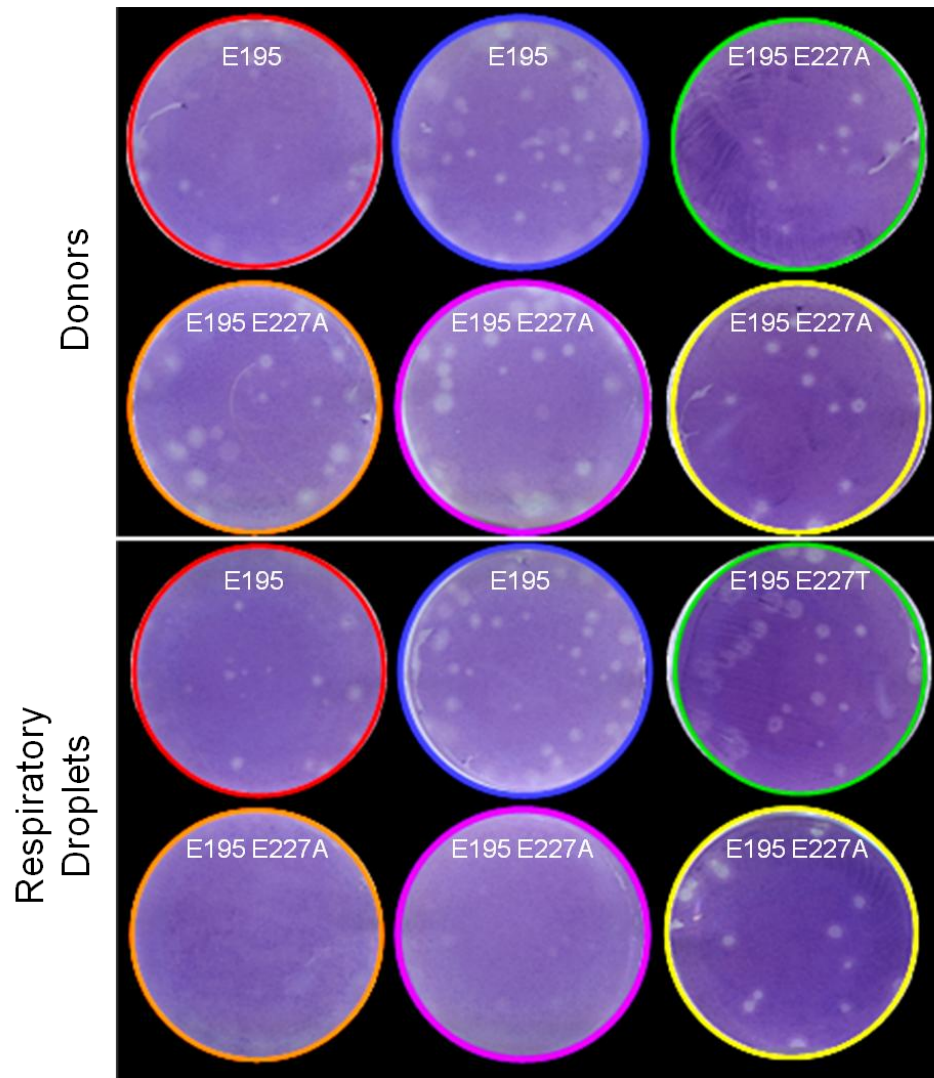


Figure 4.11 Plaque morphology phenotype of influenza virus isolated from nasal washes obtained from ferrets infected with wild type E195, E195 E227A or E195 A227T

All isolates were titred on MDCK cells and stained after 3 days. A representative example of plaque phenotype is shown for every ferret. All pairs are colour-coded per pair as in *Figure 4.10*; red and blue = E195; orange, purple and yellow = E195 E227A; green = E195 E227A (donor) and E195 A227T (RD).

formation by the viruses isolated from two RD sentinels, explaining the low average radius observed in *Figure 4.10a* and *b*. Again it is observed that the mutated virus E195 A227T has an increased radius compared to most other viruses, although it is now only significant in a few cases (*Figure 4.10c*, *Figure 4.11* and *Table 4.2*).

	D E195	D E195	D E195 E227A	D E195 E227A									
D E195					D E195 E227A								
D E195 E227A						D E195 E227A							
D E195 E227A	X	X	X				RD E195						
D E195 E227A			X					RD E195					
D E195 E227A				X	X				RD E195 E227A				
RD E195				X	X					RD E195 E227A			
RD E195				X		X					RD E195 E227A		
RD E195 E227A	X	X	X	X	X	X	X	X				RD E195 A227T	
RD E195 E227A	X	X	X	X	X	X	X	X					
RD E195 E227A				X					X	X			
RD E195 A227T	X	X	X			X	X	X	X	X	X		

Table 4.2 Statistical differences between plaque sizes of influenza virus from different origins.

The significance was determined by ANOVA one-way and Tukey's test, $p < 0.05$ was noted (X).

The different donor and RD sentinel ferret pairs are coloured as in *Figure 4.10*.

4.3 Discussion

In this chapter, the transmissibility of a prototypic A(H1N1)pdm09 virus wild type E195 and mutant E195 E227A was investigated in the ferret model. We showed that the subtle switch detected in HAE binding and infection caused by mutation E227A did not abrogate the transmissibility of the virus in the ferret model used here, suggesting that more dramatic changes in sialic acid specificity than are conferred by the E227A mutation lie behind the acquisition of transmissible phenotype shown by pandemic virus. Nonetheless, selective pressure on the virus to adjust this residue was likely; one aerosol sentinel was found to be infected with E195 E227A virus which was mutated at position 227 (A to T). No differences in severity of disease were found between wild type E195 and E195 E227A-infected ferrets.

A recent study showed that a natural isolate of A/Ohio/02/07, a virus with an identical HA sequence to Ohio01, was able to transmit between co-housed ferrets, but not by RD transmission (Belser et al. 2011a). Similar to these results, RG virus E195 E227A virus was shed robustly from donor animals and transmitted readily to 100% contact animals (*Figure 4.4*). However, unlike natural isolate A/Ohio/02/07 (Belser et al. 2011a), the E195 E227A mutant virus also transmitted to all of the exposed sentinels through the RD route, although it can be argued transmission was slightly attenuated. It is possible that other genetic difference in either the HA gene or other genes of A/Ohio/02/07 preclude the transmissibility through the air.

The HA proteins of Ohio01/02 and E195 have an overall identity of 93.7%, differing in 36 amino acids. When the residues of the RBS were compared (the 130-loop (residues 134-138), 190-

helix (residues 188-194), 220-loop (residues 221-228) and highly conserved residues (98Y, 153W, 183H and 195Y), H3 numbering) only residue 227 was different, suggesting the other amino acids do not directly affect receptor binding. The multi-cycle replication kinetics in HAE of the E195 point mutant E227A and of the 7:1 HA reassortant virus Ohio01 were similar (Section 3.2.4), suggesting that the other 35 amino acid differences in Ohio01 HA did also not significantly affect virus replication in this system.

A/Ohio/01 and A/Ohio/02 wild type viruses differ further from A(H1N1)pdm09 viruses in the origin of their NA and M gene segments; whereas A(H1N1)pdm09 acquired its NA and M genes from an Eurasian source, Ohio01 and Ohio02 contain the typical TRIG constellation and their NA and M gene originate from classical swine influenza (Zhou et al. 1999, Ma et al. 2010). It is not clear from Belser's study whether lack of RD transmission of Ohio02 was due to the NA and M genes in the Ohio02 virus. If this caused the lack of RD transmission, this would not be mirrored using the 7:1 reassortment virus Ohio01, as this virus contains the NA and M genes from an Eurasian source (identical to E195 NA and M genes). The importance of the NA and M genes has been suggested in the literature. Yen *et al.* (Yen et al. 2011a, Yen et al. 2011b) investigated the transmissibility of different human and swine-origin influenza viruses, including one virus isolated in 2004 (A/swine/HK/915/04, sw915) which contained all gene segments of the TRIG virus, but for the M gene which was of Eurasian origin. This virus is therefore similar to A(H1N1)pdm09, but does not contain the Eurasian NA gene. In ferret transmission experiments sw915 transmitted to 1/5 RD sentinels, whereas other swine-origin influenza viruses did not

transmit through the air. Introduction of the Eurasian NA gene in sw915 allows for transmission to 3/3 RD sentinels (Yen et al. 2011a, Yen et al. 2011b). These findings are confirmed in the guinea pig model (Chou et al. 2011). Furthermore, a recent study showed that the Eurasian NA gene is important in the quantity of release of infectious A(H1N1)pdm09; viruses containing the Eurasian-origin NA and M gene segments released influenza viral RNA-containing particles into the air consistently and this correlated with increased NA activity of these viruses (Lakdawala et al. 2011).

Although the sample size was small (n=4) it was observed that in two of the RD sentinels exposed to E195 E227A virus, infection was delayed and peak titres were distinctively lower (*Figure 4.4*). In one of these animals the transmitted virus was found to contain a T at the site of manipulation, A227T. Five out of five plaques picked from the nasal wash of this sentinel contained the A227T mutation, yet this mutation was not present in any of five plaques picked from virus shed from the paired donor animal, suggesting that it had been selected for at the transmission bottleneck or within the sentinel animal after transmission. Although T is not a charged residue like E, they are both polar amino acids. T at position 227 is occasionally observed in HA sequences of swine or human H1N1 influenza isolates, but not in human isolates of H5 or H3 influenza virus. To investigate the relevance of mutation A227T, the molecular surface of wild type E195, E195 E227A and E195 A227T was compared (*Figure 4.9*). One of the most apparent differences besides residue 227 is the differential orientation of residue 145 in E195 E227A compared to wild type E195. Leucine at position 145 in E195

E227A seems to bend away from the receptor binding pocket creating a more open RBS than what is observed in wild type E195. Secondly, residue 136, part of the 130 loop which forms one side of the RBS (Gamblin et al. 2004), bulges into the RBS. The size of the RBS is measured between the 130 and 220 loop, and therefore the RBS of E195 E227A virus could be smaller due to the position of residue 136. Avian HAs are thought to have a smaller RBS than human HAs, as the relatively large α 2,6-linked sialic acid needs a larger pocket to be able to bind the receptor (Stevens et al. 2004, Ha et al. 2001). Furthermore, residue 136 is important in receptor binding (Ha et al. 2001) and the changed position of this residue could alter the binding profile of A(H1N1)pdm09. Both the changes in the positions of residue 136 and 145 seem to be reversed by mutation into T at position 227. Overall, the molecular surface of the wild type E195 RBS resembles the RBS of E195 A227T, whereas the RBS of E195 E227A is unique. Although it is very difficult to make conclusions based on molecular surface images, this seems to suggest the slightly wider RBS observed in wild type E195 is preferable for transmission in ferrets, hence the mutation from A to T in E195 E227A, reversing the RBS pocket size. Further studies are needed to investigate the significance of T at position 227.

Two aerosol sentinels infected with E195 E227A or E195 A227T showed a reduced shedding profile combined with a shortened length of shedding (*Figure 4.4*). It is conceivable that although obviously initial transmission from donor to aerosol sentinel occurred, transmission of influenza virus from the primary aerosol sentinel animal to a second aerosol sentinel will be impaired due to the decrease in amount of infectious virus shed by the primary aerosol sentinel.

To investigate this possibility, so-called transmission chains could be used, in which the primary aerosol sentinel becomes the donor, and a secondary aerosol sentinel is introduced. Exposure of a new aerosol sentinel could be repeated over several rounds, and would provide additional information on the ability of the virus to spread within a population.

Importantly, the other two animals who acquired E227A virus through the RD route showed a shedding profile likely to support a chain of transmission. When variables such as days of shedding, peak titre and area under the curve (AUC) were compared between the groups, no significant difference could be observed.

It was observed that average peak titre and total amount of virus shed by the ferrets inoculated with E195 E227A was higher than those inoculated with E195. The former were inoculated with a 10x higher viral dose, which might have caused this difference. It is possible that this had aided the transmission of E195 E227A, and a more severe attenuation would have been found if the ferrets were all infected with the same lower dose (10^3 PFU/ferret).

The increase in ciliated cell binding and infection of E195 E227A compared to wild type E195 suggests a more avian-like binding profile, which could mean that the virus is capable of penetration deeper into the respiratory tract of the ferret or switches tropism to ciliated cell types, which in turn could lead to higher pathogenesis through defective clearance of virus and cell debris (Liu et al. 2010). A difference in tissue tropism favouring the LRT was for example observed when the binding profile to human respiratory tract tissue of a prototypic wild type A(H1N1)pdm09 and A(H1N1)pdm09 D225G were compared by Chutinimitkul *et al.*

(Chutinimitkul et al. 2010). The G225D mutation is known to shift the viral binding profile to include more α 2,6-linked sialic acid and infect more non-ciliated cells (Liu et al. 2010), and has been identified as one of the necessary molecular changes in the HA protein of H1N1, just before or during the emergence of the devastating 1918 virus (Tumpey et al. 2007, Stevens et al. 2006a). In these studies, Chutinimitkul *et al.* found that binding of A(H1N1)pdm09 D225G was more prominent on type II pneumocytes and alveolar macrophages than binding of wild type A(H1N1)pdm09, suggesting an increase potential for LRT infection (Chutinimitkul et al. 2010). These findings could explain the association of this mutation with a higher morbidity and mortality in humans (Kilander et al. 2010). It should however be noted that in this study no difference in either pathogenesis or tissue tropism was found in a ferret transmission experiment (Chutinimitkul et al. 2010).

Avian H5N1 influenza viruses have also been associated with a LRT infection, and consequently a significant increase in pathogenicity in ferrets (Zitzow et al. 2002). For these reasons, we closely observed the symptoms displayed by both the donor ferrets infected with E195 E227A, as well as the donor ferrets infected with wild type E195. However, no obvious differences were found between the activity profiles, number of sneezes and coughs or the weights of wild type E195 infected ferrets compared to E195 E227A infected ferrets. This further underlines our earlier conclusion that no significant difference is caused by the mutation E227A in the infection of ferrets. A difference could be found in the weight of the donor ferrets versus the RD and contact sentinels; the donor ferrets lost more weight on average than the sentinel

animals did. This is quite likely caused by the relatively large amount of virus which is used to infect the donors, 10^3 and 10^4 PFU/animal for wild type E195 and E195 E227A respectively. It is very unlikely that the sentinel animals were infected with a similar dose; the ID_{50} of influenza virus is estimated to be as low as 1.1 PFU (Roberts et al. 2011, Gustin et al. 2011). This relatively high dose of virus could have triggered a larger response of the immune system, consequently having a greater impact on the weight of the donor animals. Nonetheless, total PFU shed (AUC) was very similar for donor and RD sentinel animals. There were no uninfected animals included in this experiment, and therefore no negative control was available. As such, it is possible that infected animals gained less weight than an uninfected animal would have gained and it is therefore not possible to claim influenza infection had no effect on body weight.

Two out of four RD sentinels exposed to E195 E227A did not become positive for virus in their nasal wash until day 9 and day 12 (*Figure 4.4*), which is counterintuitive as no virus was detected in the nasal wash of the paired donor animals after day 6. Similar late shedding events have been reported by our group (Roberts et al. 2012, in press) and others (Pappas et al. 2010, Wan et al. 2008). It is not clear what causes this increase in the incubation period.

A sharp tail in the trajectory of infectious virus in the nasal wash of all ferrets was noted (*Figure 4.3, Figure 4.4*) in contrast to other transmission studies (Brookes et al. 2010, Koster et al. 2012). This phenomenon is suspected to be caused by the assay used to detect virus in nasal wash; whereas in this study plaque assays were used, in the other studies qRT-PCR was used.

This hypothesis is strengthened by a similar fast decline in virus from nasal wash in other studies detecting infectious virus instead of viral RNA (Munster et al. 2009, Belser et al. 2011a).

The plaque size of a virus is linked to the ability of the virus to spread from cell to cell; a small plaque size is associated with limitations in spread, whereas a large plaque shows an increase in viral spread. The mechanisms underlying the cell-to-cell spread of influenza are poorly understood, but thought to be important for virulence (Doceul et al. 2010). Our analysis showed a significant difference in plaque size of E195 E227A compared to wild type E195, which was smaller in plaque size, as well as E195 A227T, which was larger in plaque size than wild type E195. This suggests the A227T mutation improved the fitness of the virus. However, as E195 E227A was still able to transmit between ferrets via respiratory droplets, this mutation was not necessary to allow the virus to transmit. More experiments will be necessary to determine the difference in fitness the A227T mutation made.

Based on the experiment described above, we conclude that the acquisition of the A227E mutation in a precursor of the 2009 pandemic virus is unlikely on its own to account for the emergence of human transmissibility phenotype. Future work should be aimed at investigating the potential of a transmission chain with E195 E227A compared to wild type E195. Here, infected aerosol sentinels would function as a donor to a second aerosol sentinel, which would function as a donor animal once infected, and so forth.

5 Differential cell tropism and in vitro replication of human and avian H7 virus

5.1 Introduction

The first description of avian influenza dates back to 1878 in Italy, and was then termed classical fowl plague (Perroncito 1878). It was not until 1950 that the cause of classical fowl plague was found to be influenza A virus (Krauss and Webster 2010). No real attention was paid to this discovery until after the H2N2 pandemic in 1957. In an effort to identify the source of H2N2, the antigenic relationship between human influenza virus and influenza virus from other sources was investigated. Cross-reactivity of antisera raised against the 1957 pandemic strain with an avian influenza virus isolated from turkeys provided the first link between avian and human influenza and highlighted birds as a possible reservoir of influenza A virus (Pereira, Tumova and Webster 1967). We now know that these viruses both possess N2 neuraminidase. This finding spiked an interest in influenza virus infection of birds, and consequently influenza virus was found in a wide variety of birds, specifically aquatic waterfowl such as wild ducks (Asplin 1970, Easterday et al. 1968, Dasen and Laver 1970, Winkler et al. 1972, Downie and Laver 1973, Slemons et al. 1974, Stallknecht and Shane 1988). Phylogenetic analysis revealed that all influenza viruses from different hosts originated from an avian source and that the primary reservoir of influenza A virus are the aquatic bird orders; the Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and waders) (Krauss and Webster 2010,

Gorman et al. 1991, Gorman et al. 1990a, Gorman et al. 1990b, Okazaki et al. 1989, Schafer et al. 1993, Kawaoka et al. 1989). Longitudinal surveillance studies found almost all influenza A subtypes in American and Eurasian birds (Aymard-Henry et al. 1973, Krauss et al. 2007, Krauss et al. 2004, Obenauer et al. 2006, Sharp et al. 1993, Krauss and Webster 2010, Olsen et al. 2006, Munster et al. 2007, Wallensten et al. 2007) with the exception of H14 and H15 which are exclusively found in birds in Eurasia (Krauss and Webster 2010).

Avian influenza causes an asymptomatic to mild disease in its natural host, but can cause a range of symptoms in poultry where it is defined as low (LPAI) or highly pathogenic avian influenza (HPAI). LPAI causes a localised infection of mucosal surfaces on the digestive and respiratory tract and is characterized by low mortality and mild clinical signs. In contrast, highly pathogenic avian influenza (HPAI) is a systematic disease characterized by high mortality rate which may be as high as 100% (Suarez 2010). In nature the HPAI phenotype has been restricted to subtypes H5 and H7 hitherto, but subtypes H2, H4, H6, H8, H9 and H14 were shown capable of acquiring the HPAI phenotype using *in vitro* and *in vivo* studies (Munster et al. 2010, Soda et al. 2011, Lee et al. 2009b). Insertion of a multibasic cleavage site into H3N2 did not result in increased pathogenicity in ferrets (Schrauwen et al. 2011), whereas insertion of a multibasic cleavage site in H1, H3, H10, H11 and H15 did not convert the low pathogenic influenza viruses to high pathogenic influenza viruses when investigated in chickens (Lee et al. 2009b).

Although 16 different HA subtypes are found in aquatic birds, retrospective seroepidemiological and virological studies of influenza pandemics that occurred in humans before isolation of the first influenza virus in 1933 (Smith, Andrewes and Laidlaw 1933) suggest these pandemics were caused by H2 and H3 (Davenport et al. 1964, Davenport et al. 1969, Marine and Workman 1969, Marine, Workman and Webster 1969, Masurel 1969, Mulder and Masurel 1958, Shope 1936). This led to the concept of a limited number of influenza subtypes able to replicate in the human host, and the consequent recycling of H1, H2 and H3. Avian influenza viruses of other subtypes were therefore not perceived as a threat. This presumption changed when in May 1997 a 3-year-old boy in Hong Kong was hospitalised and eventually died of HPAI H5N1 infection (Subbarao et al. 1998, Claas et al. 1998) and six months later, another 17 cases were diagnosed with HPAI H5N1 resulting in a further five fatalities (Chan 2002). Epidemiologic and molecular evidence suggests poultry as the source of the HPAI H5N1 virus (Shortridge 1999). Since then many human infections with H5N1 influenza virus have been documented in various locations worldwide, with a reported 60% fatality rate (*Table 5.1*) (WHO Accessed 26-01-2012). Although H5N1 forms the obvious avian influenza pandemic threat, other potential pandemic viruses including H7 and H9 subtypes should not be overlooked. H7 has been found worldwide in wild waterfowl and poultry (Stegeman et al. 2004, Rohm et al. 1996a, Selleck et al. 2003, Li et al. 2006b, Abbas et al. 2010, Pasick et al. 2005, Berhane et al. 2009, Ferro et al. 2010, Baumer et al. 2010).

Country	2003		2004		2005		2006		2007		2008		2009		2010		2011		2012		Total	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	3	0	6	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	8	8	3	3	21	19
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	1	1	2	1	43	28
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	39	15	10	5	168	60
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	12	10	6	6	189	157
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	0	0	12	4
Vietnam	3	3	29	20	61	19	0	0	8	5	6	5	6	6	7	2	0	0	4	2	123	61
Total	4	4	46	32	98	43	115	79	88	59	44	33	73	32	48	24	62	34	28	17	576	339

Table 5.1 Cumulative number of human cases of H5N1 avian influenza infection reported to World Health Organization, 2003-June 2012

Table amended from WHO website (WHO Accessed 26-01-2012).

A clear phylo-geographic pattern has been observed in the H7 subtype; distinct genetic lineages are found in North-America and Eurasia (Lebarbenchon and Stallknecht 2011, Banks et al. 2000).

Outbreaks caused by H7 subtypes in poultry include HPAI H7N3 in Pakistan in 1994 (Naeem and Hussain 1995), in Chile in 2002 (Rojas et al. 2002) and in Canada in 2004 (Bowes et al. 2004, Hirst et al. 2004); LPAI H7N3 in England in 2006 (Nguyen-Van-Tam et al. 2006); HPAI H7N1 in Italy in 1999 (Mutinelli et al. 2003) and HPAI H7N7 in the Netherlands in 2003 (Stegeman et al. 2004, Fouchier et al. 2004, Koopmans et al. 2004). Several of these outbreaks have resulted in human infections. In 1996, the first human-origin H7 influenza virus strain was isolated from a 43-year-old female patient who had cleaned a duck house one day before she developed conjunctivitis (Kurtz et al. 1996). This incident was the first described natural event involving transmission of an avian H7 directly to a human from a presumed avian source. The H7N7 outbreak in poultry in the Netherlands in 2003 resulted in a record number of human cases identified by RT-PCR (89) including one fatal case (Koopmans et al. 2004). Furthermore, serological analysis of serum samples from persons directly exposed to poultry and persons exposed to H7-positive cases revealed A(H7) specific antibodies in 49% and 64% of samples respectively (Meijer et al. 2006). H7N3 in Canada in 2004 led to two human cases (Hirst et al. 2004, Tweed et al. 2004) and a poultry worker in England contracted H7N3 in 2006 (Nguyen-Van-Tam et al. 2006). These incidences of human infection highlight the potential of H7 influenza virus to jump the host species barrier. Furthermore, evidence of anti-H7 antibodies

was found in 3.8% of serum samples obtained from poultry workers active between 1999 and 2003 in Italy (Puzelli et al. 2005). Nonetheless, antibodies do not necessarily reflect an active infection with influenza; the immune system could have responded solely to exposure to the virus, for example via chicken dust during their work with poultry. This is occasionally observed during influenza transmission experiments in ferrets; although no infectious virus is detected in obtained nasal washes, ferrets do seroconvert to the influenza virus tested (Maines et al. 2011). Human infections with the H5 subtype often cause severe disease and have a reported ~60% fatality rate (WHO Accessed 26-01-2012), although this is thought to be an overestimation as only hospitalized cases are included. In contrast, infection with the H7 subtype causes milder influenza-like symptoms and serologic evidence even suggests many infections are asymptomatic (Meijer et al. 2006, Puzelli et al. 2005). The single reported fatal case caused by H7 infection in the Netherlands was associated with 14 amino acid substitutions found in five different genes (Fouchier et al. 2004). The pathogenesis of H7 influenza virus infection in humans is unique in that in addition to causing only a mild influenza-like illness in most cases, conjunctivitis is typically reported and virus can be isolated from ocular samples (Fouchier et al. 2004, Nguyen-Van-Tam et al. 2006, Wong and Yuen 2006). In contrast with the cells lining the human respiratory tract, corneal, conjunctival and lacrimal duct epithelial cells of the human eye express abundant α 2,3-linked sialic acid on their surface (Paulsen et al. 1998, Royle et al. 2008). Furthermore, whereas mucins in the sialylated secretions of the upper respiratory tract, which are important in initial defense against influenza virus, contain mostly α 2,3-linked sialic

acid, ocular secretions contain mostly α 2,6-linked sialic acid (Couceiro et al. 1993, Berry, Ellingham and Corfield 1996, Thale et al. 2001, Ellingham et al. 1999). H7 viruses with avian-like receptor properties might be able to preferentially bind α 2,3-linked sialic acid expressed by epithelial cells on the conjunctiva and avoid capture by mucins in the ocular secretion. H5 viruses are much less likely to infect the eye (Chan 2002), suggesting a subtle divergence in receptor binding preference beyond a shared α 2,3-linked sialic acid preference. It has been documented that the balance between receptor binding protein HA and receptor destroying protein NA is important in viral growth (Wagner et al. 2000). It is therefore also possible that the balance between H7 HA and NA allows for viral replication in human conjunctiva, whereas this balance within the H5 subtype is suboptimal for viral growth in conjunctival cells.

In 2008, Belser *et al.* (Belser et al. 2008) used glycan arrays to demonstrate that several recent isolates of the North American lineage of H7 were able to bind both α 2,3-linked sialic acid and α 2,6-linked sialic acid, in contrast to older H7 isolates which maintained the classic avian-like receptor binding to α 2,3-linked sialic acid. Similar binding patterns of individual viruses (A/Netherlands/219/2003, A/Netherlands/230/2003, A/New York/107/2003 and A/Turkey/Virginia/4529/2002) were observed in a much larger screen of H7 influenza viruses from different sources (Gambaryan et al. 2012). In a subsequent transmission experiment in ferrets, one such isolate associated with human respiratory infection (A/New York/107/2003) which showed a particular increase in α 2,6-linked sialic acid binding combined with a decrease

in α 2,3-linked sialic acid binding, was transmitted from infected animals to sentinel animals placed in direct contact (3/3). RD transmission in ferrets was not detected (Belser et al. 2008).

Additional glycosylation of HA and deletions in the NA stalk region have been identified in H7 viruses which have crossed the species barrier from wild birds to domestic poultry (Banks et al. 2001), however the molecular determinants for H7 infection of humans are not clear. During the outbreak of H7N7 in Netherlands in 2003, the avian virus was not human adapted and a typical non-fatal human case showed only a single amino acid difference from the avian precursor strain in the NS1 protein, K126R. However, the virus isolated postmortem from a veterinarian who was exposed to H7N7 on 2 April 2003 and succumbed to the infection of 17 April 2003 was shown to have adapted to the human host over 15 days. Indeed, 26 nucleotide changes were found resulting in 14 amino acid substitutions in comparison to the avian precursor virus (Fouchier et al. 2004). These substitutions include E627K in PB2, which is associated with higher virulence of H5N1 avian influenza virus in mice (Hatta et al. 2001), and an additional potential glycosylation site near the tip of HA, which is associated with higher virulence in seals (Naeve and Webster 1983) and chickens (Perdue, Latimer and Crawford 1995). However, even though the fatal human isolate had accumulated 14 amino acid substitutions (five in PB2, four in NA, three in HA and one each in PA and NS1) this virus did not change its receptor binding preference and was unable to transmit even via direct contact in ferrets (Belser et al. 2008).

Although human-to-human transmission of H7 influenza virus is only rarely detected and so far has not been sustained (Koopmans et al. 2004), the ability of H7 influenza virus to transmit

within the ferret model (at least by direct contact) implies a molecular predisposition for human-to-human transmission. Furthermore evidence of H7 associated conjunctivitis suggests that the eye can form an additional portal of entry for H7 influenza virus. Indeed, H7 positive samples were isolated from both the eye and throat of a human case of H7N3 infection in the UK in 2006 (Nguyen-Van-Tam et al. 2006). There is also evidence that viral loads are higher in the eye than in the respiratory tract (Fouchier et al. 2004). We hypothesize that the ability of H7 viruses to infect humans by binding to the α 2,3-linked sialic acid receptors in the eye allows the virus to breach the initial host species barrier permitting a window of opportunity for adaptation of the virus to the human host and selecting for viral mutants with human receptor binding ability, potentially resulting in a wider cell tropism and infection of other sites such as the throat.

Between March and April 2006, two free range layer chicken flocks and a broiler breeder chicken flock were infected with LPAI H7N3 in Norfolk, England. As a precautionary measure, all birds on the farms were culled. Nonetheless, several poultry workers and one veterinarian presented with influenza-like illness, including symptoms such as conjunctivitis and fever (Nguyen-Van-Tam et al. 2006). Swabs were taken from all patients, but found negative by RT-PCR with the exception of one poultry worker where samples taken from both his left eye and throat were found positive for H7N3.

In this chapter, we set out to investigate whether the H7 virus that infected the above mentioned human patient and poultry differed from the Eurasian avian H7 virus currently circulating in the avian population by comparing cell tropism and replication in different models. Avian influenza

virus A/mallard/Sweden/105/02 (M105) was selected, as this virus was isolated in Europe and was readily available. Nonetheless, this virus might not be an ideal match as it is subtype H7N7, and not subtype H7N3. As the HA sequence of a chicken isolate (A/chicken/England/4054/06, E4054) from the outbreak was available, we also investigated whether specific mutations in the HA allowed for the zoonotic jump from chicken to a human host. Finally, we hypothesized that the different sites of replication in the patient (throat vs. eye) could have selected for mutations important in site-specific adaptations. Genotypic differences between throat or eye-H7N3 isolates were therefore investigated using a whole genome sequencing assay and phenotypic differences were investigated using conjunctival cells and HAE cultures. For this purpose, H7N3 viruses A/England/480/06 (isolated from the throat) and A/England/481/06 (isolated from the eye) were selected. Please refer to *Table 5.2* for more details on all four viruses.

Virus name	Abbreviation	Host	Isolation site	Subtype
A/England/480/06	E480	Human	Throat	H7N3
A/England/481/06	E481	Human	Eye	H7N3
A/Chicken/England/4054/06	E4054	Chicken (<i>Gallus gallus domesticus</i>)	N/A	H7N3
A/Mallard/Sweden/105/02	M105	Mallard (<i>Anas platyrhynchos</i>)	N/A	H7N7

Table 5.2 List of virus strains

List of all virus strains used in this chapter.

5.2 Results

5.2.1 Molecular studies of LPAI H7N3 isolated from the eye and throat of a human case

In this chapter two viruses isolated from specimens taken from different anatomical sites of a single human case of H7N3 infection were investigated. A/England/480/06 (E480) was isolated from the throat and A/England/481/06 (E481) was isolated from the left eye of a single H7N3 infected human case. The patient was a poultry worker infected during an outbreak of H7N3 in the UK in 2006 (*Table 5.2*). Both viruses were isolated and passaged in embryonated hen's eggs. In this chapter virus obtained from egg passage 4 is studied unless stated otherwise.

5.2.1.1 *Development of a whole genome sequencing assay for H7N3 influenza virus*

At the Health Protection Agency Microbiology Services (HPA MS) Dr. Monica Galiano has developed a whole genome sequencing (WGS) assay for human influenza A based on primers developed by Hoffmann *et al.* and Rod Daniels (Hoffmann *et al.* 2001, Baillie *et al.* 2012). To investigate whether this assay was suitable for H7N3 whole genome sequencing, all primers used in Dr. Galiano's WGS assay (WGS primers) were aligned against the internal genes of A/Mallard/Netherlands/9/05 (H7N7). This virus was chosen as the reference sequence because all internal genes were available online (Cypel *et al.* 2008) (Cypel *et al.* 2008) (Cypel *et al.* 2008) (Cypel *et al.* 2008) (Cypel *et al.* 2008) (Cypel *et al.* 2008) (Cypel *et al.* 2008), the isolate falls into the Eurasian

Virus name	Subtype	Accession number
A/Chicken/England/4054/2006	H7N3	EF467826
A/Chicken/England/4266/2006	H7N3	EF467825
A/Turkey/Italy/214845/2002	H7N3	AJ627491
A/Chicken/Italy/267/2000	H7N1	AJ493215
A/Chicken/Italy/1081/1999	H7N1	AF202255
A/Turkey/Italy/3889/1999	H7N1	AJ493466
A/Mallard/Netherlands/12/2000	H7N1	GU053030
A/Netherlands/33/2003	H7N7	AY338457
A/Netherlands/219/2003	H7N7	AY338459
A/Turkey/England/1963	H7N3	CY015065
A/African Starling/England_Q/983/1979	H7N1	AF202232
A/Chicken/Pakistan/CR2/1995	H7N3	GU053057
A/Avian/NY/7729-6/2000	H7N2	AY240882
A/Avian/NY/74211-2/2000	H7N2	AY240879
A/Avian/NY/118353-1/2000	H7N2	AY240877
A/Chicken/NJ/608/2002	H7N2	AY240888
A/Chicken/British Columbia/2004	H7N3	AY611524
A/Turkey/Chile/4418/2002	H7N3	AY303635
A/Chicken/Chile/4322/2002	H7N3	AY303631
A/Equine/Prague/1956	H7N7	X62552 S96523

Table 5.3 List of viruses used to design H7 HA primers

A multiple sequence alignment of the haemagglutinin genes was produced using MegAlign (DNASTAR Lasergene 9 Core Suite software) of the listed viruses was used to design sequencing primers for the HA gene of E480 and E481.

lineage similar to the UK isolates and it was isolated within one year of E480 and E481. WGS primers designed by Dr. Galiano with more than 3 mismatches when compared to A/Mallard/Netherlands/9/05 were excluded from the assay in the first instance.

Neuraminidase (NA) N3 primers were designed in-house at the HPA MS by Dr. Catherine Thompson. These primers were used to obtain part of the N3 sequence of E480. Using NCBI BLAST technology it was then determined that the influenza isolate closest in phylogeny to the E480 N3 sequence was A/Duck/Singapore/3/97 N3 NA. The WGS primers designed to sequence NA by Dr. Galiano were aligned against A/Duck/Singapore/3/97 NA. WGS NA primers designed by Dr. Galiano with more than 3 mismatches when compared to A/Duck/Singapore/3/97 NA were excluded from the assay in the first instance.

The HA gene of E480 and E481 was sequenced using primers designed based on an alignment of 20 different H7 HA genes (*Table 5.3*).

Original WGS primers that were excluded from the assay due to mismatches were replaced by newly designed primers based on the sequence of A/Mallard/Netherlands/9/05, A/Duck/Singapore/3/97 or the H7 HA alignment. Initially, 15 out of 50 primers were replaced; one primer used to sequence PB2, six primers used to sequence HA, two primers used to sequence NP, four primers used to sequence NA and two primers used to sequence NS (*Table 5.4, 1st round*). The entire genome of E480 virus was then sequenced using this strategy.

Sequence chromatographs were analyzed to determine whether the primers were suitable for H7 sequencing. Where primers were found unsuitable because sequencing was unsuccessful,

WGS primers MG	WGS primers H7 1 st round	WGS primers H7 2 nd round	WGS primers H7 3 rd round (Final)	Mism.
PB2				
Bm-PB2-1	Bm-PB2-1	Bm-PB2-1	Bm-PB2-1	N/A
HPB2F398	HPB2F398	HPB2H7F724	HPB2H7F724	3
HPB2R421	HPB2R421	HPB2R421	HPB2R421	2
HPB2R960	HPB2R960	HPB2R960	HPB2R960	1
HPB2F1441	HPB2F1441	HPB2F1441	HPB2F1441	0
PB2-1105F	PB2-1105F	PB2-1105F	PB2-1105F	2*†
HPB2F1785	HPB2H7F1907	HPB2H7F1907	HPB2H7F1907	3
Bm-PB2-2341R	Bm-PB2-2341R	Bm-PB2-2341R	Bm-PB2-2341R	N/A
PB1				
Bm-PB1-1	Bm-PB1-1	Bm-PB1-1	Bm-PB1-1	N/A
HPB1F370	HPB1F370	HPB1F370	HPB1F370	1
HPB1R389	HPB1R389	HPB1R389	HPB1R389	1
PB1-1262R	PB1-1262R	PB1-1262R	PB1-1262R	0
PB1-1123F	PB1-1123F	PB1-1123F	PB1-1123F	0
HPB1F1711	HPB1F1711	HPB1F1711	HPB1F1711	4*
HPB1R1730	HPB1R1730	HPB1R1698	HPB1R1698	1
Bm-PB1-2341R	Bm-PB1-2341R	Bm-PB1-2341R	Bm-PB1-2341R	N/A
PA				
Bm-PA-1	Bm-PA-1	Bm-PA-1	Bm-PA-1	N/A
HPAR620	HPAR620	HPAR620	HPAR620	1
HPAR984	HPAR984	HPAH7R984	HPAH7R984	0
PA-1498R	PA-1498R	PA-1498R	PA-1498R	3
PA-745F	PA-745F	PA-745F	PA-745F	2
HPAF1435	HPAF1435	HPAF1435	HPAF1435	0
HPAR1824	HPAR1824	HPAR1824	HPAR1824	2
Bm-PA-2233R	Bm-PA-2233R	Bm-PA-2233R	Bm-PA-2233R	N/A
HA				
HAFUc	HAFUc	HAFUc	HAFUc	N/A
H1A1F1	ND_seqHAH7_F2	ND_seqHAH7_F2	ND_seqHAH7_F2	N/A
H1HAR623	ND_seqHAH7_F4	ND_seqHAH7_F4	ND_seqHAH7_F4	N/A
H1HAR1087	ND_seqHAH7_R1	ND_seqHAH7_R1	ND_seqHAH7_R1	N/A
H1HAF594	ND_seqHAH7_F2	ND_seqHAH7_F2	ND_seqHAH7_F2	N/A
H1HA2F1	ND_seqHAH7_R2	ND_seqHAH7_R2	ND_seqHAH7_R2	N/A

WGS primers MG	WGS primers H7 1 st round	WGS primers H7 2 nd round	WGS primers H7 3 rd round (Final)	Mism.
HA				
H1A2R1	ND_seqHAH7_R3_rep	ND_seqHAH7_R3_rep	ND_seqHAH7_R3_rep	N/A
HARUc	HARUc	HARUc	HARUc	N/A
NP				
NPFUc	NPFUc	NPFUc	NPFUc	N/A
NPUF645-68	NPUF645-68	HNP7R594	HNP7R594	2
HNP7R986	HNP7R1017	HNP7R1017	HNP7R1017	0
NPUR664-83	NPUR664-83	NPUR664-83	NPUR664-83	1
HNP7R1010	HNP7R1109	HNP7R1109	HNP7R1109	0
NPRUc	NPRUc	NPRUc	NPRUc	N/A
NA				
FLN1_For	NAFuc	Bm-N3-F	NAFuc	N/A
FLN1_Rev	HNAH7F736	N3-555F	H7NAF615	1
AN1-392_For	HNAH7R569	N3-275R	H7NAR664	3
AN1-742_For			H7NAF1105	0
AN1-974_Rev			H7NAR1145	1
AN1-487_Rev	NARuc	Bm-N3-R	NARuc	N/A
M				
MFUc	MFUc	MFUc	MFUc	N/A
HMF581	HMF581	HMF581	HMF581	4*
HMR400	HMR400	HMH7R366	HMH7R366	1
MRUc	MRUc	MRUc	MRUc	N/A
NS				
NSFUc	NSFUc	NSFUc	NSFUc	N/A
HNSF481	HNSH7F355	HNSH7F355	HNSH7F355	7*
HNSR504	HNSH7R474	HNSH7R474	HNSH7R474	5*
NSRUc	NSRUc	NSRUc	NSRUc	N/A

Table 5.4 List of WGS primers.

The first column contains all WGS primers designed by Dr. Monica Galiano, HPA MS. The second column contains all approved WGS primers (no more than three mismatches, no mismatches nearby or at starting point elongation) and additional H7 specific primers (blue).

The third column includes additional H7 specific primers (green), designed after other primers were found unsuitable (no sequencing result was obtained). The fourth column contains all primers used for the final H7 WGS strategy. New primers are coloured red. The last column represents the number of mismatches between the named primers in column 4 and the consensus of alignments of sequences named in *Table 5.5*. This analysis was a theoretical approach to determine whether primers would be suitable for WGS of other H7 viruses. Mism. = Number of mismatches; * = unsuitable primer; † = last nucleotide of primer is mismatch; N/A = not applicable (no mismatches were found).

replacement primers were designed based on the sequence of A/Mallard/Netherlands/9/05 or A/Duck/Singapore/3/97. At this stage, nine primers were replaced; one primer used to sequence PB2, one primer used to sequence PB1, one primer used to sequence PA, one primer used to sequence NP, all four primers used to sequence NA and one primer used to sequence M (*Table 5.4, 2nd round*).

A second round of sequencing of E480 was performed using the newly designed primers, after which only the NA gene was not fully sequenced. Four additional primers were developed (*Table 5.4, 3rd round*), which resulted in a functioning WGS assay for both E480 and E481.

To determine whether these primers would be suitable for WGS of other H7 viruses, 10 H7 sequences per gene segment were aligned using Clustal W in DNASTAR Lasergene 9 Core suite software (*Table 5.5*). Virus strains from the North American and Eurasian lineages were compared. The final H7 WGS primers were matched to the consensus alignment for each gene. The number of mismatches is stated in column five of *Table 5.4*. Where there were more than 3 mismatches or the last nucleotide of the primer was a mismatch, primers were deemed unsuitable. From 52 primers, only five were found to be unsuitable for WGS of other H7 viruses. This *in silico* investigation indicated that the H7 WGS sequencing strategy would be suitable for sequencing other H7 viruses of the five subtypes (H7N1, H7N2, H7N3, H7N7, H7N9) that were compared; however the strategy remains to be validated experimentally.

Virus name	Subtype	Gene segment
A/Chicken/MD/MinhMA/2004	H7N2	PB2, PB1, PA, NP, M
A/Chicken/New York/14714-9/1999	H7N3	NS
A/Chicken/NY/12273-11/1999	H7N3	PB2, PB1, PA
A/Chicken/NY/21665-73/1998	H7N2	NS
A/Chicken/PA/9701524/1997	H7N2	NS
A/Chicken/PA/9801289/1998	H7N2	PB2, PB1, PA, NP, NS
A/Chicken/Pakistan/34669/1995	H7N3	NA
A/Duck/Alberta/49/1976	H7	M, NS
A/Duck/France/02166/2002	H5N3	NA
A/Duck/Germany/1215/1973	H2N3	NA
A/Duck/Hokkaido/Vac-2/2004	H7N7	PB1, PA, NP, M
A/Duck/Italy/4692-9/2004	H7	PA, NP, M, NS
A/Duck/Jiangxi/1760/2003	H7N7	PB2, NS
A/Duck/Jiangxi/1786/2003	H7N7	PB1, NP, M
A/Duck/New Zealand/164/1976	H11N3	NA
A/Laughing gull/Delaware/34/1994	H2N3	NA
A/Mallard/France/2518/2001	H9N3	NA
A/Mallard/Italy/250/2002	H7N1	PB2, PA, M, NS
A/Mallard/Italy/299/2005	H7N7	PB2, NP, M
A/Pintail/Alberta/21/1979	H7	PB1
A/Pintail/MN/423/1999	H7N3	PB2, PB1, PA
A/Ruddy turnstone/DE/1538/2000	H7N9	PB2, PA, NP
A/Ruddy turnstone/NJ/195/2006	H7	NP, M
A/Shorebird/Delaware/2696/1988	H7N3	NA
A/Teal/France/2546/2001	H1N3	NA
A/Tern/Astrakan/775/1983	H13N3	NA
A/Tern/South Africa/1961	H5N3	NA
A/Tundra Swan/NC/671-058/2006	H7	NP, NS
A/Turkey/Italy/121964/2003	H7N3	PB1
A/Turkey/Italy/68819/2003	H7N3	PB1, M
A/Turkey/Italy/97500/2003	H7N3	PB2, PA, M
A/Turkey/VA/66/2002	H7N2	NS
A/Turkey/VA/Sept67/2002	H7N2	PB2, PB1, PA, NP

Table 5.5 Virus strains used in multiple sequence alignment to analyze suitability of final H7 WGS primers for WGS of other H7 viruses

For each gene segment, ten sequences were aligned using the ClustalW method in DNASTAR software (Lasergene 9 Core suite). The resulting consensus sequence was used for comparison with the final H7 WGS primer sequences. The numbers of mismatches are stated in *Table 5.4*, primers which were deemed unsuitable are marked (*).

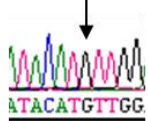
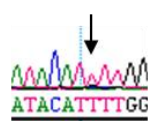
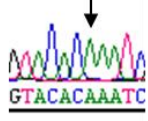
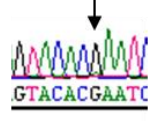
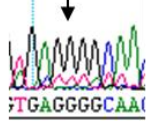
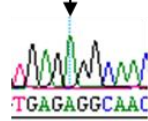
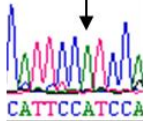
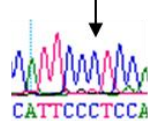
Gene	Position	E480	E481
PB2	650		
	895		
	1982		
NS	592		

Figure 5.1 Chromatographs of PB2 and NS1 genes of E480 and E481

Using the H7 WGS assay, all gene segments of E480 and E481 were sequenced. Multiple sequence alignments using DNASTAR Lasergene 9 Core suite software revealed four mutational differences; three in the PB2 gene and one in the NS gene. Representative chromatographs of these four mutations are depicted here, stratified per gene segment.

Gene	Nucleotide			Amino acid		
	<i>Position</i>	<i>E480</i>	<i>E481</i>	<i>Position</i>	<i>E480</i>	<i>E481</i>
PB2	650	G	T	206	Methionine	Isoleucine
	895	A	G	288	Glutamine	Arginine
	1982	G	A	-	-	-
NS	529	G	C	-	-	-

Table 5.6 Mutational differences between H7N3 isolates E480 and E481

Nucleotide and amino acid differences are listed. Nucleotide mutation 1982 in PB2 was synonymous. Nucleotide mutation 529 in NS was synonymous in both the NS1 and NS2 open reading frame.

	Human isolates (%)	Avian isolates (%)
E480 (M206)	2253 (99.96)	2962 (99.80)
E481 (I206)	0 (0)	3 (0.10)

Table 5.7 Comparative analysis of identity of amino acid 206 in H7N3 isolates E480 and E481 against available avian and human PB2 sequences

The percentages of isolates with amino acid M or I at position 206 in PB2 were calculated using 2254 human PB2 sequences, and 2968 avian PB2 sequences. Sequences were downloaded from the NCBI influenza database on 23rd November 2011 using the following search criteria: Type A; Host *Human* or *Avian*; Region *Worldwide*; Full-length; Collapse identical sequences

	Human isolates (%)	Avian isolates (%)
E480 (Q288)	2240 (99.38)	2895 (97.54)
E481 (R288)	5 (0.22)	61 (2.06)

Table 5.8 Comparative analysis of identity of amino acid 288 in H7N3 isolates E480 and E481 against available avian and human PB2 sequences

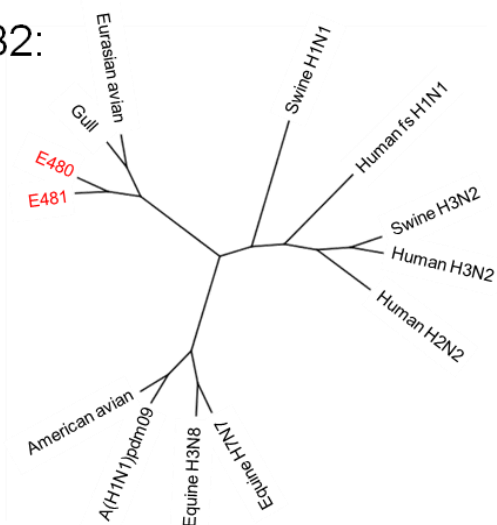
Percentages of isolates with amino acid Q or R at position 288 in PB2 were calculated using 2254 human PB2 sequences, and 2968 avian PB2 sequences from the NCBI Influenza Virus Database. Sequences were downloaded from the NCBI influenza database on 23rd November 2011 using the following search criteria: Type A; Host *Human* or *Avian*; Region *Worldwide*; Full-length; Collapse identical sequences

5.2.1.2 Molecular comparison of H7N3 isolates E480 and E481

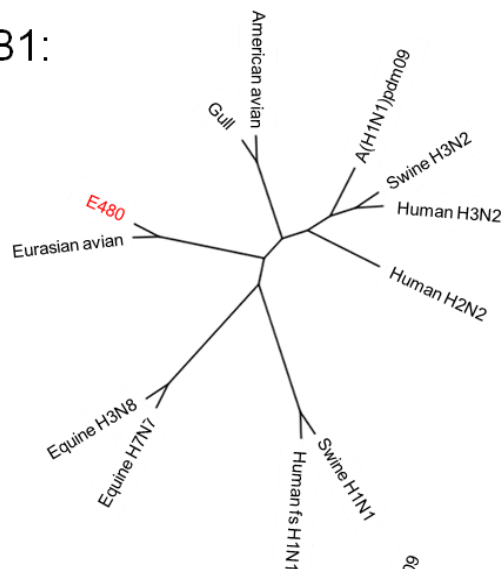
After successful whole genome sequencing of both E480 and E481, alignments were made using ClustalW (MegAlign, DNASTAR Lasergene 9 Core suite software) to compare the gene segments of the two virus isolates. In gene segment PB2, three differences were found (E480→E481; G650T, A895G and G1982A). In gene segments NS one difference was found (G529C) (*Figure 5.1*). No differences were found in the other gene segments. Both G650T and A895G mutations in PB2 were non-synonymous (M206I and Q288R respectively) whereas the other two mutations were synonymous (*Table 5.6*).

To investigate whether the non-synonymous mutations were commonly found in PB2, multiple protein sequence alignments were performed using MAFFT (<http://mafft.cbrc.jp/alignment/server/>) of all full-length human (2254) and all full-length avian (2968) PB2 sequences from the NCBI Influenza Virus Sequence database (<http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database>) downloaded in May 2011. Identical sequences were collapsed; the earliest entry into the database was used. Interestingly, both amino acids found in E480 (throat isolate) were more frequently found than those in E481 (eye isolate). In human viruses, 2253 (99.96%) virus strains contained methionine at position 206, and 2240 (99.4%) virus strains contained glutamine at position 288 similar to E480. No human viruses contained I at position 206 and 5 (0.2%) viruses contained arginine at position 288 similar to E481. In avian viruses, 2962 (99.8%) virus strains contained methionine

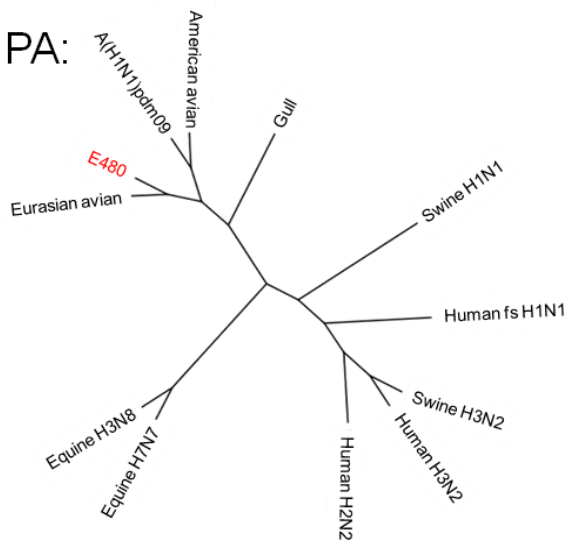
PB2:



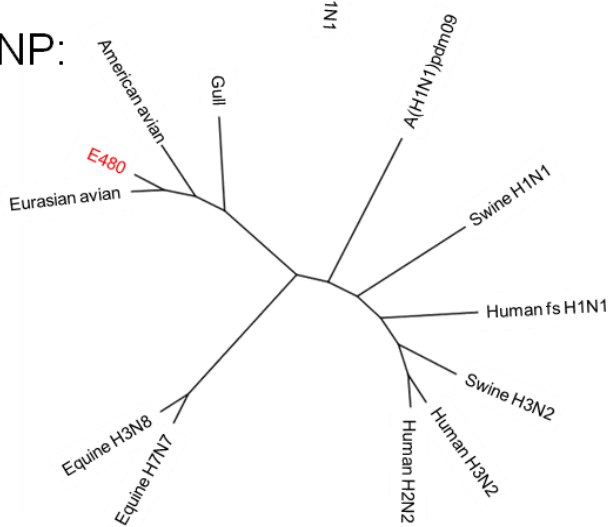
PB1:



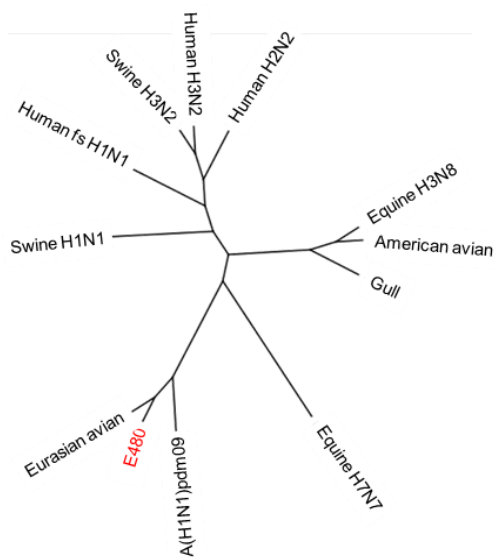
PA:



NP:



M:



NS:

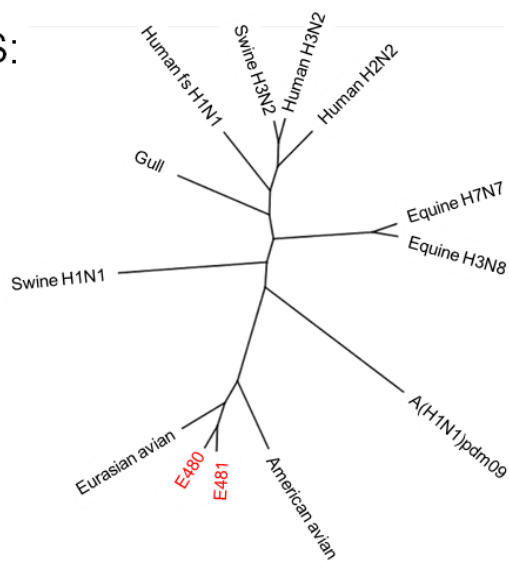


Figure 5.2 Phylogenetic trees of the internal genes of H7N3 isolates E480 and E481 compared to reference isolates of known origin

All internal genes of E480 and E481 were found to be closely related to Eurasian avian isolates. Nucleotide sequences analysed were chosen from Fouchier *et al.* (Fouchier et al. 2004) and were aligned using Geneious software followed by generation of maximum likelihood trees. Bootstrap analyzing parameters: 1000 trials and 111 random seeds. Fs = former seasonal

at position 206 and 2895 (97.5%) virus strains contained glutamine at position 288 like E480. Only 3 (0.1%) avian viruses contained I at position 206 and 61 (2.1%) viruses contained arginine at position 288 like E481 (*Table 5.7 and Table 5.8*).

5.2.1.3 Phylogenetic analysis of internal genes from E480 and E481

Due to a segmented genome, influenza viruses are capable of gene reassortment where one or more gene segments from one virus are swapped for the gene segments of another virus. This phenomenon has been well-described and is thought to have caused the pandemics in both 1957 and 1968 (Guan et al. 2010). A simple way to examine whether such reassortment occurred is by creating phylogenetic trees of all gene segments, comparing them to gene segments from a known source, such as different hosts and geographical origin (Suzuki 2010). This was done by Fouchier *et al.* for the H7N7 influenza virus that caused an outbreak in the Netherlands in 2003 (Fouchier et al. 2004). Using the same reference nucleotide sequences, phylogenetic trees were constructed of all internal genes of E480 (and E481) and reference sequences using DNASTAR software as described in Materials and Methods (*Figure 5.2*). As expected and comparable to the result by Fouchier *et al.* (Fouchier et al. 2004), all gene segments were most similar to Eurasian avian influenza, which suggests no reassortment with non-Eurasian avian influenza strains took place. Reassortment events are relatively rare, especially between different hosts and with viruses from a different geographical origin. It should

be noted that this analysis does not exclude reassortment between influenza viruses from Eurasian avian origin.

5.2.1.4 Molecular comparison of HA genes of H7N3 isolates E480 and E4054

A/Chicken/England/4054/2006 (E4054, H7N3) was isolated from poultry on 26 April 2006 in England as part of the same outbreak as E480 and E481 and the HA gene was partially sequenced (accession number EF467826). The HA protein sequence of this isolate was compared to the HA protein sequence of E480 using Megalign (DNASTAR Lasergene 9 Core Suite software). Two amino acid substitutions were found in the signal peptide of E480 HA compared to E4054 HA; T3I and A9V (H7 numbering). The signal peptide of H7 influenza virus, which is 18 amino acids long, is generally not conserved, differences are found across all amino acids. 826 H7 HA sequences were downloaded from the NCBI Influenza Database on 15th April 2012 using the following search criteria: Type A; Subtype *H7N**; Collapse identical sequences. Residue 3 was not represented in 40 out of 826 sequences, but found to be T in 66% (520/786) of the remaining sequences and I in 30% (237/786) of the remaining sequences. Valine (V) at position 9 was not found in any of the 826 H7 HA sequences, whereas A was present in 39% (322/826) of HA sequences. Therefore the E4054 HA signal peptide sequence was more representative of a typical H7 virus. To investigate whether the signal peptide of E480 was likely to be functional, a signal peptide prediction program for eukaryotes (SignalP 4.0) was used (Petersen et al. 2011) using the first 30 amino acids of both E480 and E4054. This program

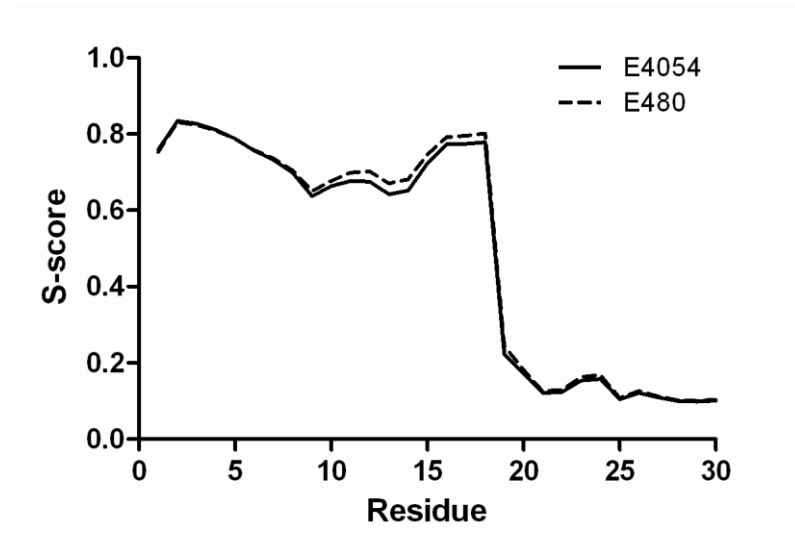


Figure 5.3 Signal peptide analysis of avian and human H7 sequences E4054 and E480

Signal peptide recognition program SignalP 4.0 was used to analyse the S-score of the first 30 amino acids of avian H7N3 HA sequence E4054 and human H7N3 HA sequence E480. A high S-score is allocated to an amino acid that is predicted to be part of the signal peptide, whereas a low S-score is allocated to an amino acid that is predicted to be part of the mature protein. Both E4054 and E480 were predicted to contain a signal peptide (residue 1-18) with very similar S-scores.

calculates both the cleavage site and the S-score per amino acid. A high S-score is assigned to amino acids that are part of the predicted signal peptide, whereas low S-scores are assigned to amino acids that are part of the mature protein. *Figure 5.3* shows a graphical representation of the S-score. Both proteins were predicted to be cleaved at residue 19. Furthermore, the overall S-scores were very similar (0.73 for E4054 vs. 0.75 for E480); suggesting the two mutations found in the signal peptide of E480 HA would have no effect on its function. The mutation at residue three (T3I) had no effect on the S-score. The mutation at residue nine (A9V) slightly increased the S-score starting at residue nine continuing to residue 20. This most likely does not have an effect on the function of the signal peptide of E480.

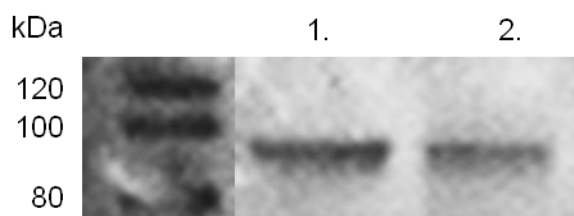


Figure 5.4 Western blot of recombinant HA proteins expressed in SF9 cells

Protein levels of 1) E480 and 2) M105. Proteins were run on a 10% Bis/Tris gel and stained with anti-human Fc. Molecular protein mass was predicted to be 87.25 kDa for E480 and 87.14 for M105 using the Protein Molecular Weight Calculator from Molecular Gateway (ScienceGateway). Two lanes between the ladder and lane 1 were removed from the original image, causing a difference in background colour.

5.2.2 Cell tropism of E480

5.2.2.1 Cell tropism of E480 in human airway epithelium

HAE cells containing both non-ciliated and ciliated cells were used to investigate whether the binding characteristics of E480 HA were human- or avian-like. To achieve this E480 HA protein was expressed using a recombinant baculovirus expression system. First the E480 HA protein was codon optimized for expression in insect cells by GeneArt (Life Technologies, UK). This step was necessary, as the original HA sequence was found to be toxic in bacterial culture and cloning of this sequence was unsuccessful. The optimized HA sequence was cloned using the pAc3CFc His Sfi1 expression vector and HA protein was expressed in SF9 insect cells as described in section 3.2.2 and *Figure 3.6*. Detection of binding was facilitated by fusion to the human Fc tag (Shelton et al. 2011).

The HA of avian H7N7 isolate A/Mallard/Sweden/105/2002 (M105, Genbank accession number AY999989) was expressed similarly. Protein production was confirmed with WB against the His tag (*Figure 5.4*).

These two recombinant H7 HA proteins were used to investigate whether the cell type in HAE cultures used for receptor binding varied between an avian H7 isolate and the human isolate E480. If a differential cell tropism was found, this could indicate either that the receptor binding preference changed within the human host or that a slightly different receptor binding preference increased the chance of the initial zoonotic jump from poultry to humans. An equivalent amount of each HA protein, based on visual inspection of the strength of WB bands,

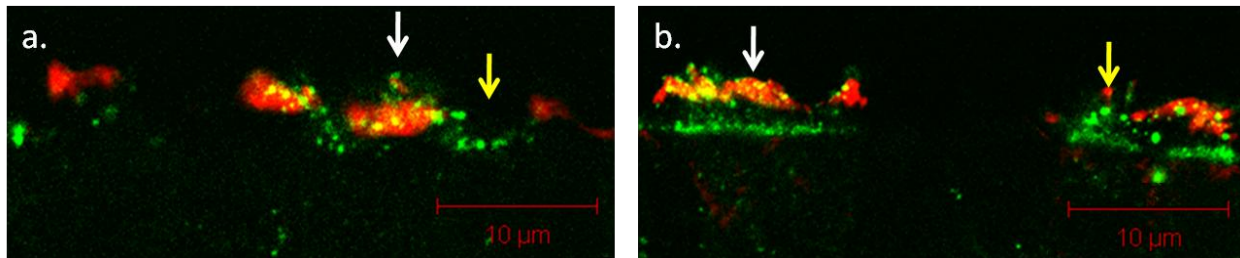


Figure 5.5 Binding of recombinant H7 HA proteins to HAE sections

HAE culture sections obtained from Professor Ray Pickles, University of North Carolina, were probed with HA-Fc recombinant proteins from a) E480, an H7N3 virus isolated from a human case, and b) M105 avian influenza virus. Ciliated cells were identified using anti-acetylated α -tubulin (AMS Biotechnology) (red) and HA-Fc proteins were identified using anti-human Fc antibody (Sigma) (green). Images are representative of multiple probed sections. Both ciliated (white arrow) and non-ciliated (yellow arrow) cells were found in HAE sections.

was used to probe HAE sections (*Figure 5.4*). Ciliated cells in the formalin fixed HAE sections were identified using anti-acetylated α -tubulin antibody (AMS Biotechnology) (red) and HA protein binding to specific cells was detected by anti-human Fc antibody (Sigma) (green) (*Figure 5.5*). In total HA protein of E480 bound to 46.8% of available cells. HA protein of E480 did not show a clear preference for ciliated or non-ciliated cells, 45.1% of the bound cells were ciliated and 54.9% were non-ciliated. In comparison to E480 HA, the HA protein of M105 bound to a similar percentage of total cells (56.8%) but a higher proportion of them were ciliated cells (71.9% ciliated vs. 38.1% non-ciliated). Consequently, the non-ciliated/ciliated cell binding ratio was 1.2 for E480 and 0.6 for M105, which reflects a decreased ciliated cell binding of E480 compared to a prominent ciliated cell binding for M105 (*Figure 5.6*). This result was compared to the ratios of cell binding obtained by HA proteins of wild type E195 and E195 E227A (*Figure 3.9*). Whereas E195 HA protein showed preferential binding to non-ciliated cells (ratio 3.9), the ratio obtained by E195 E227A was very similar to that of E480 (1.2 in both cases). As discussed in section 4.2.1, E195 E227A was able to transmit between ferrets. The E480 HA protein binding result suggests that E480 has an increased human-like receptor binding profile, which could explain why this virus was able to jump the host species barrier.

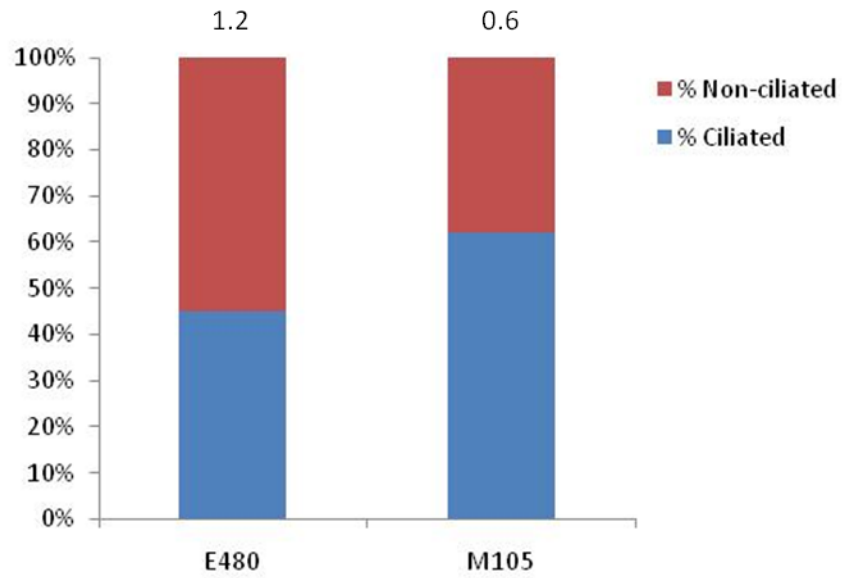


Figure 5.6 Quantification of recombinant HA protein binding to HAE sections

The percentages of non-ciliated and ciliated cells were quantified by blind counting >70 cells per sample. The non-ciliated/ciliated cell binding ratio is displayed above each bar.

5.2.2.2 Cell tropism of E480 in the IOBA-NHC cell line

E481 was isolated from the left eye of a human case, whereas E480 was isolated from the throat of the same human case (*Table 5.2*). Because the initial cell tropism of influenza is dictated by the virus HA receptor binding preference, and no molecular differences were found between the HA genes of E480 and E481 (see paragraph 5.2.1), all cell tropism experiments were done with E480 virus only. To investigate whether E480 can infect conjunctival cells, we used the IOBA-NHC cell line, kindly provided by Dr. Virginia Calder (Institute of Ophthalmology, UCL). This cell line originates from normal human conjunctival cells and retains many important morphological and functional conjunctival epithelial characteristics *in vitro* such as CK-3 expression (Diebold et al. 2003). Both α 2,3-linked and α 2,6-linked sialic acid were expressed on IOBA-NHC cells (*Figure 5.7*).

IOBA-NHC cells were seeded on coverslips, infected with virus at an MOI of 0.1 and incubated at 37°C for 16 hrs. After fixation with ice-cold methanol/acetone 50:50, the cells were co-stained for NP protein (green) and with either MAA or SNA lectin (red). These lectins are specific for α 2,3-linked sialic acid and α 2,6-linked sialic acid respectively. Images were taken using an inverted fluorescence microscope (Zeiss Axiovert 200). Using ImageJ software (<http://rsbweb.nih.gov/ij/>) brightness and contrast was adjusted in exactly the same way for every image. NP-positive and lectin-positive images were merged. Lastly, both the red and green images, adjusted for brightness and contrast, were converted such that all green or red pixels were now white and all dark pixels were converted into black pixels. This resulted in a

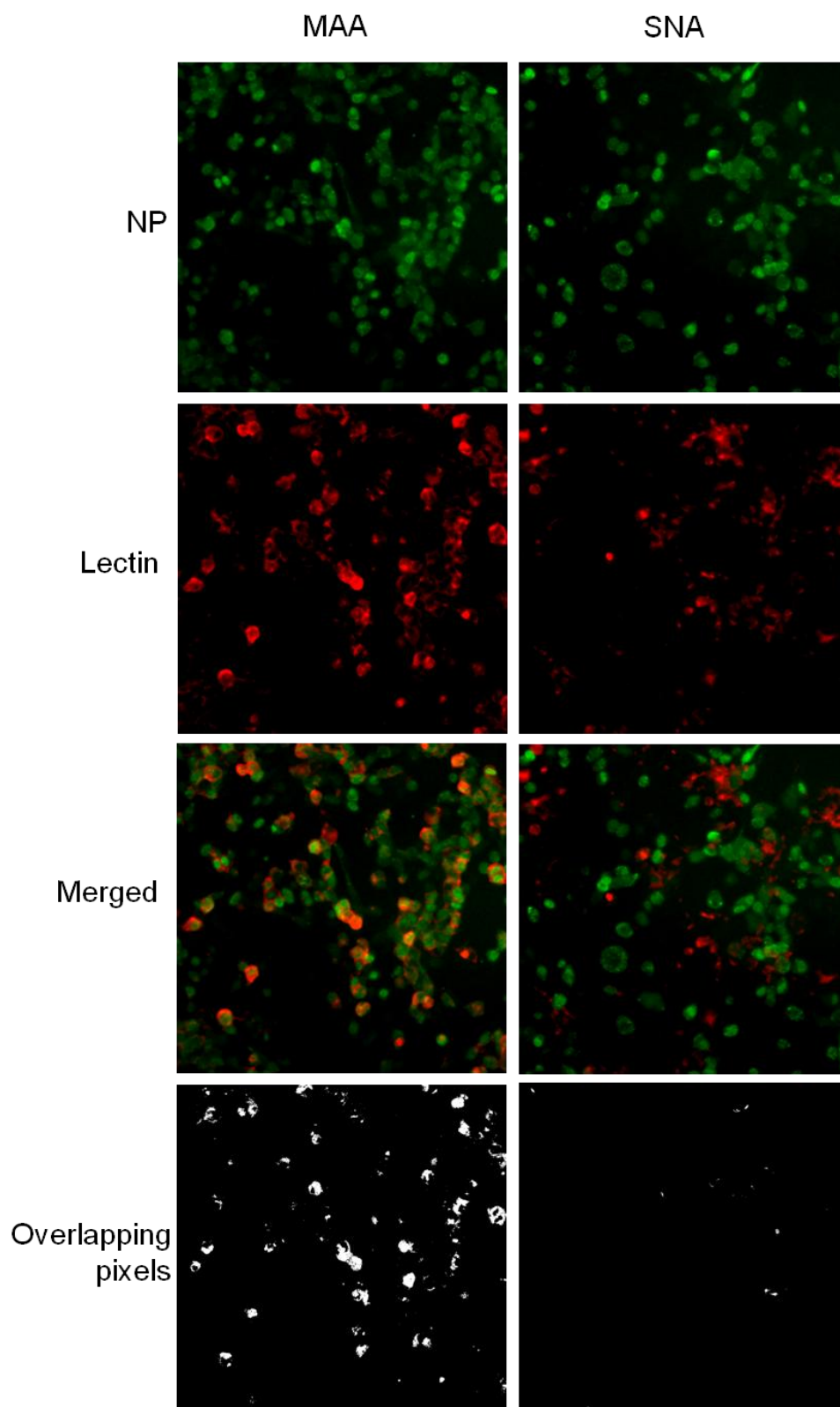


Figure 5.7 Infection of IOBA-NHC cells with E480

Cells were mounted on coverslips, infected with E480 (MOI 0.1) and incubated at 37°C for 16 hrs. After fixation with acetone/methanol (50:50), the cells were probed with anti-NP protein antibody (green, panel 1) and either MAA or SNA lectins (red, panel 2). Using ImageJ software (<http://rsbweb.nih.gov/ij/>) NP-positive images were merged with matching lectin-positive images (panel 3). Lastly, co-location was visualized by converting pixels that were positive in both the NP-positive image and lectin-positive image to white, whereas all other pixels were converted to black (panel 4). N=3, representative images of all three experiments were used.

binary image. The NP-positive image was then 'multiplied' by the lectin-positive image using ImageJ image calculator. This resulted in an image with white pixels where the primary picture pixels were white in both images, and black pixels where the primary picture pixels were black in both images or only one of the images contained white pixels. This allowed overlapping NP and lectin positive cells to be identified. Using these methods, it was determined that E480 had a preferential cell tropism for conjunctival epithelial cells positive for α 2,3-linked sialic acid (*Figure 5.7*). Almost no infected cells were found to be positive for α 2,6-linked sialic acid, whereas most cells that were infected were also α 2,3-linked sialic acid positive. Although primary conjunctival cells are permissive for influenza virus infection (Belser et al. 2011c), to our knowledge this is the first time a conjunctival cell line (IOBA-NHC) has been shown to be infected by influenza A virus. H7 is known to have an ocular cell tropism, and it remains to be investigated whether other subtypes in addition to H7 are also able to infect IOBA-NHC.

5.2.3 Replication of E480, E481 and M105 in IOBA-NHC cell lines and HAE cultures

In the previous sections (5.2.2.1 and 5.2.2.2) it was shown that E480 has an increased preference for non-ciliated cells, but still bound to α 2,3-linked sialic acid-expressing IOBA-NHC cells. M105 showed a preference for binding ciliated cells in HAE cell sections, however cell tropism of M105 was not investigated in IOBA-NHC cells. Replication of E480, E481 and M105 viruses in cells derived from the potential target tissues (eye and respiratory tract) was investigated. By examining virus replication changes in HA affecting cell tropism as well as any effect of differences between internal proteins could be investigated. Firstly, it was investigated whether the human virus isolates replicated better than the avian M105 virus in human conjunctival and airway epithelial cells. Secondly as differences were found in the PB2 sequences of E480 and E481 which might affect virus replication it was investigated whether E480 and E481 differ in viral replication. PB2 is thought to be the second most important protein, after HA, in determining influenza virus host range. For example, the residue at position 627 in PB2 has been associated with tissue cell tropism; E at position 627 is commonly found in avian influenza viruses whereas lysine at this position is commonly found in human influenza viruses. A human influenza virus reassortment with an avian PB2 gene segment was found to display a restricted replication profile in mammalian cells, however a single amino acid substitution in PB2 (E627K) fully restored replication (Subbarao, London and Murphy 1993).

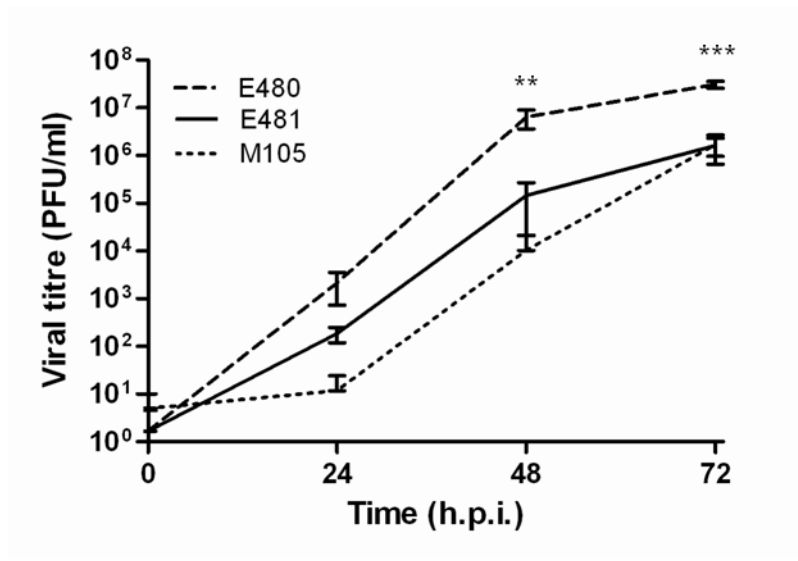


Figure 5.8 Multi-cycle growth curve of human H7N3 isolates E480, E481 and avian isolate M105 in IOBA-NHC cell line

IOBA-NHC cells were infected with E480, E481 or M105 at an MOI of 0.001 and incubated at 37°C. Samples were taken from the supernatant at t=24, 48 and 72 h.p.i. and checked for the presence of influenza virus using plaque titration on MDCK cells. Data shown represent the mean and standard deviation of 3 wells. Statistical differences between E480 and E481 are represented and were determined using one-way ANOVA. ** = $p < 0.01$, *** = $p < 0.001$

Another PB2 residue at position 701 is associated with increased activity of avian origin virus polymerases in human cells. A mutation from D to asparagine (N) at this position was linked to replication of avian influenza virus H5 in mice and guinea pigs (Gao et al. 2009, Li et al. 2005). Combining both the E627K and D701N mutation was found to increase avian influenza virus H5 replication in the guinea pig model (Steel et al. 2009). Interestingly, A(H1N1)pdm09 did not encode the human-like signatures at position 627 or 701. A third amino acid change (Q591R) in A(H1N1)pdm09 PB2 is linked to its ability to replicate in the human host (Yamada et al. 2010, Mehle and Doudna 2009).

Therefore, it was of interest to investigate whether the two amino acid differences in PB2 between E480 and E481 (M206I and Q288R) had an effect on viral replication in different cell lines, even though the mutations found in PB2 in E481 were rare and not specific for human H7 PB2 (*Table 5.7* and *Table 5.8*).

5.2.3.1 Replication of E480, E481 and M105 in IOBA-NHC cell lines

IOBA-NHC cells were infected with E480, E481 and M105 at an MOI of 0.001 and incubated at 37°C and 5% CO₂. Samples were taken from the supernatant at different time points post infection (24, 48, 72 hrs) and checked for the presence of influenza virus by plaque titration on MDCK cells (N=3). All viruses were capable of replication in IOBA-NHC cells, but avian influenza virus M105 replicated to a lower viral titre than both E480 and E481, which was

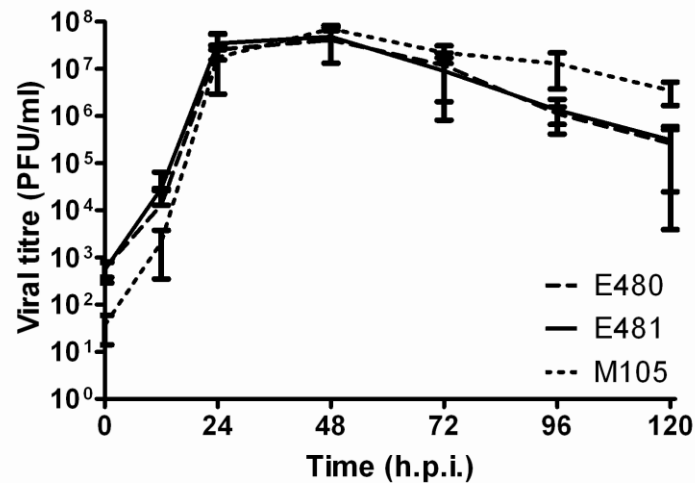


Figure 5.9 Multi-cycle growth curve of human H7N3 isolates E480, E481 and avian isolate M105 in HAE cultures at 37°C

HAE cultures were infected with E480, E481 or M105 at an MOI of 0.01 and incubated at 37°C. Samples were taken from the supernatant at t=12, 24, 48, 72, 96 and 120 h.p.i. and checked for the presence of influenza virus using plaque titration on MDCK cells. Data shown represent the mean and standard deviation of 3 wells. Statistical differences between E480 and E481 or M105 were determined using one-way ANOVA.

significant in comparison to E480. Replication of M105 was delayed; there was no increase in viral titre between 0 and 24 h.p.i. This suggests that both E480 and E481 were better adapted to human IOBA-NHC cells. Interestingly, throat isolate E480 reached higher viral titres at each time point compared to E481 and M105, which compared to E481 was found significant at t=48 h.p.i. (in 2 out of 3 performed growth curves) and t=72 h.p.i. (in 3 out of 3 performed growth curves) by one-way ANOVA (*Figure 5.8*).

It should be noted that M105 was grown in a mammalian cell line (MDCKs) which could have positively influenced its ability to grow in mammalian cells such as IOBA-NHC cells.

5.2.3.2 Replication of E480, E481 and M105 in HAE cultures

Viral replication was then tested in HAE cultures, which are thought to represent the human upper respiratory tract. HAE cultures were infected with E480, E481 and M105 at an MOI of 0.01 and incubated at 37°C. Samples were taken from the apical surface at different time points and checked for the presence of influenza virus using plaque titration on MDCK cells (N=1). All viruses were able to replicate in HAE cultures, maximum viral titres were reached rapidly at t=24 h.p.i. Similar to the observation in IOBA-NHC, avian influenza virus M105 replication was delayed at early time points compared to both human isolates of H7N3, although these differences were not found to be significant. No differences were observed between viral titres of E480 and E481 at different time points (*Figure 5.9*). Again, it should be taken into account that M105 was grown in a mammalian cell line. Sequence comparison to the original isolate will

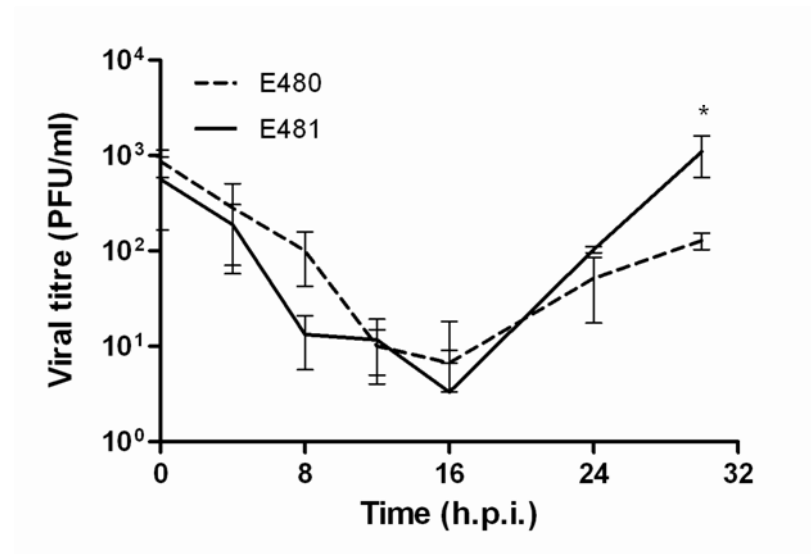


Figure 5.10 Multi-cycle growth curve of human H7N3 isolates E480 and E481 in HAE cultures at 32°C

HAE cultures were infected with E480 or E481 at an MOI of 0.01 and incubated at 32°C. Samples were taken from the apical surface at t=4, 8, 12, 16, 24 and 30 h.p.i. and checked for the presence of influenza virus using plaque titration on MDCK cells. Data shown represent the mean and standard deviation of 3 wells. Statistical differences between E480 and E481 were determined using Student's t-test. * = $p < 0.05$

reveal whether the stock of M105 used in these experiments has adjusted to growth in mammalian systems.

As maximum titres were found at such an early time point, the growth curve was repeated at 32°C with E480 and E481 up to 30 h.p.i. and samples were taken at earlier time points (N=1). Here, viral replication was slow compared to the growth curve performed at 37°C and the plateau of viral replication was not yet reached at t=30 h.p.i. Moreover, at t=30 h.p.i., E481 displayed increased viral replication compared to E480 which was found to be significant by Student's t-test (*Figure 5.10*). Temperature-dependent attenuation of viral replication has been observed previously in avian influenza viruses, and been attributed to the PB2 gene. Indeed, obtaining the humanizing E627K mutation in PB2 re-established viral replication at lower temperatures (Massin et al. 2001).

Virus	Egg passage	Nt at position 650	Nt at position 895	Identifier
E480	4	G	A	N/A
E480	1	G	A	E480, 0.5ml, 08-05-2006
E480	2	G	A	E480, 250µl, 01-05-2006
E480	3	G	A	E480, H0617800001, T/S, egg pass1
E481	4	T	G	N/A
E481	1	G	A	E481, 01-05-2006, 500µl, E7
E481	2.1	T	G	E481, 20-10-2006, 0.5ml, MK+LH
E481	2.2	G	A	E481, H0617800623, L/Eye, egg pass 1

Table 5.9 Summary of sequence results E480 and E481 egg passage stocks

The PB2 gene segment (nt 1-1473) of egg passage 1, 2 and 3 of E480 and E481 was sequenced using H7 WGS primers. A multiple sequence alignment using MegAlign (DNASTAR Lasergene 9 Core suite software) was performed to compare the nucleotide sequences. The viral stocks used throughout this chapter are displayed in green, the egg-passaged stocks used to grow these viral stocks are displayed in blue.

5.2.4 Sequencing of earlier egg passages of E480 and E481

The E480 and E481 influenza viruses used in this study were serial-passaged through eggs four times. A well-known artifact of serial passage of virus in eggs is adaptive mutations in influenza virus to this environment (Gambaryan, Robertson and Matrosovich 1999, Robertson et al. 1987, Robertson et al. 1993). To ensure the mutations we found in E480 and E481 egg passage 4 were representative of the consensus of the original isolates, the PB2 gene (nucleotide 1 to 1473) of earlier passages was sequenced. For E480, egg passage 1, 2 and 3 was sequenced. In this project, egg passage 3 was passaged once in eggs to grow a high viral titre stock. For E481, egg passage 1 and two times an egg passage 2 (2.1 and 2.2) was sequenced. In this project, egg passage 2.1 was used to grow a viral stock and passaged twice in eggs to obtain a high viral titre stock.

All earlier E480 egg passages contained the same nucleotide sequence as E480 egg passage 4. However, only one of the earlier egg passages of E481 (egg passage 2.1) contained the same nucleotide sequence as E481 egg passage 4. Both egg passage 2.1 and egg passage 4 were unusual compared to all other egg passages in containing the G650T and A895G mutations. This suggests that either the non-synonymous mutations found in PB2 were egg-adaptive mutations, or egg passage 2 represents a quasi-species found in the original isolate (*Table 5.9*).

E480(E481) versus M105		
<i>Experiment</i>	<i>Result</i>	<i>Significance</i>
HA protein binding on HAE cultures	E480: 45.1% ciliated, 54.9% non-ciliated M105: 71.9% ciliated, 38.1% non-ciliated	N/A
Virus replication in IOBA-NHC cells	M105 is attenuated compared to E480 and E481	p>0.01
Virus replication in HAE cultures	M105 is attenuated compared to E480 and E481	NS
E480 versus E4054		
<i>Experiment</i>	<i>Result</i>	<i>Significance</i>
Multiple alignment of HA protein	Two non-synonymous changes in signal peptide	N/A
E480 versus E481		
<i>Experiment</i>	<i>Result</i>	<i>Significance</i>
Multiple alignment of all segments	One synonymous mutation in PB2 One synonymous mutation in NS Two non-synonymous mutations in PB2	N/A
Virus replication in IOBA-NHC cells	E481 is attenuated compared to E480	p>0.01
Virus replication in HAE cultures at 37°C	No difference	NS
Virus replication in HAE cultures at 32°C	E480 is attenuated compared to E481	p>0.05
E480		
<i>Experiment</i>	<i>Result</i>	<i>Significance</i>
Cell tropism in IOBA-NHC cells	Clear preference for α 2,3-linked sialic acid expressing cells	N/A

Table 5.10 List of all experimental results

All experimental results were summarized in above table. N/A = Not applicable; NS = Not significant

5.3 Discussion

In this chapter, we showed that HA protein from E480 bound more non-ciliated cells in HAE cultures than HA protein from M105. Furthermore, replication of M105 in both IOBA-NHC and HAE cultures was attenuated compared to E480 and E481, although this difference was not significant in HAE cultures. These small differences could explain the ability of the virus to make the zoonotic jump. The HA protein of E480 and chicken virus E4054 differed only in the signal peptide, which was not thought to have an effect on cell tropism. Finally, two non-synonymous mutations were found in the PB2 protein of E480 compared to E481, which potentially accounted for a difference in replication in HAE cultures and IOBA-NHC cells. Unfortunately due to a lack of materials it was not possible to confirm whether these mutations were genuine.

After successful whole genome sequencing of both E480 and E481 using a newly developed WGS assay, the HA gene segment of E480 was compared to poultry isolate E4054. Two amino acid substitutions, T3I and A9V, were found in the HA signal peptide of E480. These mutations are not thought to have an effect on receptor binding properties, as the signal peptide is absent from the mature protein. Using a signal peptide prediction program, it was shown that the 'new' E480 signal peptide is still recognized as such, and the mutations are not thought to have an effect on expression of the HA protein in eukaryotic cells. Multiple sequence alignment using H7 HA sequences, showed that I at position 3 was common (30% of sequences), but valine at position 9 was not observed in any H7 HA sequences. In the signal peptide prediction outcome however, this mutation had no effect other than elevating the S-score, which in any case is high

for signal peptide linked amino acids. It is unclear whether this would affect the expression levels of HA in the eukaryotic cell.

Next, all gene segments of E480 and E481 were compared. As only the HA sequence of E4054 was known, no further comparison could be made between the poultry and human viruses. Two synonymous changes (one in NS, one in PB2) and two non-synonymous changes (both in PB2) were identified. None of the other gene segments had any differences.

Both HA and PB2 are thought to be key proteins in influenza virus that need to adapt to the host to allow replication of the virus and transmission between hosts. As the receptor binding protein, the HA protein is mostly important in the selection of which cells will be entered by influenza virus and thereby dictates initial cell tropism. Avian influenza viruses bind preferentially to $\alpha 2,3$ -linked sialic acid, whereas human influenza viruses preferentially bind $\alpha 2,6$ -linked sialic acid and this differential receptor binding preference is reflected in a divergence in cell tropism; avian influenza virus infects mostly ciliated cells, whereas human influenza virus infects non-ciliated cells in the HAE cell model (Matrosovich et al. 2007, Matrosovich et al. 2000, Matrosovich et al. 1997, Matrosovich et al. 2004a, Thompson et al. 2006, Ayora-Talavera et al. 2009, Shelton et al. 2011).

Together with PB1 and PA, PB2 forms the RdRp. Host-specific adaptations in this protein are thought to be mostly important for the replication of influenza virus in either avian or mammalian cells. Several changes in PB2 have been identified that result in such an adaptation and have been discussed previous. A mutation at position 627 from E to lysine (E627K) is known to

facilitate adaptation to the mammalian host (Hatta et al. 2001, Subbarao et al. 1993). A second mutation (D701N), which was identified in a natural H5N1 isolate, caused high pathogenicity in a mouse model (Li et al. 2005). Both of these mutations were found to contribute to efficient propagation in the human upper respiratory tract (Le et al. 2009) and be a factor in transmission in mammalian models (Van Hoeven et al. 2009b, Steel et al. 2009). A recently identified mutation is thought to have played a major role in the adaptation of A(H1N1)pdm09 to the human host, which has the avian signature amino acids at position 627 and 701. Here residue 590 and 591, which are closely located to residue 627 on the surface of PB2, are mutated from G to serine and glutamine to arginine respectively (Yamada et al. 2010).

Two possible scenarios would explain the adaptive effect of the PB2 mutations; either it is possible that a stimulatory host-specific co-factor is necessary for efficient replication of influenza virus, or alternatively a host-specific inhibitory factor in human cells is dysfunctional when these mutations occur. Recent research in our laboratory using heterokaryons formed by avian and human cells suggests a stimulatory host-specific co-factor is necessary for efficient replication in human cells, such as importin α (Moncorge, Mura and Barclay 2010).

The identification of the two amino acid mutations in PB2 is a potential adaptation of H7N3 to the human host. The influenza polymerase does not have any proofreading activity, and therefore tens of thousands of new viral mutants (quasispecies) are produced during replication of the virus (Drake 1993). Neither of the amino acids found in E481 (isolated from the eye) are regularly found in avian or human PB2 gene segment sequences. This could suggest that E480

represents an earlier consensus of H7N3, whereas E481 represents a quasispecies with a favorable fitness in the human host, allowing increased replication of this 'mutant virus'.

The mutations found in PB2 in earlier research linked to host adaptation are localised in the C-terminal part of the protein and are found adjacent in the crystal structure of PB2 (Yamada et al. 2010) at what is very likely to be the interaction point of PB2 and the (stimulatory or inhibitory) host-specific co-factor. In fact, the area in which the two mutations found in this study are located have so far not been linked to any function of PB2. It is therefore difficult to predict whether they are likely to affect viral replication.

The growth curves performed in IOBA-NHC cells and HAE cultures did show a difference; whereas E480 grew better in conjunctival epithelial cell line IOBA-NHC, E481 outgrew E480 in HAE cultures when incubated at 32°C, the temperature of the human upper respiratory tract, which is slightly lower than the temperature of the eye at 34°C (Fujishima et al. 1996). This suggests that amino acids 206I and 288R in PB2 result in increased replication in the human URT. However the molecular analysis does not support this, as these mutations are found in 2.06% and 0.22% of avian PB2 sequences respectively versus 0.1% and 0% in human PB2 sequences. It is possible that cells in the URT express co-factors important for influenza virus replication which are expressed to a lesser extent in conjunctival epithelial cells and that the presence of 206I and 288R result in a better exploitation of these factors. To further explore the adaptive formation of E481 over E480, growth curves should be performed in avian cells and compared to replication in human cells, although it should be noted that any potential adaptation

advantage to the human host does not necessarily come at a cost for the avian host. It would also be informative to increase the length of growth curves in HAE cultures performed at 32°C; a greater difference could potentially be found at later time points.

Development of the H7 WGS strategy during this project allowed retrospective analysis of earlier passages of E480 and E481 which had not been selected for study previously due to limited volumes. WGS of earlier passages of E481 revealed a different consensus in two out of three earlier egg passages. It is therefore possible that the mutations M206I and Q288R described in this chapter are the result of egg adaptations, a well-known artifact of egg passage (Gambaryan et al. 1999, Robertson et al. 1987, Robertson et al. 1993, Matrosovich et al. 1997, Lugovtsev, Vodeiko and Levandowski 2005). An alternative possibility is that the original clinical isolate of E481 contained a range of quasispecies, including E481 206M/288R as well as E481 206I/288R. As a consequence, in two egg passages the former quasispecies dominated, whereas in one egg passage the latter quasispecies dominated. To resolve this question next-generation sequencing of the original clinical isolates could be used, which will not just give the consensus of E480 and E481, but also allow analysis of the quasispecies content. This experiment would reveal whether E481 (and possibly E480) naturally contain virus with the 206I/288R combination or whether these mutations are purely egg-adaptations.

Cell tropism experiments performed with E480 revealed a very avian-like binding profile. In IOBA-NHC cells, the virus displayed a preferential infection pattern for α 2,3-linked sialic acid expressing cells. This preference for α 2,3-linked sialic acid was also reflected in its HA protein

binding profile on HAE cells; almost 50% of bound cells were ciliated. Compared to M105 recombinant HA protein binding a slight decrease in ciliated cell binding was found. This could potentially explain the ability of E480 to jump the zoonotic barrier. However, when this binding profile is compared to the A(H1N1)pdm09 E195 recombinant HA protein binding profile on HAE, there is still much more ciliated cell binding than would be expected with an influenza virus able to transmit between humans. E480 was able to infect a human but there was no evidence of human-to-human transmission during this outbreak. This binding profile suggests further adaptation to a more human-like non-ciliated cell binding profile would be required before this could happen.

Finally, phylogenetic trees were created to investigate whether reassortment of E480/E481 had occurred with non-Eurasian avian gene segments. However, all internal genes were found to be closely related to Eurasian avian lineages and therefore it was concluded that no reassortment occurred prior to the zoonotic jump.

Future research should focus on the 'missing link', by investigating replication and cell tropism of the chicken virus E4054. This will reveal whether differences between M105 and E4054, or between E4054 and E480 were responsible for the zoonotic jump. Importantly, cell tropism of M105 in IOBA-NHC was not yet investigated. It is possible that this virus is attenuated in initial infection of human conjunctival cells compared to E480, as suggested by the delay in viral replication as observed in *Figure 5.8*. Furthermore, the effect of single mutations in the PB2 of H7 as revealed via WGS assay should be investigated using a reverse genetic system. Finally,

although no virus was recovered from samples taken from patients presenting with influenza-like illness after exposure to H7-infected poultry, it should be attempted to isolate RNA and sequence the entire genome. This will provide us with a much larger spectrum of H7 viruses which are able to infect the human host and might provide more information on which amino acids are of particular importance in H7 influenza virus zoonosis.

6 Infection of ex-vivo whole porcine lungs with A(H1N1)pdm09

6.1 Introduction

In the middle of 2011, the opportunity arose to investigate a new model of influenza infection via collaboration with the Royal Brompton Hospital, London. The possibilities of ex-vivo lung perfusion (EVLP), a method developed by Steen *et al.* (Steen *et al.* 2003) to assess the quality and facilitate the reconditioning of rejected human donor lungs to increase the donor pool available for transplantation, have been further investigated by Dr. Mark Griffiths and Dr. Alastair Proudfoot at the Royal Brompton Hospital. Here, whole lungs are ventilated and perfused using an albumin-based medium, potentially with red blood cells (*Figure 6.1*). Applying this methodology, lung function and histology have been preserved for up to twelve hours (Cypel *et al.* 2008). While developed for clinical transplant, increasingly investigators are realising the application of this system as a tractable model in respiratory research; EVLP combines the 3D dimensions of a real lung with the physical factors involved in breathing. Furthermore, the immune system could potentially be included in this model, as it might be possible to include immune cells in the perfusate. However, EVLP experiments require a very specific expertise, are relatively challenging and expensive. As a pilot study, we investigated whether porcine lungs used in an EVLP experiment were able to support influenza replication. Within this chapter, several different readouts of influenza-infected porcine lungs in the EVLP setup are assessed, and uncontrolled factors are revealed.

A(H1N1)pdm09 was a very interesting candidate for the pilot study, as the first pandemic of the 21st century did not originate from birds, as was expected by many scientists in the field since 1997 when H5N1 directly infected humans, but from a swine-origin. Traditionally, the swine host has been seen as a possible mixing vessel for avian and human influenza, allowing for reassortment between these viruses and subsequent release of a potentially pandemic virus. This hypothesis was based on findings that the pig exhibits a relatively low species barrier towards infection with avian and human influenza viruses. Indeed, isolation of human influenza virus (Ottis et al. 1982, Katsuda et al. 1995) and avian influenza virus (Guan et al. 1996, Karasin et al. 2000) from pigs as well as documented reassortment events involving avian and human influenza viruses within pigs (Brown et al. 1998, Castrucci et al. 1993, Zhou et al. 1999) underline the possibility of the pig as a mixing vessel. Furthermore, phylogenetic analyses of the NP gene of avian, human and swine influenza viruses showed that swine influenza NP sequences were scattered across the phylogenetic tree, whereas avian and human influenza NP sequences were found to form two distinct branches (Scholtissek 1994), suggesting NP genes of swine influenza viruses have both avian and human origin. As discussed previously, avian influenza virus has HA that preferentially binds to α 2,3-linked sialic acid, whereas human influenza HA prefers α 2,6-linked sialic acid as its receptor. Interestingly, expression of both of these terminal sugars is found in the porcine trachea (Ito et al. 1998) although recent data suggests the distribution of α 2,3-linked sialic acid is restricted to lower LRT (bronchioles and alveoli) whereas α 2,6-linked sialic acid is found abundantly throughout the respiratory tract (Van

Poucke et al. 2010, Nelli et al. 2010). After circulation in the swine host, the HA of swine influenza viruses of avian origin have acquired a more human-like receptor binding profile, potentially preparing them for a zoonotic jump (Ito et al. 1998, Bradley et al. 2011). Finally, pigs are similar to the human host in regards of the physiology of the respiratory tract. To sum up, the hypothesis of the pig serving as a mixing vessel for generation of viruses with human pandemic potential has a thorough factual grounding. Nonetheless, for decades no such reassortant virus emerged from the swine population and hence people focused on H5N1 zoonoses which have occurred relatively frequently since 1997. Although A(H1N1)pdm09 is not a reassortant between an avian and human influenza virus but rather between two distinct swine lineages (Garten et al. 2009), the recent pandemic has again put pigs in the spotlight as a possible source for pandemic influenza. Therefore, porcine *in vitro*, *ex vivo* and *in vivo* models to investigate influenza pathogenesis, replication and transmission are vital research tools. Furthermore, swine influenza virus infections cause significant economic losses within the pig industry (Bennett, Christiansen and Clifton-Hadley 1999), illustrating the importance of research on this subject.

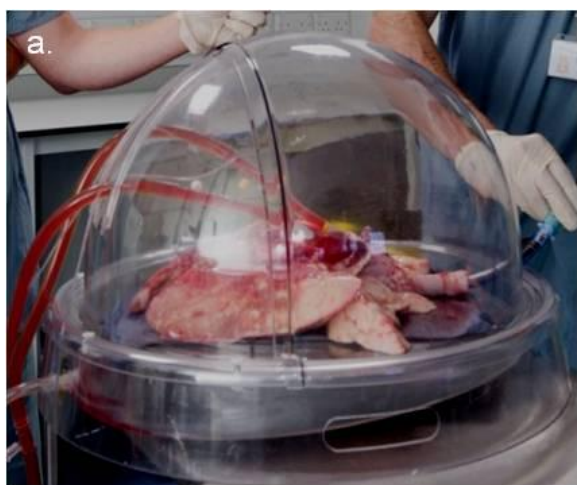
Other porcine infection models have been developed over the years. Primary porcine cells, developed either from alveolar epithelial cells (Daidoji et al. 2008) or respiratory epithelial cells derived from distal tracheal/proximal primary bronchial airway (Busch et al. 2008), were able to support replication of avian, human and swine influenza virus. In earlier published research, Busch *et al.* showed an H3N2 virus pair of either swine or human-lineage to have different

infectivity and replication kinetics in swine; swine-lineage H3N2 infected more cells and showed higher viral replication than human-lineage H3N2 in 7-week-old pigs (Landolt et al. 2003). Interestingly, this was mirrored in their primary cell model where increased infection of cells by the swine-lineage H3N2 isolate was found compared to the human-lineage H3N2. This suggests this particular model could have a good predictive value of infection in the host. The different infection profile was shown to depend on amino acids in the HA protein (Busch et al. 2008). However, cell lines usually lack several important physiological characteristics of the respiratory tract, such as cell polarization and the mucociliary escalator system.

Porcine and human *ex vivo* organ explants have been used extensively to investigate infectious disease such as *Bordetella bronchiseptica* and *Bordetella pertussis* as these tissue sections do reproduce the physiological characteristics and cellular complexity of the respiratory tract (Dugal, Girard and Jacques 1990, Soane et al. 2000). Not surprisingly, similar models using porcine explants to investigate influenza infection have been developed. Nunes *et al.* (Nunes et al. 2010) maintained porcine tracheal explants with an air-liquid interface and showed the cellular architecture of these explants was intact for 7 days during which time mucus was produced and cilia stayed active. Furthermore, this model was able to support viral replication of a swine H1N1 virus. A second group developed methods to culture *ex vivo* organ explants extracted from porcine nasal, tracheal, bronchial and lung sites. All of these tissue explants could support influenza replication, and interestingly showed limited growth of avian influenza in nasal and tracheal explants compared to swine and human influenza viruses. This result

correlated with the sialic acid expression in the different explants (Van Poucke et al. 2010, Glorieux et al. 2007). These models represent an interesting application, but unlike *in vivo* models exclude the involvement of the immune system (more specifically immune cells) in infection kinetics due to an absence of blood supply, and lack the physical complexity of the whole organ or the involvement of dynamic mechanic forces created by continuous breathing.

In contrast, *in vivo* models allow for investigation of numerous infection kinetics parameters within the host, such as cytokine expression, vaccine efficacy and transmission. Pigs have been proven useful in *in vivo* experiments to study replication of influenza virus and subsequent pathogenesis (Van Reeth et al. 2002, Hinshaw et al. 1981, Kida et al. 1994, Shortridge et al. 1998, Landolt et al. 2003, Weingartl et al. 2009) or influenza transmission between pigs and occasionally from pigs to ferrets (Choi et al. 2005, De Vleeschauwer et al. 2009, Brown et al. 1994, Zhu et al. 2011). Transmission experiments using pigs are challenging, as pigs are relatively large animals and difficult to handle. Furthermore, the price of experiments is significantly increased when sourcing 'high health status herd' pigs, which are extensively tested to ensure they are free of certain pathogens, including swine influenza. Accidental infection of laboratory workers after collecting nasal swabs from pigs experimentally infected with swine H1N1 has been reported, even though experiments were carried out using BSL3 containment level practices (Wentworth et al. 1997). Furthermore, outcome of disease is not always similar to that found in humans; pigs found to be infected with avian influenza virus H5N1 did not display clinical symptoms (Nidom et al. 2010) and swine experimentally infected with highly



c.

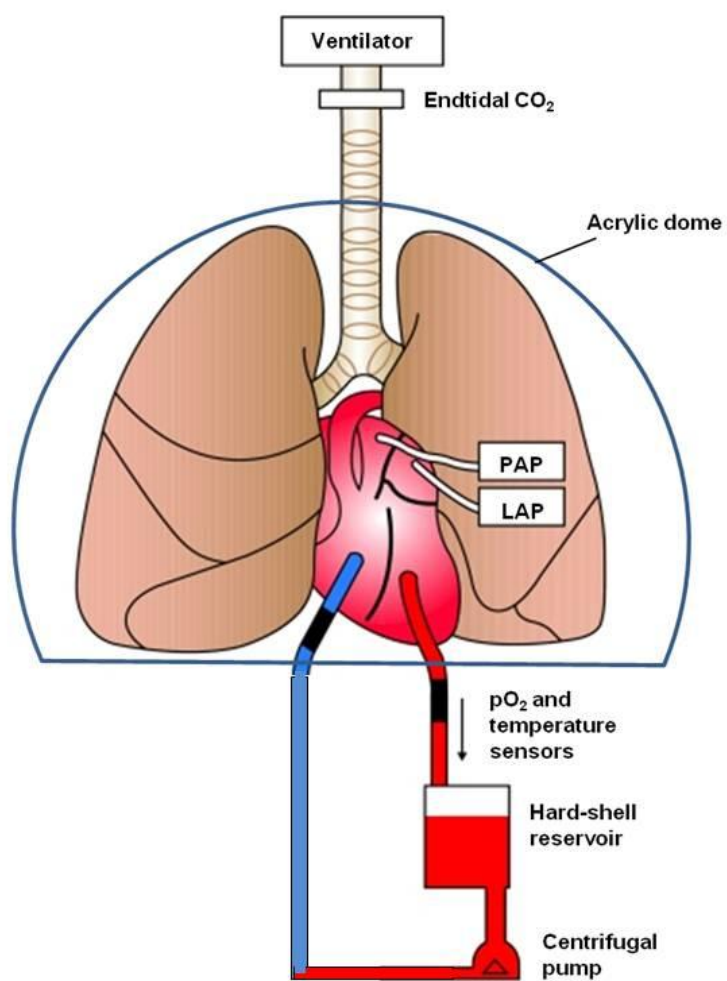


Figure 6.1 The ex-vivo lung perfusion model

The lungs were perfused with Steen solution circulated via a centrifugal pump. The trachea is attached to a ventilator protected by a filter. This closed system allows for ventilation of the lungs and is expected to maintain them for approximately 12-18 hrs. The lungs were kept in an unsealed dome to provide an environment with a high humidity a), connected to the centrifugal pump by cannulae placed in the pulmonary artery and vein and connected to the ventilator by a cannula placed in the trachea b). A schematic overview of the EVLP system is displayed in panel c (Scheme modified from Proudfoot *et al.* (Proudfoot et al. 2011)). PAP = pulmonary artery pressure, LAP = left atrial pressure

pathogenic H5N1 avian influenza virus typically develop mild disease compared to mouse and ferret animal models (Lipatov et al. 2008, Shortridge et al. 1998).

A new high-tech model, a biomimetic microsystem that reconstitutes the alveolar-capillary interface of the human lung measuring just 1-2 cm in length, has been described recently by Huh *et al.* (Huh et al. 2010). This model was able to reproduce the host immune response to *Escherichia coli* including inflammatory cytokines such as TNF- α . However, although this model might represent an option for future experiments, the current availability does not yet allow us to investigate influenza infection.

In this chapter, the possibility of infecting porcine lungs in the EVLP setup with influenza virus A(H1N1)pdm09 was investigated. Several virological and physiological parameters were examined, such as viral replication and Pmax. In the experiments described here lungs of porcine origin were used as a proof of principle prior to future study in whole human lungs.

Please refer to the supplied CD or DVD to view the set-up of the EVLP experiments on video.

6.2 Results

6.2.1 Infection of *ex vivo* porcine lung tissue sections

Prior to infection of an entire porcine lung, the ability to grow influenza virus in tissue sections obtained from porcine lungs routinely used by Dr. Alastair Proudfoot, Royal Brompton Hospital, for EVLP was investigated. To this end, two viruses with an expected differential lung tropism were used to infect *ex vivo* porcine lung tissue sections; E195 is a prototypic A(H1N1)pdm09 virus which has been discussed before, E195 D225G was constructed using the reverse genetics system. The D225G mutation in the HA protein of A(H1N1)pdm09 was first described by Kilander *et al.* They investigated the amino acid distribution at residue 225 in 205 mild and 61 severe/fatal cases and detected the 225G mutation in 11/61 severe cases, but none of the mild cases. This led to the hypothesis that the 225G mutation increased pneumotropism (Kilander *et al.* 2010) via suspected increased α 2,3-linked sialic acid binding (Stevens *et al.* 2006a) subsequently resulting in severe outcome of disease. Indeed, successive research showed that the 225G mutation in A(H1N1)pdm09 greatly increases infection of ciliated cells and results in an increase in HA binding to α 2,3-linked sialic acids (Liu *et al.* 2010). Interestingly, in pigs inoculated with a mixture of 225D/G A(H1N1)pdm09 both mutations were found in influenza virus obtained from the nasal wash, but influenza virus obtained from 2/4 middle lung lobe sections contained only A(H1N1)pdm09 with mutation 225G whereas the other 2 middle lung lobe sections contained a mixture (Brookes *et al.* 2010). Therefore, it was hypothesized

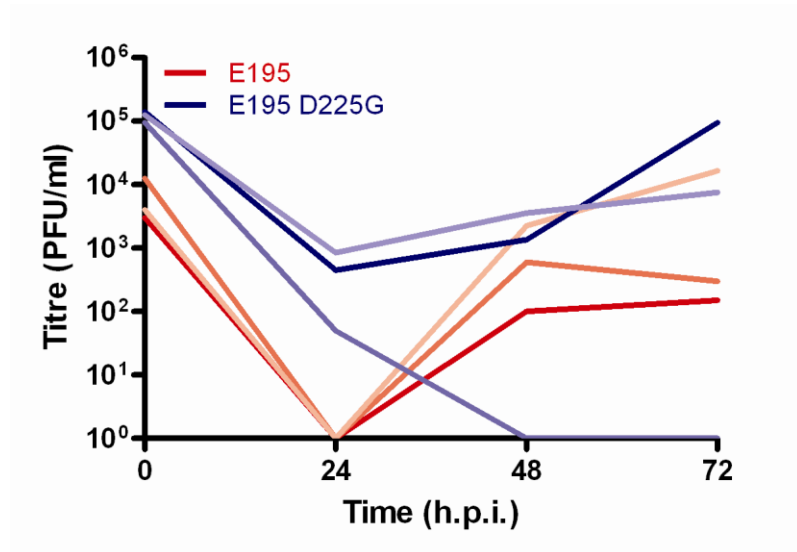


Figure 6.2 Infection of ex vivo porcine lung tissue sections with influenza virus

Each section was infected with 10⁶ PFU of either wild type E195 or E195 D225G. Medium samples were taken every 24 hrs after infection and infectious viral titre was detected on a monolayer of MDCK cells. Viral titres of ex-vivo porcine lung tissue sections (EVPLTS) infected with wild type E195 are depicted in different shades of red, viral titres of EVPLTS infected with E195 D225G are depicted in different shades of blue.

that E195 D225G might have an advantage over wild type E195 to grow in lung tissue.

Ex vivo porcine lung tissue sections (EVPLTS) were divided into different sections of equal size (approximately 5 by 5 mm) and washed with DMEM (Gibco). Hereafter, EVPLTS were incubated in triplicate at 37°C for 1 hr in 250 µl DMEM containing 10⁶ PFU of either E195 or E195 D225G. After inoculum was removed, the EVPLTS were washed thrice with PBS, and incubated at 37°C in DMEM with 50 U/ml penicillin and 50 µg/ml streptomycin. No trypsin was added throughout the experiment and media was replaced every 24 hr followed by titration on MDCK cells to detect infectious virus.

Directly after infection, a high viral titre was observed despite the wash step after removal of the inoculum. Due to the complex three-dimensional structure of the EVPLTS, it is deemed impossible to remove all virions that did not infect cells during the incubation step. Hence, using a lower viral titre as inoculum might be recommended. E195 D225G was detected at a higher viral titre than E195 at 0 h.p.i., but backtitration of the inoculum showed a similar viral input for both strains (10⁶ PFU). This may suggest that less E195 D225G was bound and internalized than wild type E195. Such early time point differences were also noted by Scull *et al.* when titrating avian and human influenza virus in HAE cultures (Scull *et al.* 2009).

Twenty-four hrs after infection, no infectious virus was detected in EVPLTS infected with wild type E195. However, at this time point all three EVPLTS infected with E195 D225G were positive for influenza virus; the average detected viral titre was 450 PFU/ml. It should be noted

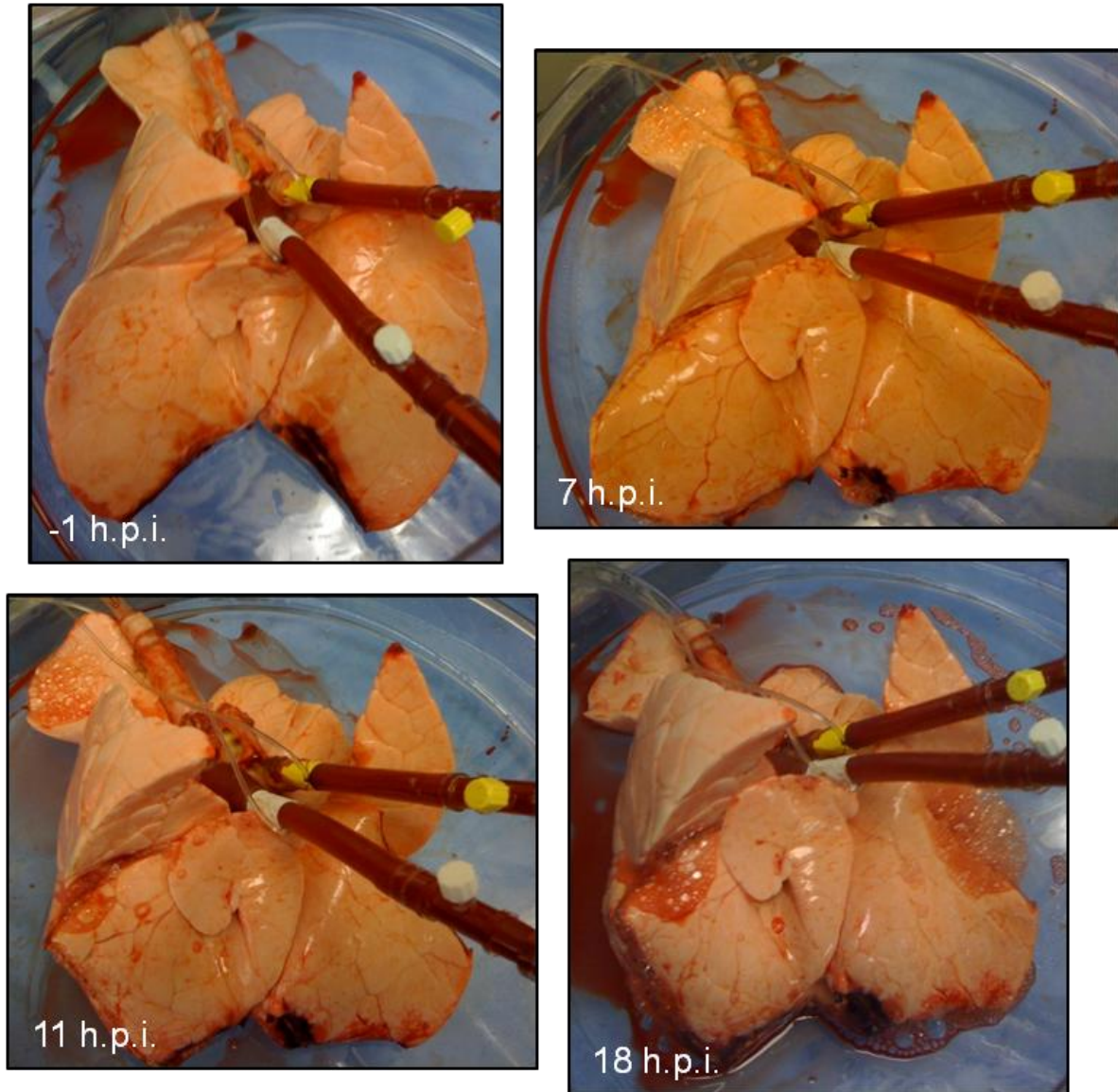


Figure 6.3 Photographs of ex-vivo lung perfusion experiment

Throughout the experiment, photographs were taken at -1, 7, 11 and 18 h.p.i. The air bubbles observed at 18 h.p.i. are caused by air leaks. On the posterior side of the lungs, tissue samples were taken. Photographs courtesy of Dr. Alastair Proudfoot.

that this titre is lower than the viral titre found at 0 h.p.i. suggesting that the virus detected here might be partly the remains of input that has not yet decayed. However, wild type E195 has decreased by 4 logs at this time whereas E195 D225G has decreased with only 2 logs. It is possible that virus detected at this time point is newly generated virus. Indeed, hereafter 2/3 EVPLTS infected with E195 D225G continued to increase in infectious viral titre over time. Likewise, 3/3 EVPLTS were able to support replication of wild type E195. It is unclear why one EVPLTS was unable to support viral replication. Division into the EVPLTS was done randomly and it is possible that there was a difference in the number of cells susceptible to influenza infection available in each section (*Figure 6.2*).

This pilot experiment proved that EVPLTS were susceptible to influenza infection and increases the likelihood that EVLP could be used as a model to study influenza virus infection.

6.2.2 First infection of whole porcine lungs with E195

The following experiments were only performed with clinical E195, and not with mutant E195 D225G. En bloc lungs were extracted from a 5-month old large white pig by Dr. Alastair Proudfoot according to published protocols and transported on wet ice. The lungs were coupled to the EVLP circuit as described in Proudfoot *et al.* (Proudfoot et al. 2011). In short, cannulae were placed in the pulmonary artery and left atrial cuff after which the lungs were perfused with two litres of albumin-based perfusate called Steen solution (Steen et al. 2003) combined with 200 ml of autologous porcine red blood cells to increase oncotic pressure and buffer the

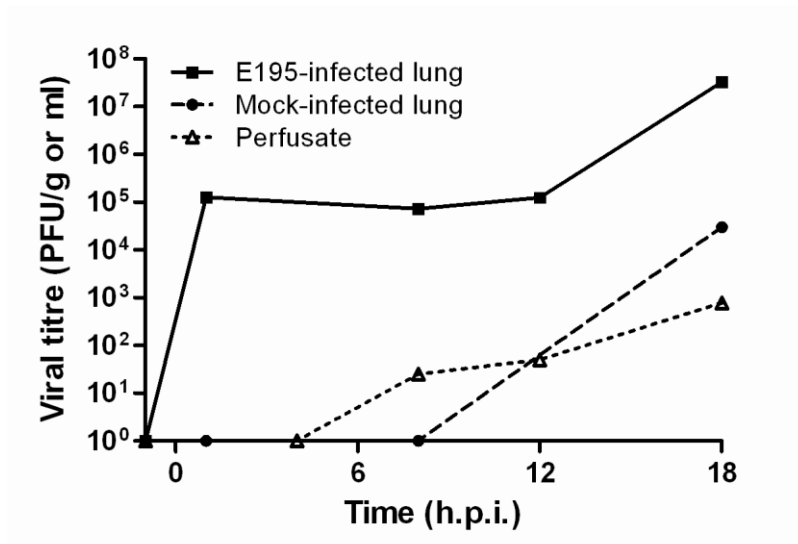


Figure 6.4 Influenza virus detection in porcine lung tissue samples obtained from first EVLP experiment

Presence of infectious virus was detected by standard plaque assay on a confluent monolayer of MDCK cells in PFU per gram of lung tissue or millilitre (perfusate).

inevitable lactic acidosis as the lung anaerobically metabolises over time. The perfusate was circulated via a centrifugal pump at a fixed flow rate of 1 litre/minute. Lastly, the trachea was attached to a standard clinical-grade ventilator protected by a filter which ventilates the porcine lungs. This closed system allows for ventilation of the lungs and was expected to maintain them for approximately 12-18 hrs (*Figure 6.1*). Following rewarming of the lungs to 37°C via a heat-cooler device, 50 ml of 10^7 E195 isolate/ml was introduced into the right lower lobe via a bronchoscope. The other lung was left uninfected and was termed the mock-infected lung. Physiological parameters were measured throughout the experiment. Tissue samples were taken at -1, 1, 8, 12 and 18 h.p.i. of the infected lung and -1, 1, 8 and 18 but not 12 h.p.i of the mock-infected lung from the distal parts of the lower lobes, which were thought to be the tissue sites most likely to be infected with influenza virus. Perfusate samples were taken at 4, 8, 12 and 18 h.p.i. Air leaks developed after 14 h.p.i in both lungs and the experiment was terminated at 18 h.p.i. Images were taken at different time points by Dr. Alastair Proudfoot to illustrate the appearance of the porcine lungs over time (*Figure 6.3*).

Viral titres in homogenised porcine lung tissue samples were measured by standard plaque assay on a confluent MDCK cell monolayer. In samples obtained from the lung infected with the E195 isolate, high viral titres were found immediately after inoculation (10^5 PFU/g lung tissue). Unlike when a confluent cell monolayer is infected with influenza virus, the lungs could not be washed after infection. Therefore, the detected virus is most likely virus originating from the inoculum which had not entered cells. No increase in viral titre was observed in tissue samples

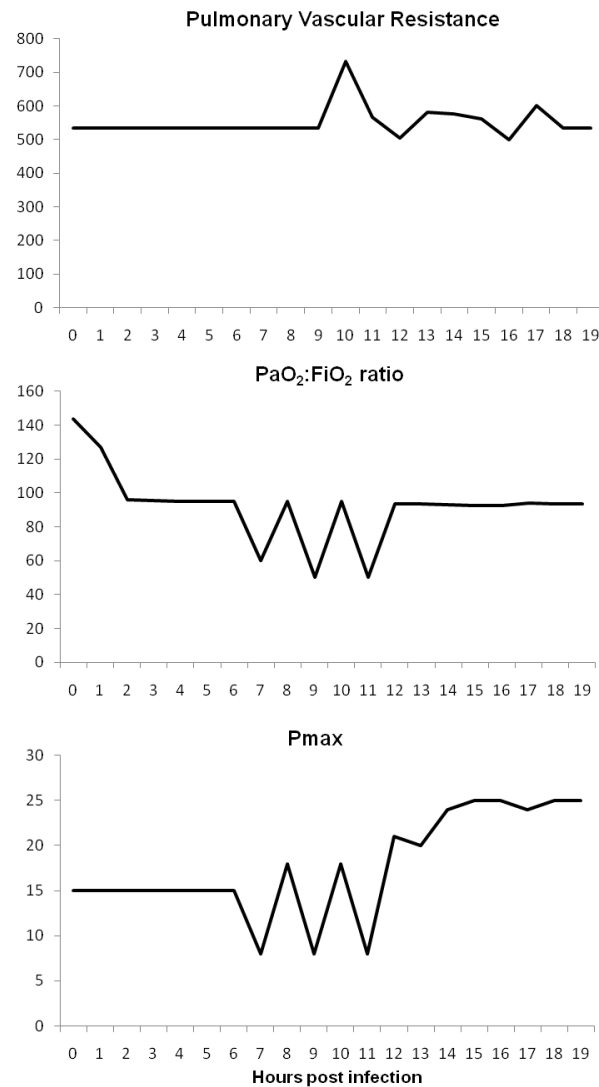


Figure 6.5 Physiological parameters of the porcine whole lung after infection

The pulmonary vascular resistance was calculated by extraction of the mean pulmonary arterial wedge pressure (LAP, mmHg) of the mean pulmonary arterial pressure (PAP, mmHg) divided by the perfusate flow (L/min) x 80. The PaO₂:FiO₂ ratio was calculated by multiplying the flow of inhaled oxygen (FiO₂) with the mean airway pressure (MAP), which is divided by the arterial partial pressure of oxygen (PaO₂). Lastly, the peak inspiratory pressure (Pmax) was measured directly from the ventilator.

taken from the E195 inoculated lung at 8 and 12 h.p.i., but at 18 h.p.i. an increase in viral titre of 2.5 logs over the original titre was observed, which likely represents viral replication and release. No infectious virus was detected in the mock-infected lung at 1 and 8 h.p.i., but at 18 h.p.i. a viral titre of 10^4 PFU/g was present. This increase in viral titre likely represents viral replication and release.

Interestingly, infectious virus could also be detected in the perfusate from 12 h.p.i. onwards. As the perfusate was circulated throughout the entire pair of lungs and as such does not discriminate between the mock-infected and E195 infected lung, this could be a potential source of infection of the mock-infected lung. However, the fact that the increase in viral titre in the mock-infected lung occurs at the same time and with a similar increase to that found in the E195-infected lung (2.5 logs and 4 logs between 8-12 and 18 h.p.i.), may suggest viral replication started at the same time in both lungs. It is therefore thought to be most likely that the infection of the mock-infected lung started with a low viral titre which entered the mock-infected lung at 0 h.p.i., either during infection of the other lung with E195 isolate or via aerosolized E195 virus mobilized via ventilation. It can however not be excluded that the perceived stagnation in growth between 1 and 12 h.p.i. is in fact decay of virus that did not initially infect cells in the lung in combination with progeny virions. In that case, the infected perfusate might be the source of infection of the mock-infected lung (*Figure 6.4*).

Functional assessment of porcine lungs was performed in accordance with techniques applied clinically in human ex-vivo lung transplantation (Cypel et al. 2011). Three physiological variables

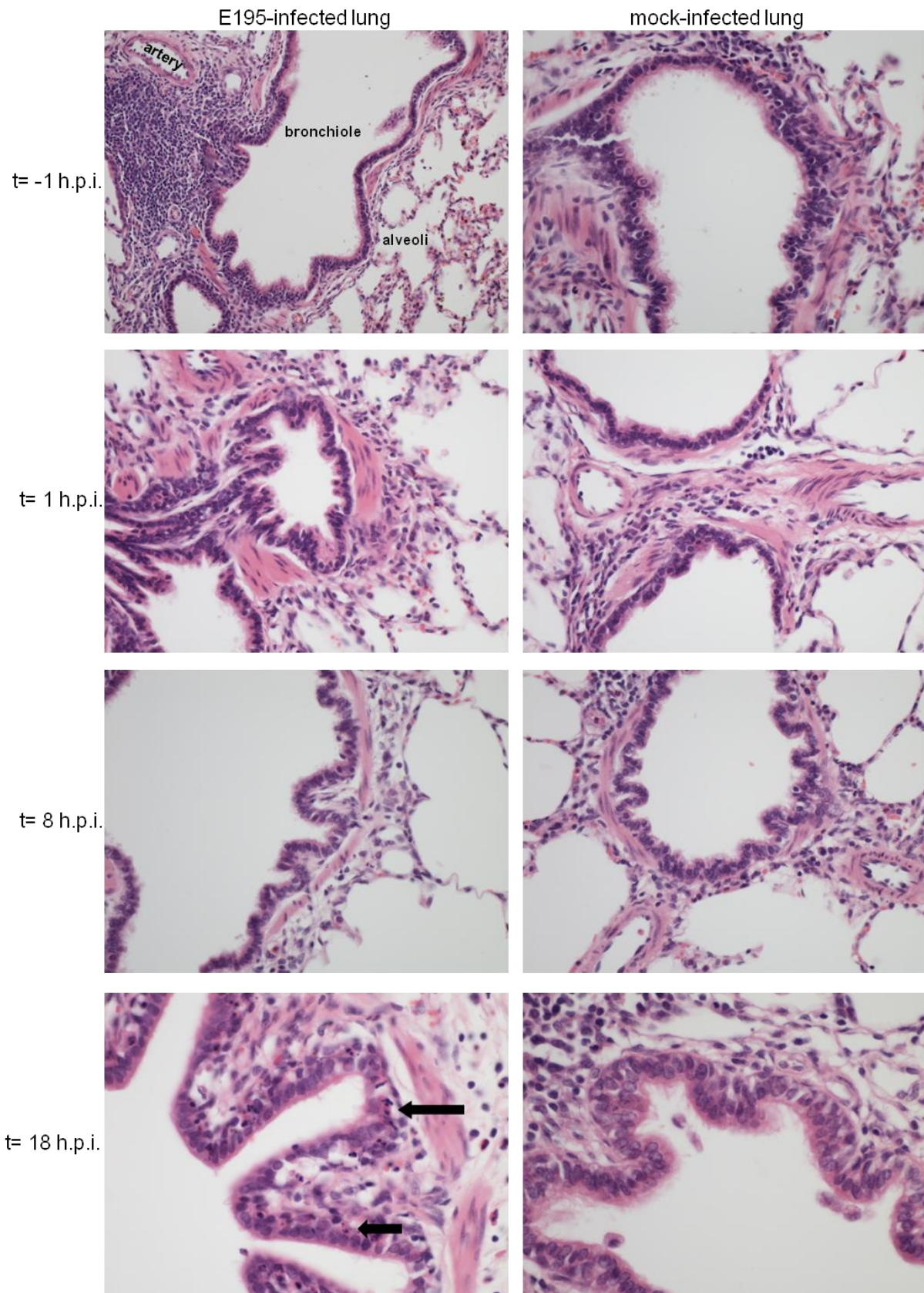


Figure 6.6 Hematoxylin and eosin staining of tissue sections extracted from the E195- and mock-infected porcine lung

Staining of different tissue sections at different time points was performed to score the pathology, as in *Table 6.1*. One image is annotated to show the bronchioli, alveoli and arteries. The black arrows point out apoptotic cells. All images are courtesy of Dr. Alexandra Rice.

were measured hourly; pulmonary vascular resistance (PVR), $\text{PaO}_2\text{:FiO}_2$ ratio and peak inspiratory pressure (Pmax). Pulmonary vascular resistance ($\text{dyn}\cdot\text{s}\cdot\text{cm}^{-5}$) as a marker of pulmonary vascular endothelial function was calculated:

$$\frac{80 \times (\text{pulmonary artery pressure} - \text{left atrial pressure})}{\text{perfusate flow}}$$

No consistent changes in PVR were observed during the experiment. $\text{PaO}_2\text{:FiO}_2$ ratio provides an assessment of the ability of the lungs to diffuse oxygen across the alveolar capillary membrane and was calculated as follows:

$$\frac{\text{FiO}_2 \times \text{MAP}}{\text{PaO}_2}$$

In which the concentration of inhaled oxygen is FiO_2 , the mean airway pressure is MAP and the arterial partial pressure of oxygen is PaO_2 , measured in the circulating perfusate. Again no consistent changes in this parameter were observed during the experiment. Pmax reflects the driving pressure required to inflate the lungs to a given volume and is therefore a surrogate measure of parenchymal lung injury for example secondary to interstitial oedema due to acute lung injury. In this setting, the deterioration in lung compliance at 13 h.p.i. (*Figure 6.5c*) could reflect virus-induced injury to the alveolar capillary membrane albeit that this was not associated with any deterioration in PVR or the $\text{PaO}_2\text{:FiO}_2$ ratio. This hypothesis is supported by an

increase in viral titre observed by standard plaque assay from 12 h.p.i. to 18 h.p.i. Assessment of regional interstitial oedema in the virus-injured lobe could have been undertaken by x-ray imaging however, this was logistically challenging for our model system (*Figure 6.5*).

Lastly, pathogenesis caused by influenza infection in porcine lungs was scored unblinded by pulmonary pathologist Dr. Alexandra Rice, Royal Brompton Hospital. Lung tissue samples were fixed in 10% formalin for 24 hrs and then embedded in wax. Sections cut at 5 μ m were stained with haematoxylin and eosin and then examined by light microscope for pathological changes (*Figure 6.6*). Semi-quantitative assessment of acute lung injury was performed using interstitial, alveolar and septal oedema and interstitial and alveolar inflammation. Each parameter was scored for severity using a 4 point scale; 0 = absent, 1 = mild, 2 = moderate, 3 = severe. The sum of scores for each parameter gave the Total Lung Injury Score (TLIS) with a value of 0 to 24. Bronchial damage was divided into epithelial damage and cellular influx driven by inflammation. No differences were observed in the TLIS between the mock-infected or E195-infected lungs and the inflammatory cell influx is similar at all time points between both lungs. However, alveolar epithelial cell and neutrophil apoptosis was evident at 18 h.p.i. in the E195-infected lung combined with an indurated epithelium, which was absent at 1 and 8 h.p.i. and the mock-infected lung. Furthermore, the general appearance of the E195-infected lung at 18 h.p.i. was described as resembling an acute bronchiolitis. In conclusion, at 18 h.p.i. the E195-infected

E195-infected lung

	Oedema			Inflammation		TLIS	Bronchial	
	I	A	S	I	A		E	F
Pre-infection	0	0	2	0	0	2	0	1
1 h.p.i.	0	1	2	1	0	4	0	1
8 h.p.i.	0	0	2	0	0	2	0	1
12 h.p.i.	1	0	2	1	0	4	0	1
18 h.p.i.	1	0	1	1	0	3	2-3 apoptosis++	2

Mock-infected lung

	Oedema			Inflammation		TLIS	Bronchial	
	I	A	S	I	A		E	F
Pre-infection	0	0	2	0	0	2	0	1
1 h.p.i.	0	0	2	1	0	3	0	1
8 h.p.i.	1	0	2	1	0	4	0	1
18 h.p.i.	1	0	2	1	0	4	0	2

Table 6.1 Pathogenesis score of ex vivo porcine lung infected with E195, experiment 1

Oedema and inflammation were scored for the interstitium (I) and alveoli (A), oedema was also scored for the septum (S). Each parameter was scored for severity using a 4 point scale; 0 = absent, 1 = mild, 2 = moderate, 3 = severe. The sum of scores for each parameter gave the Total Lung Injury Score (TLIS) with a value of 0 to 24. No differences were observed in TLIS over time or between the E195-infected and mock-infected lungs. Overall bronchial damage was divided into epithelial damage (E) and cellular influx driven by inflammation (F). Although no differences were found in cellular influx during the experiment or between the E195-infected and mock-infected lungs, an increased apoptosis of epithelial cells and neutrophils was observed in the E195-infected lung at 18 h.p.i.

lung showed clinical symptoms of infection, whereas this was not found in the mock-infected lung (*Table 6.1*).

6.2.3 Second infection of whole porcine lungs with E195

In the previous experiment porcine whole lungs were infected with a high dose of E195; the lungs were inoculated with 5×10^8 PFU/lung. Consequently, a high viral load was found in this lung at 1 h.p.i. Hypothetically, this could have been the cause of virus replication in the neighbouring mock-infected lung; aerosolised virus potentially could have gained access to the mock-infected lung via recirculation of air. Therefore to prevent cross-infection and a high viral titre at earlier time points, the experiment was repeated with a much lower dose of the E195 inoculum, namely 4×10^6 PFU/lung (virus titre was 2×10^5 , four lobe segments were infected with five ml each). Both lungs were again extracted from a 5-month old large white pig by Dr. Alastair Proudfoot and transported on wet ice, after which the lungs were coupled to the EVLP system as described in section 6.2.2. After one hr to allow the lungs to warm-up to approximately 37°C, one lung was mock-infected with serum-free medium and one lung was infected with 20 ml of 2×10^5 PFU of clinical E195/ml. Physiological parameters were measured throughout the experiment. Tissue samples were taken at -1, 1, 4, 8, 12 and 18 h.p.i. of the infected lung and -1, 1, 4, 8, 12 and 18 h.p.i. of the mock-infected lung at the distal part of the lower lobe. This site was argued to be most likely to be infected by influenza virus, as virus inoculum was targeted at this tissue.

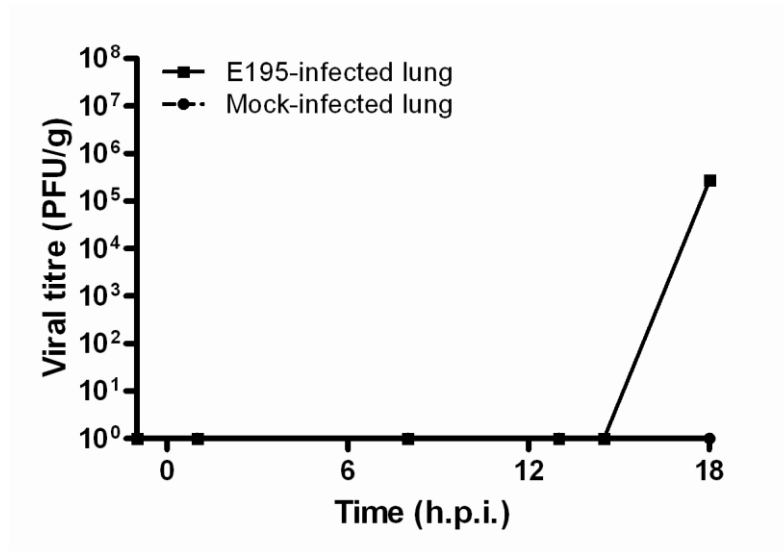


Figure 6.7 Influenza virus detection in porcine lung tissue samples obtained from second EVLP experiment

Presence of virus was detected by standard plaque assay on a confluent monolayer of MDCK cells in PFU per gram of lung tissue.

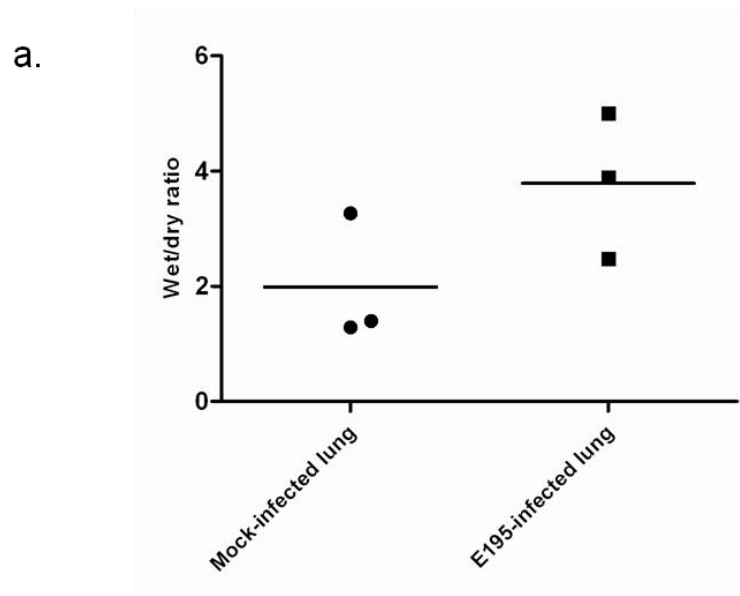


Figure 6.8 Wet/dry ratio of E195- and mock-infected ex-vivo porcine lungs

a) Samples taken at 18 h.p.i. from the E195- and mock-infected porcine lung were weighed. After incubation at 65°C for two hrs on a hot plate, the tissue samples were weighed again. The ratio was calculated by dividing the weight before heating by the weight after heating of the samples. All samples were performed in triplicate and statistical analysis was done on Excel by Student's t-test. b/c) Heating of the whole lung samples on a hot plate in a CL2 safety cabinet.

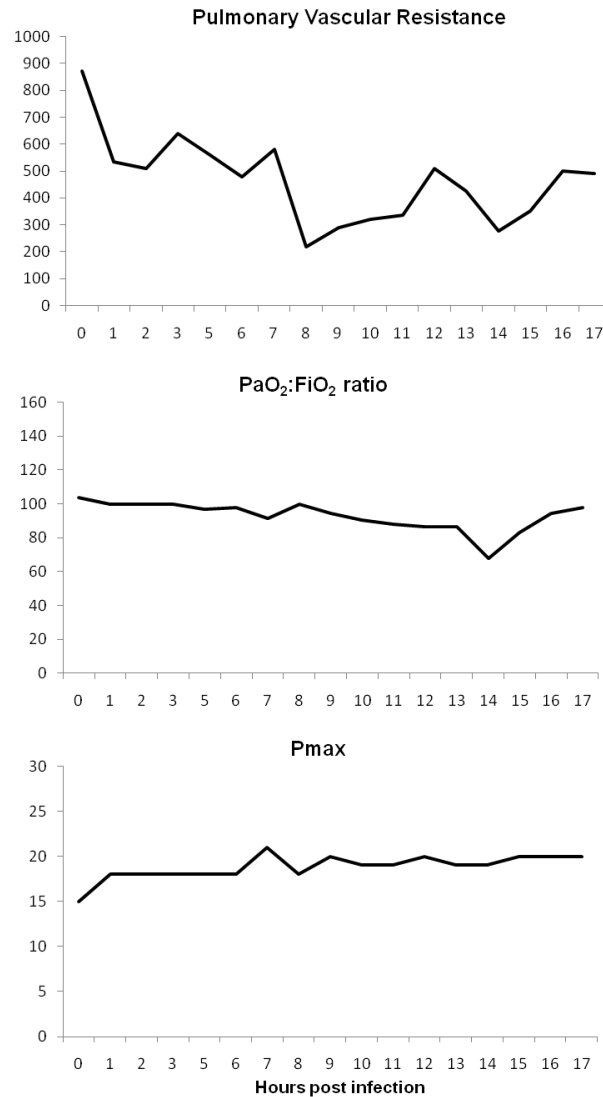


Figure 6.9 Physiological parameters of the porcine whole lung after infection

The pulmonary vascular resistance was calculated by extraction of the mean pulmonary arterial wedge pressure (LAP, mmHg) of the mean pulmonary arterial pressure (PAP, mmHg) divided by the perfusate flow (L/min) x 80. The PaO₂:FiO₂ ratio was calculated by multiplying the flow of inhaled oxygen (FiO₂) with the mean airway pressure (MAP), which is divided by the arterial partial pressure of oxygen (PaO₂). Lastly, the peak inspiratory pressure (Pmax) was measured directly from the ventilator.

Viral titres in homogenised porcine lung tissue samples were measured using standard plaque assay on a confluent MDCK cell monolayer. In samples obtained from the lung infected with E195, viral titres were not found until 18 h.p.i. (2.7×10^5 PFU/g lung tissue). No infectious virus was detected in the mock-infected lung at any time point throughout the experiment (*Figure 6.7*).

In the clinical setting, injured lungs contain a higher proportion of water than healthy lungs. To measure lung injury after influenza infection, the wet/dry ratio of tissue samples obtained from the E195- and mock-infected lungs at 18 h.p.i. was investigated by homogenization in sterile PBS followed by heating the samples at 65°C for two hrs. Indeed, the mean wet/dry ratio of samples from the E195-infected lung was 3.79, whereas this was 1.99 for samples from the mock-infected lung. However, this difference was not significant as calculated by Student's t-test ($p=0.14$) (*Figure 6.8*).

Functional assessment of porcine lungs was performed as in section 6.2.2 in accordance with techniques applied clinically in human ex-vivo lung transplantation (Cypel et al. 2011); PVR, $\text{PaO}_2:\text{FiO}_2$ ratio and Pmax were calculated. No consistent changes in either of these parameters were observed during the experiment (*Figure 6.9*). This is in line with the lack of infectious virus as detected by plaque assay until 18 h.p.i.

Lastly, pathogenesis caused by influenza infection in porcine lungs was scored again by pulmonary pathologist Dr. Alexandra Rice as described in section 6.2.2. In line with the other

E195-infected lung

	Oedema			Inflammation		TLIS	Bronchial	
	I	A	S	I	A		E	F
Pre-infection	0	0	3	1	0	4	0	0
1 h.p.i	0	0	1	1	0	2	0	0
8 h.p.i	0	0	1	0	0	1	0	0
13 h.p.i	0	0	1	1	1	3	0	0
14.5 h.p.i	0	0	1	1	0	2	0	0
18 h.p.i	0	0	2	1	0	3	0	0

Mock-infected lung

	Oedema			Inflammation		TLIS	Bronchial	
	I	A	S	I	A		E	F
Pre-infection	0	0	3	1	0	4	0	0
1 h.p.i	0	0	2	1	0	3	0	0
8 h.p.i	0	0	2	1	0	3	0	0
13 h.p.i	0	0	2	0	0	2	0	0
14.5 h.p.i	0	0	1	0	0	1	N/A	N/A
18 h.p.i	0	0	1	0	0	1	0	0

Table 6.2 Pathogenesis score of ex vivo porcine lung infected with E195, experiment 2

Oedema and inflammation were scored for the interstitium (I) and alveoli (A), oedema was also scored for the septum (S). Each parameter was scored for severity using a 4 point scale; 0 = absent, 1 = mild, 2 = moderate, 3 = severe. The sum of scores for each parameter gave the Total Lung Injury Score (TLIS) with a value of 0 to 24. Overall bronchial damage was divided into epithelial damage (E) and cellular influx driven by inflammation (F). No differences were observed over time or between the E195-infected and mock-infected lungs.

experimental outcomes of the second infectious experiment, no differences were observed between the mock-infected and E195-infected lungs at any time point (*Table 6.2*).

6.3 Discussion

In this chapter, the possibility of using EVLP as a porcine infection model was investigated. It was shown that whole porcine *ex vivo* lungs were able to support viral replication, which resulted in measurable viral and pathological parameters. It seems therefore feasible to use this model to study influenza infection in the context of a whole organ.

It should be noted that the investigated virus (A(H1N1)pdm09) is known to replicate in pigs (Brookes et al. 2010); indeed A(H1N1)pdm09 has a porcine origin. It is not clear whether this model would also be suitable for the study of other influenza viruses such as seasonal H3N2 of human origin or H5N1 of avian origin. It would be of interest to investigate whether EVLP of a porcine lung is suitable for these influenza A viruses, as well as influenza B viruses, or whether the use of a human lung would be required to extend the model for this purpose. Both human and avian influenza viruses have been shown to be able to grow in *ex vivo* porcine tissue section originating from the respiratory tract (Van Poucke et al. 2010, Punyadarsaniya et al. 2011) suggesting it is likely that viruses from both origins will be able to replicate in EVLP of a porcine lung. However, avian influenza replication was generally delayed in *ex vivo* cultures, and might be too slow to be investigated using EVLP of porcine lungs.

No increase in viral titre was observed until 18 h.p.i. in two independent experiments. Even though it was noted which lobes were inoculated with influenza virus, translating that to the right location for biopsies is complex, due to the three-dimensional structure of the whole lung. It is therefore possible that the viral replication cycle within this model took the expected 8 hours, but

that this occurred at sites that were not used to take biopsies. The increase in viral titre observed at 18 h.p.i. is most likely caused by multi-cycle events, and the growth curves (*Figure 6.4* and *Figure 6.7*) are not thought to represent a single cycle of replication. However, it cannot be excluded that viral replication is slightly delayed. Before the lungs were attached to medium and oxygen supply, the tissue was transported on ice to increase preservation. Although warm-up time of one hour was allowed, it is possible that the cell machinery was still relatively slow. Unfortunately, it is not possible to increase the length of the warm-up phase, as the life expectancy of the lungs is relatively short (Cypel et al. 2008) and already restricts the length of the experiment. Interestingly, viral growth does seem slow in the *ex vivo* porcine tissue sections; no increase in viral titre is observed until 48 h.p.i. This does not mean however that viral replication did not take place until 48 h.p.i., a high viral titre at 0 h.p.i. might hide viral replication at time points before 48 h.p.i.

The interpretation of data on viral titre is relatively difficult, as the current model includes some variables. Firstly, the isolated tissue will vary between time points and contains an unknown mixture of different cell types. It is therefore possible that one sample contains a large portion of cells that support replication of influenza, whereas another sample contains a small portion of these cells potentially resulting in differences in detected infectious virus titre. Secondly, the porcine lungs are relatively large organs and it is not clear where influenza replication is taking place, potentially leading to under- or overestimation of viral titre per lung due to sampling at the

wrong location. Finally, as the number of cells is not known, it is not possible to calculate a MOI.

This can lead to variation between experiments.

Future experiments using this model should be done to refine the methodology. Ideally, the length of the experiment should be increased to 48 or 72 h.p.i. However, the condition of the lungs at the end of both experiments was poor and perceived as a potential safety hazard due to air leaks. Therefore, to increase the length of the experiment either the quality of the lung needs to be improved or the rate of decay needs to be decreased. The porcine lungs used were directly isolated from a pig, time between isolation and start of the experiment was minimized where possible. During this time, the lungs were kept on ice to minimize decay. It seems therefore improbable that the quality of the lungs can be improved. To decrease the rate of decay, maintenance variables such as medium should be improved, however the current state of knowledge does not allow for further improvement. An interesting alternative way to increase the length of the experiment would be to move to the use of so-called precision-cut lung slices (PCLS). Initial infection would then occur in the context of the whole lung, thereby taking advantage of the whole three-dimensional physiology of the lung as well as mimicking the dynamic mechanical forces normally experienced in the lung, which are potentially important in the enhancement of the inflammatory response (Huh et al. 2010). Physiological parameters can be measured during this part of the experiment, followed by wet/dry ratios when proceeding to the second part of the experiment. In the PCLS extension, the (mock-)infected porcine lungs would be filled with warm (37°C) low-melting agarose, followed by extracting cylindrical portions

of tissue of interest (e.g. where the lung was inoculated), which is subsequently cut into slices with a thickness of approximately 250 μm as described in Punyadarsaniya *et al.* (Punyadarsaniya *et al.* 2011). PCLS slides can be maintained in medium for up to seven days, which significantly increases the time span of growth curves.

An ideal viral inoculum titre needs to be defined by infecting lungs with serially diluted virus. In the first experiment performed, the viral inoculum titre was perhaps too high, in the second experiment the viral inoculum titre was perhaps too low.

It would be interesting to investigate viral growth of mutant E195 D225G in the porcine lungs. This virus has been found to be selected for *in vivo* in pigs (Brookes *et al.* 2010) and grew better in EVPLTS. Hypothetically, this virus would grow better in the porcine lung than wild type E195 and could therefore present a relatively simple method to test the tissue-specificity of the EVLP model.

Finally, other measurement such as x-ray imaging and electron microscopy would greatly improve the output of each experiment.

Naturally, this model is too complex and expensive to simply look at how influenza viruses grow within the porcine lung. It is therefore important to think of niche experiments that this model is ideally suited for. One such example would be to test a novel antiviral therapy; simultaneously antiviral efficacy and toxicity to lung physiology would be tested. Alternatively, the effect of blood-borne immune cells on influenza infection could be investigated via addition to the media.

Moreover, the effect of therapies that target the immune response could be tested in that context.

Initially, EVLP was developed for the maintenance of human lungs. These experiments could be extended to the human model, enabling performance of such antiviral testing in human lungs. It can even be envisioned to study the effect of avian influenza virus such as H5 and H7 in a human lung.

7 General Discussion

There is no doubt that there will be another influenza pandemic, but the timing and nature of the emerging virus is still difficult to predict. Very recent work looking at the H5N1 avian influenza virus described genetic changes the avian virus would need to undergo before acquiring transmissibility between mammals. These changes occurred in two genes, the receptor-binding protein HA and polymerase complex unit PB2 protein (Herfst et al. 2012, Imai et al. 2012). The mutations described for H5N1 adaptation are not necessarily predictive of the amino acid substitutions that will be found in the next pandemic virus. Instead, it is more likely that certain properties of the virus, such as receptor binding and stability of the HA protein and efficiency of the polymerases need to change in order to produce a pandemic virus (Sorrell et al. 2011).

The work presented in this thesis aimed to identify adaptations in the HA protein primarily but also in the PB2 protein that had an effect on the zoonotic and pandemic potential of influenza A virus. The viruses studied in this thesis included the recently emerged A(H1N1)pdm09 virus that caused the 2009 swine flu pandemic, and an H7N3 virus associated with zoonotic infections in the UK.

7.1 Why did the A(H1N1)pdm09 virus that emerged in 2009 cause a human pandemic?

It remains unclear why the particular virus that emerged in 2009 in Mexico from pigs caused a pandemic whereas other swine viruses did not. One hypothesis was that this virus was more able to bind human forms of the sialic acid receptor. Using *in vitro* methods, it was shown in this thesis that residue 227, which was unique to A(H1N1)pdm09 had a significant effect on cell tropism. Whereas A(H1N1)pdm09 with the original residue 227E in HA preferentially bound and infected non-ciliated cells in human airway epithelium cultures, this profile is shifted to include more ciliated cells when the HA gene is mutated at this position to contain 227A, the amino acid found in swine-origin isolates. Nonetheless, this mutation did not abrogate transmission in a RD transmission experiment in ferrets; four out of four sentinel animals were infected with E195 E227A. Interestingly, when comparing the virus shedding patterns of the RD sentinels of the E195 E227A transmission experiment with those of the wild type E195 transmission experiment, subtle differences were observed. The average duration of shedding was considerably shorter for E195 E227A compared to E195 (3.5 days vs. 5.3 days). Furthermore, for two out of four animals peak titre as well as the total number of plaque forming units shed during the experiment was considerably lower, whereas this was relatively uniform for animals infected with E195 (*Table 4.1*). If such characteristics were to be reflected in the human host, it is possible that the E227A mutant virus would have increased difficulty initiating a chain of transmission, as both the period and amount of shedding are decreased. In fact, the basic

reproduction number R_0 , which defines the number of secondary cases produced by a single infection, is based on three variables; the average rate of contact between a susceptible and infected person (c), the transmissibility of the agent (t) and the duration of contagiousness (d): $R_0 = t * c * d$. As the A227E mutation decreased both variables t and d in the ferret transmission experiment and assuming transmissibility depends on amount of virus shed, the R_0 number will decrease accordingly. Simplistically, if the value of R_0 were to drop below 1 no sustained transmission pattern will be reached. Different studies to provide an estimate of the R_0 of A(H1N1)pdm09 based on early epidemic growth data determined an array of different outcomes, ranging from 1.25 to 3.1 (Roberts and Nishiura 2011, Boelle, Bernillon and Desenclos 2009, Fraser et al. 2009). If indeed the true R_0 value of the earlier isolates of A(H1N1)pdm09 is found in the lower range, it is possible that the presence of the E227A mutation would lower its value sufficiently to prevent a transmission chain. Experiments to investigate transmission chains in ferrets have not yet been described in the literature, but could provide the key to the effect of residue 227 on the pandemic potential of A(H1N1)pdm09.

It should be noted that ferret transmission experiments are designed in such a way that aerosol transmission of influenza virus is more likely to occur than in a natural situation. Sentinel ferrets are continuously exposed to infected animals under permissive conditions, for example the air flow is directed from the infected ferret to the sentinel ferret thereby increasing the chance of transmission. Furthermore, in general viral doses used to infect ferrets (10^4 - 10^6) are much higher than the viral dose expected to naturally infect a human. This can significantly alter the

shedding profile as shown by Roberts *et al.* (Roberts *et al.* 2011). It is possible that when investigating less restricted influenza viruses, such as E195 E227A, the unnatural shedding profile caused by a high viral inoculum pushes the equilibrium towards transmission. Lastly, due to the scale of ferret transmission experiments the number of ferrets per experiment is always relatively small. Therefore, no statistical analysis can be performed on the results and the outcome is presented in a very black and white fashion: aerosol transmission occurred or did not occur. If it would be possible to use larger numbers of ferrets, properties such as 'total amount of virus shed', 'days of shedding' and 'time to infection' could be more reliably compared between different influenza viruses and might alter the current conclusions on the transmission potential of E195 E227A.

It should be noted that it is likely that the A227E mutation has occurred on several occasions in other swine influenza viruses, however none of these viruses went on to cause a pandemic. The start of the A(H1N1)pdm09 chain of transmission in humans was most likely a chance encounter between one swine host and one human host. Furthermore, it was not investigated whether the A227E mutation increases viral fitness in the porcine host, but if this is not the case it is unlikely the mutant virus would outgrow other quasispecies variants.

A second role the A227E mutation might have played in starting the A(H1N1)pdm09 pandemic involved the avidity of the HA protein for sialic acid. In *Figure 3.8* it can be observed that the E227A mutation seemed to increase recombinant HA protein binding to HAE sections whereas in comparison E195 showed weaker binding. A similar effect of this mutation was found in

glycan assay studies by de Vries *et al.* and recently by Xu *et al.* (de Vries *et al.* 2011, Xu *et al.* 2012). Xu *et al.* relate the relative weak binding of A(H1N1)pdm09 HA to the equally weak receptor-destroying activity of A(H1N1)pdm09 NA and find that the HA and NA of all earlier pandemic strains were equally balanced (1918 H1N1, 1957 H2N2 and 1968 H3N2); both 1957 H2N2 and 1968 H3N2 have strong HA and NA activity, whereas 1918 H1N1 displays an intermediate HA and NA activity. In contrast, 2 out of 3 swine progenitor viruses tested show a clear mismatched HA/NA. The balance between HA and NA therefore seems to represent another prerequisite of human influenza, but not necessarily swine influenza. The importance of the HA NA balance for transmission of A(H1N1)pdm09 in ferrets was also described by Yen *et al.* (Yen *et al.* 2011b).

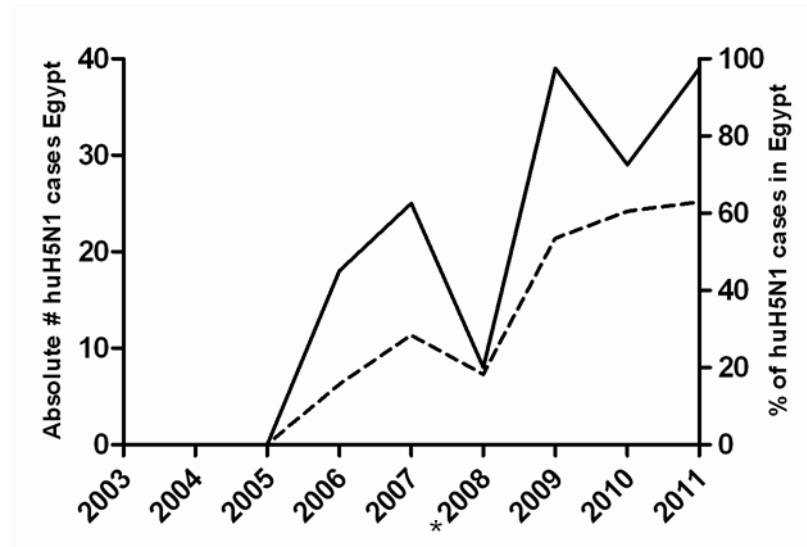


Figure 7.1 Human H5N1 virus infection cases in Egypt

Absolute number of human H5N1 virus infections in Egypt (solid line, left Y axis) and relative number of human H5N1 virus infections in Egypt compared to the number of worldwide infections (dashed line, right Y axis) is displayed. The year in which the H5N1 influenza virus sublineage with increased α 2,6-linked sialic acid binding emerged is marked with a star (*). Information obtained from WHO website (WHO Accessed 26-01-2012).

7.2 Why have some avian influenza viruses jumped into the human host, whereas others have not (yet) done so?

Transmission of an avian influenza virus to a human host is in most cases associated with direct contact with infected animals and hence exposure to a relative high quantity of virus and this is suspected to be the direct cause of most human cases. Nonetheless, some properties of the virus seem to increase the chance to make a zoonotic jump. A pertinent example is the emergence of new sub-lineages of H5N1 genotype Z (for example clade 2.2.1) in birds in Egypt in 2008 which display an enhanced binding affinity for α 2,6-linked sialic acid in combination with residual binding affinity for α 2,3-linked sialic acid (Watanabe et al. 2011). Interestingly, the emergence of these sub-lineages coincided with an increase in the number of human H5N1 infection cases in Egypt and consequently since 2009, Egypt has seen much higher numbers of human H5N1 infections than other affected countries (*Figure 7.1*). These findings suggest that the altered receptor binding affinity of H5N1 contributed to the increased number of bird-to-human zoonotic events. In addition, clade 2.2.1 contains the E627K mutation in PB2 (Chen et al. 2005), which is characterised as a mammalian adaptation and could help increase the host range of influenza A virus (Subbarao et al. 1993), thereby further increasing the chances of this specific subtype to cause a successful infection in a human host.

Using an alternative initial site of infection might also help a virus make a zoonotic jump. Many human H7 influenza infection cases are associated with conjunctivitis (Belser et al. 2009a), whereas this outcome has only been found in a few H5N1 and A(H1N1)pdm09 influenza cases

(Dubnov-Raz et al. 2011, Chan 2002). Access to the respiratory tract via the eye has been shown for infection with respiratory syncytial virus (RSV) in both the human and murine host under experimental conditions (Hall et al. 1981, Bitko, Musiyenko and Barik 2007) and RSV has been isolated from the eye of paediatric patients with respiratory infection and conjunctivitis (Bitko et al. 2004). It is assumed that migration of the virus occurs via the lacrimal duct, which connects the eye to the respiratory tract in a wide variety of animals, including ferrets (Lewington 2007), pigs and humans. It is possible that H7 influenza virus can use a similar route, initially infecting the eye, followed by migration into the respiratory tract. Supporting that hypothesis, recent work has shown that ferrets infected with seasonal and avian influenza virus (H1, H3, H5 and H7) via the ocular route shed virus in the nasal and conjunctival wash (Belser et al. 2012). Likewise, mice infected via the ocular route were found to be positive for influenza virus in ocular tissue (H5 and H7), lung tissue (H1, H3, H5 and H7) and nasal tissue (H5 and H7) at 6 d.p.i. (Belser et al. 2009c). Bischoff *et al.* investigated the importance of the ocular portal of entry for influenza virus by exposing human volunteers wearing varying degrees of protective clothing to aerosolised $10^{6.5-7.5}$ fluorescent forming units of live attenuated influenza virus H1N1, H3N2 and influenza B. They found that even in the nasal wash of volunteers wearing a fit-tested N95 respirator, but no eye protection, influenza virus could be detected using RT-PCR in 3/5 volunteers. However, when eye protection was worn in combination with the fit-tested N95 respirator, influenza virus was detected in only 1/5 volunteers (Bischoff et al. 2011). These investigations suggest that the ocular route is a possible mechanism for viral entry

for influenza virus when the human host is exposed to a high enough viral titre. Combined with the relative ease of human ocular infection with H7 influenza virus compared to other subtypes, this route could form an alternative gateway to creating a pandemic virus of avian origin.

An outbreak of low pathogenicity H7N3 in poultry in the UK led to one confirmed human infection associated with conjunctivitis in a poultry worker, and virus was isolated from both the throat and the eye. Due to the nature of his work, this patient was most likely exposed to infected birds and potentially received a high dose of virus which facilitated this zoonotic event. Nonetheless, we were interested in why this H7N3 isolate was able to jump the zoonotic barrier, and compared the virus to an avian H7N7 virus that was not associated with human infections. It was shown that the human H7N3 isolate (E480) bound more non-ciliated cells in a human airway epithelium culture than an avian H7N7 isolate (M105). Furthermore, M105 displayed a delayed growth in both differentiated human airway epithelium cultures and conjunctival epithelial cells compared to E480. We hypothesise that due to the changed receptor binding preference, E480 was more likely to infect the human eye than M105. Further work with these viruses using glycan arrays might help elucidate the nature of the receptor change (Liu, Palma and Feizi 2009).

7.3 Viral adaptations within the human host, to the human host

In conjunction with one of the recent controversial papers on molecular determinants of H5N1 mammalian transmission (Herfst et al. 2012), Russell *et al.* published a mathematical model to estimate the possibility of an avian H5N1 influenza virus acquiring the remaining mutations to obtain a identical profile to the transmissible virus of Herfst *et al.* (Herfst et al. 2012) or Imai *et al.* (Imai et al. 2012) within a mammalian host. Several different scenarios were investigated that could improve the possibility of such a mutant arising, such as the duration of infection and the positive selection of mutations. Both of these variables (and others) were found to increase the chances of such mutations evolving within a single host (Russell et al. 2012).

It can be hypothesised that using the eye as an initial infection site prolongs the infectious period. Efficient viral replication in the eye close to a more restricted site, which can potentially positively select for mammalian-adapted influenza virus mutants, may facilitate movement from the eye to the respiratory tract. Both of those scenarios should increase the potential of obtaining a more mammalian-adapted H7 influenza virus (Russell et al. 2012). We investigated whether any such adaptation to the new host could be seen in the H7 infected individual by comparing the viruses isolated at different sites. No differences were found in the HA sequences of E480 (throat isolate) and E481 (eye isolate), but two non-synonymous changes were found in the PB2 sequence, namely M206I and Q288R. It cannot yet be excluded that these changes were not caused by serial egg-passage, but it was found that E481 containing the two unique mutations in PB2 displayed a small growth advantage over E480 in human

airway epithelial cultures at the temperature of the upper airway tract. The M201I mutation is found in a domain with an unknown function, but the Q288R mutation is found close to a functional domain associated with cap-snatching. Nonetheless, amino acid 288 was not found to be necessary for cap-snatching (Guilligay et al. 2008). Due to the relative uncertainty surrounding functional domains in PB2, it is difficult to predict what effect these mutations have. Avian polymerases are thought to be inefficient in mammalian cells, and adaptive mutations such as 627K and 701N are thought to increase the viral replication of the polymerase in mammalian cells (de Jong et al. 2006, Tarendeau et al. 2008, Hudjetz and Gabriel 2012). More work is needed to establish the origin and full effect of these mutations, but it is possible that they also represent an adaptive mechanism towards the human host.

7.4 Receptor binding assays, an essential tool to predict pandemic potential

Interestingly, when comparing HA binding profiles of E195 and E480, it was noticeable that E195 E227A had the same non-ciliated/ciliated cell binding ratio as E480 (both 1.2) whereas the ratio for M105 was much lower (0.6) and the ratio for wild type E195 was much higher (3.9). Do these two isolates represent viruses that are approaching human-like receptor binding? Receptor binding assays are an essential tool in determining whether changes in the viral HA protein increase the pandemic potential of influenza virus isolates.

Traditional receptor binding assays use red blood cells treated with sialidase followed by re-sialylation using specific sialyltransferases (Rogers and Paulson 1983, Glaser et al. 2005). Although this assay is relatively easy to use, it is perceived as inadequate to detect subtle changes in receptor binding preference as complex branched glycans are lacking. Alternatively, avian and human glycan receptor analogues can be synthesized or isolated from natural sources and receptor binding assays can be performed using solid-phase enzyme-linked immunosorbent assays (Gambaryan and Matrosovich 1992, Barclay et al. 2007), thin-layer chromatography (Masuda et al. 1999, Suzuki et al. 1992) or lipid-linked glycan probes (neoglycolipid (NGL) technology), which converts isolated oligosaccharides into multivalent probes, thereby increasing sensitivity for detection of low affinity protein-carbohydrate interactions (Childs et al. 2009, Liu et al. 2010, Feizi et al. 2003, Feizi et al. 1994, Feizi and Chai 2004). Development of printed covalent glycan arrays by the Consortium for Functional Glycomics allowed for differentiation between 611 glycan targets resulting in a detailed receptor

binding profile of HA proteins (Stevens et al. 2006a, Stevens et al. 2006b, Blixt et al. 2004, Dyukova et al. 2006, Ratner et al. 2004). These last methods have provided us with a much more detailed insight into the subtle changes in receptor binding preferences. However, critics of these methods argue that the involvement of these 611 specific sugars in HA receptor binding is unproven and that the complex types of glycans found in the URT are underrepresented (Xu et al. 2009).

A rapid glycan assay suitable for use in routine influenza virus surveillance containing just one α 2,3-linked sialic acid and one α 2,6-linked sialic acid would undoubtedly be unable to tease out small differences in receptor binding preference and choosing one glycan representing respiratory tract sialic acid expression is impossible. Furthermore, producing or obtaining sufficient quantities of quality controlled glycans suitable for use in a high throughput assay within a surveillance laboratory is challenging (Liu et al. 2009).

We and others have shown the differential preference of human and avian influenza virus or HA protein for non-ciliated and ciliated cells within HAE cultures (Ayora-Talavera et al. 2009, Shelton et al. 2011, Thompson et al. 2006, Liu et al. 2010, Matrosovich et al. 2004a). Recently, Shelton *et al.* investigated the feasibility of using paraffin-embedded HAE culture sections to examine the pandemic potential of different avian influenza virus subtypes (Shelton et al. 2011). Well-differentiated human airway epithelium (HAE) cells are available from commercial sources such as Epithelix Sàrl (Geneva, Switzerland). Using A(H1N1)pdm09 virus, we were able to show that these cultures are indeed infected by influenza virus, and a differentiation between

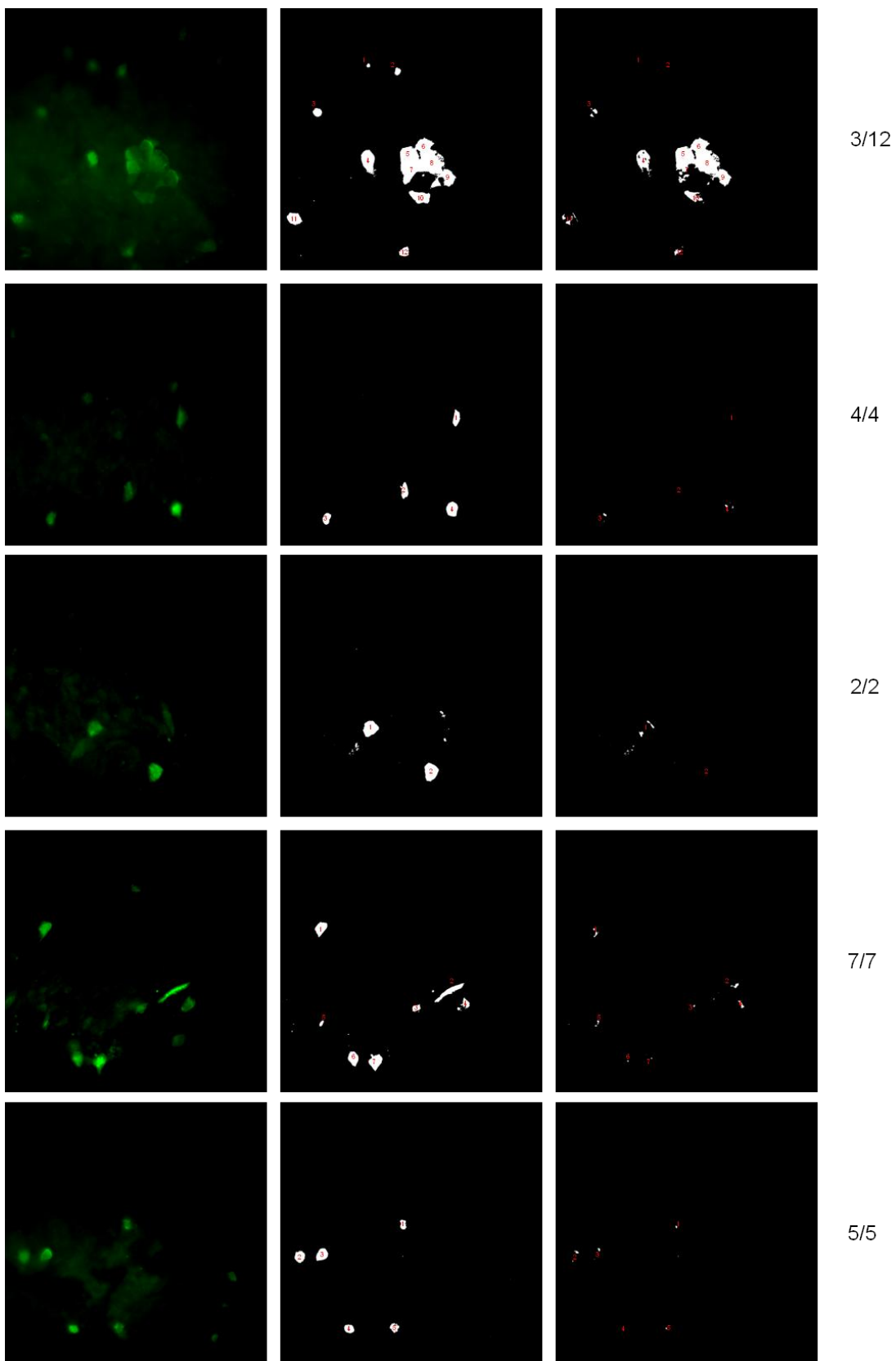


Figure 7.2 Receptor binding assay in HAE cultures using E331

HAE cultures were infected with E331 (A(H1N1)pdm09) at an MOI of 1 and incubated at 37°C for 16 hrs. After fixation with paraformaldehyde, the cells were probed with anti-NP protein antibody (green, panel 1) and anti-tubulin (red, data not shown). Using ImageJ software (<http://rsbweb.nih.gov/ij/>) co-location was visualized by converting pixels that were positive in both the NP-positive image (panel 2) and tubulin-positive image to white, whereas all other pixels were converted to black (panel 3). All infected cells were counted and termed ciliated or non-ciliated (panel 4), e.g. if 10 cells were infected of which 3 were non-ciliated panel 4 would indicate 3/10. N=1, all five images shown.

ciliated and non-ciliated cells could be made (*Figure 7.2*). Infection of HAE cells and differential quantification of infected ciliated and non-ciliated cells could potentially be developed into a receptor-binding assay, arguably utilizing 'relevant' sialic acids naturally found in the respiratory tract. Van Riel *et al.* have developed a different method of looking at receptor binding of influenza viruses; formalin-fixed, paraffin-embedded relevant tissue sections such as human trachea and alveoli were incubated with formalin-inactivated fluorescein isothiocyanate (FITC)-labelled virus using a two-step signal-amplification system (van Riel *et al.* 2006). The functionality of this assay has been proven using tissue sections from different sources. For example, A/Vietnam/1194/2005 (H5N1) virus was able to bind abundantly to epithelial cells in the duck intestine but bind rarely to human trachea tissue sections, whereas A/Netherlands/213/2003 (H3N2) was found to bind abundantly to cells in the human trachea but bound poorly to cells in the duck intestine (van Riel *et al.* 2006). This assay is an interesting alternative to the HAE receptor binding assay described above. However, if the object of investigation is clinical isolates, passaging the initial isolates to provide sufficient virus for labelling has the potential to introduce mutations in HA which might change receptor-binding preference. Furthermore, there may be difficulties in providing a reliable tissue source, especially with human-origin tissue. Finally, very subtle changes in receptor-binding preference might only be observed using HAE cultures; in our hands non-ciliated/ciliated HA binding ratios were more discriminative on HAE culture cells than on human trachea tissue (*Table 3.5*).

7.5 Which influenza virus subtype will cause the next pandemic?

The experiments described above suggest that relatively small differences in viral fitness have the potential to tip the balance and create a pandemic influenza virus. So, what influenza viruses are out there, infecting both avian and swine hosts, that need only a small change to cause the next human influenza pandemic?

Avian influenza virus H5N1 continues to circulate in wild bird and poultry populations and has caused hundreds of human infections since 1997. However, none of these infections have yet led to sustained human-to-human transmission. Recently published studies investigating the RD transmission in ferrets of a mutated recombinant H5N1 virus based on a human isolate by groups led by Dr. Yoshihiro Kawaoka and Dr. Ron Fouchier show that H5 is capable of transmission between mammals (Herfst et al. 2012, Imai et al. 2012). Herfst *et al.* achieved airborne transmission of complete A/Indonesia/5/2005 H5N1 virus between ferrets on six different occasions with a minimum of nine amino acid mutations (mutations Q226L, G228S (HA) and E627K (PB2) were introduced, mutations H103Y, T156A (HA), H99Y, I368V (PB1), R99K and S345N (NP) were found after serial passage within the ferret host, virus F5). The mutations that were introduced into the virus by mutagenesis before serial passage (Q226L, G228S (HA) and E627K (PB2)) were found in all six progeny airborne-transmissible viruses, but only two additional mutations were consistently detected; H103Y and T156A (HA), which removes a potential N-linked glycosylation site (Herfst et al. 2012). Imai *et al.* achieved transmission of their recombinant H5N1 virus (HA of A/Vietnam/1203/2004 on a backbone of

A(H1N1)pdm09) by mutating four amino acids in the HA protein (mutations N224K and Q226L were introduced by mutagenesis, mutations N158D and T318I were found after serial passage within the ferret host). It is particularly interesting that transmission of H5 occurred on a A(H1N1)pdm09 backbone, and the reassortment event leading to such a viral variant is not inconceivable; both A(H1N1)pdm09 and H5N1 can be found in swine populations in Asia (Nidom et al. 2010, Lee et al. 2009a, Zhou et al. 2011, Kim et al. 2011). It is not clear whether the complete H5N1 virus discussed in Herfst *et al.* would be transmitted through the air with just the three engineered and two consistently found additional mutations or whether the additional four mutations found in virus F5 are necessary to cause transmission. In the latter case, the chances of such a virus arising in nature are severely decreased and it seems more likely that a reassortment event with for example A(H1N1)pdm09 such as that discussed in Imai *et al.* is more likely to give rise to a future pandemic virus.

Another potential pandemic candidate that was the subject of Chapter 5 is influenza subtype H7. This virus has a distinct ocular tropism and although infection can lead to severe respiratory distress and even death (Fouchier et al. 2004) it is often associated with self-limiting conjunctivitis. This relatively mild symptomatic display could contribute to undetected viral infections. So far, there have been more than 100 detected cases of human infection with H7 including a H7N7 outbreak in the Netherlands causing 89 human infections (Belser et al. 2009a, Fouchier et al. 2004), but only limited human-to-human transmission has been reported (Koopmans et al. 2004). Recent North American H7 isolates display a more human-like receptor

binding profile and can be transmitted to contact sentinels in a ferret model (Belser et al. 2008), theoretically increasing its chances of causing a pandemic. Lastly, as discussed above H7 influenza virus has the potential to use eye tissue as an alternative infection site and move down into the respiratory tract via the tear ducts, potentially gathering adaptive mutations in the process.

Another avian influenza virus that forms a potential pandemic threat is of the H9N2 subtype. This virus has been prevalent among domestic poultry in Asia since the early 1990s (Choi et al. 2004) and has since reached other parts of the world (Tombari et al. 2011). H9N2 has caused repeated human infections in Asia (Peiris et al. 1999, Butt et al. 2005). Patients usually present with mild clinical symptoms and no fatal cases have been reported. As with H5 and H7, no sustained human-to-human transmission has yet occurred. However, H9N2 displays a more human-like receptor binding preference (Wan et al. 2008) and both contact transmission and aerosol transmission (after repeated passage in ferrets) of viruses reassorted with H3N2 or A(H1N1)pdm09 viruses in a ferret model has been reported (Sorrell et al. 2009, Kimble et al. 2011, Wan et al. 2008). Interestingly, H9N2 has been detected in pigs (Peiris et al. 2001) increasing the risk of such a reassortant virus arising in nature.

Another potential candidate for the next pandemic is swine-origin H3. Recent events in North America have demonstrated the potential of this virus to be introduced into the human population. Transmission of both A(H1N1)pdm09 and H3N2 in the swine population has resulted in a reassortment virus (H3N2v) containing all genes of swine H3N2 but for the M gene,

which originated from A(H1N1)pdm09. Thirteen human infections with H3N2v have been detected between July 2011 and April 2012, of which six cases had no history of exposure to swine suggesting that human-to-human transmission had occurred (ProMED 12-04-2012). These viruses are able to transmit through the air in the ferret transmission model, although this was not linked to the introduction of the M gene from A(H1N1)pdm09 (Pearce et al. 2012). H3N2 was introduced into the American swine population in 1998 from a human source (Zhou et al. 2000) and consequently people born before 1998 are expected to have been exposed to an antigenically similar virus. This is reflected in the age of the H3N2v-infected patients; eleven of the thirteen cases occurred in children aged below ten years. Therefore, although H3N2v is possibly able to transmit between humans (although this is not yet confirmed) its pool of susceptible cases is limited compared to that of for example H5N1. H3 influenza virus does not only circulate in pigs and humans, but can also be found in the avian population. Until the recent A(H1N1)pdm09 many scientists assumed that the human population would be sufficiently immune to H3, and other subtypes like H1 and H2 due to previous exposure and no pandemic preparedness aimed at these three subtypes was undertaken. Capua *et al.* however hypothesised that due to antigenic drift, contemporary avian influenza viruses would not be cross-reactive with contemporary human influenza viruses. Indeed, antibodies present in human sera obtained 3-5 weeks after vaccination using the seasonal 2006-2007 vaccine did not cross-react with Eurasian avian H1 and H3 viruses, and limited H2 cross-reactivity was found in

people >40 years of age (Capua et al. 2009), highlighting a potential large pool of susceptible subjects.

In my personal opinion, the influenza A subtype most likely to cause the next pandemic is H2. H2N2 has already proven its ability to cause a pandemic in 1957, something that H5, H7 and H9 might never be able to do. This virus continues to circulate in the avian host and has been isolated from birds in geographically distinct locations such as Norway, Australia, North America and Germany (Jonassen and Handeland 2007, Kishida et al. 2008, Makarova et al. 1999, Suss et al. 1994) as well as in pigs (Ma et al. 2007). Moreover, earlier isolates of H2 had the potential to bind α 2,6-linked sialic acid (Viswanathan et al. 2010) and H2N3 which has been isolated from swine in the United States was able to transmit to contact sentinels in both the ferret and swine model (Ma et al. 2007). Finally, everyone born after 1968, the last year in which H2N2 was detected in the human host, has no protective immunity against H2N2 influenza virus (Nabel, Wei and Ledgerwood 2011, Capua et al. 2009). This includes me, and an estimated 1,833,255,876 others (number based on google data as of 2010, www.google.co.uk/publicdata). During a standard worldwide proficiency panel testing in 2004-2005, A/Japan/305/1957 H2N2 influenza virus was accidentally sent out to approximately 4,000 laboratories. As this virus should be handled under biological containment level 3 conditions, and the proficiency panel is regularly handled under biological containment level 2 conditions, CDC immediately took precautions to prevent a laboratory-origin influenza pandemic. All laboratories and organisations responsible for delivery of the panels were urged to destroy the H2N2 samples, and laboratory

workers exposed to the proficiency panel were closely followed up. Although this particular incident did not cause an outbreak (Schrauder et al. 2006), the significant response of the CDC further underlines the possibility of an influenza pandemic caused by H2N2 (EditorialTeam et al. 2005).

Whichever subtype will cause the next influenza pandemic, it is vital that good global surveillance of avian, swine and human hosts is in place. Although A(H1N1)pdm09 was first detected in Mexico, Eurasian swine flu (which provided the NA and M gene of A(H1N1)pdm09) has still not been detected in this continent. Therefore, it would be more logical to conclude the actual reassortant arose in Asia, where all genetic elements are currently circulating in the swine host. If this reassortment had been detected at an earlier stage, the influenza community might have been better prepared, by for example starting vaccine development. In the future, this information can potentially save thousands of lives.

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