Zoonotic and Pandemic Influenza A Viruses: Lessons from H5N1 and H2N2

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The research described in this thesis was conducted at the Department of Viroscience of the Erasmus Medical Center, Rotterdam, The Netherlands and within the Postgraduate School of Molecular Medicine.

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Zoönotische en pandemische influenza A virussen:
leren van H5N1 en H2N2

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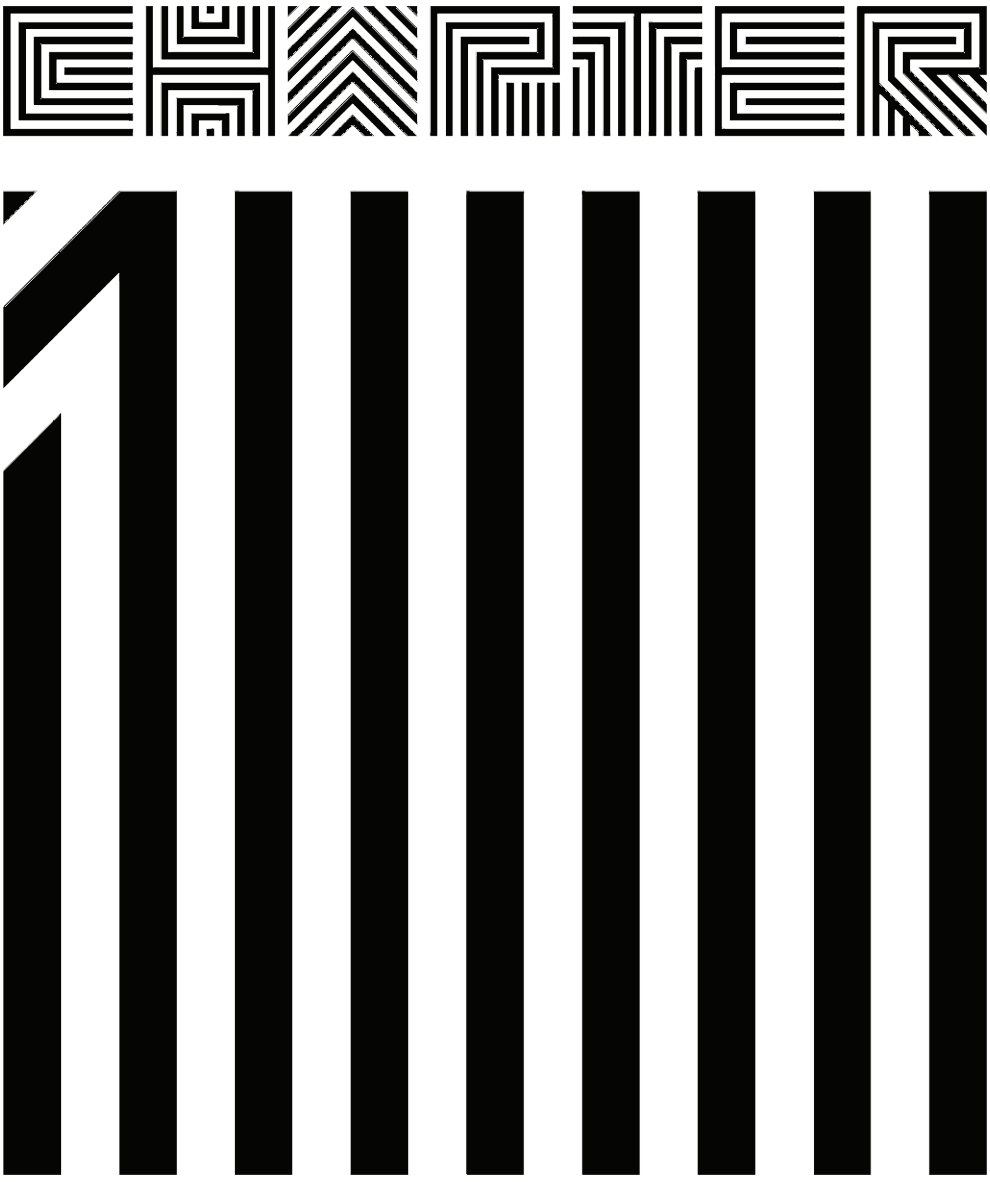

Introduction

partially based on
Predicting 'airborne' influenza viruses:
(trans-) mission impossible?
Current Opinion in Virology. 2011 (1)

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Influenza A viruses - Classification

Influenza A virus is a segmented negative stranded RNA virus within the Alphainfluenzavirus genus of the family Orthomyxoviridae. This family further contains the genera Betainfluenzavirus, Gammainfluenzavirus and Deltainfluenzavirus and the three non-influenza genera Thogotovirus, Isavirus and Quaranjavirus (2). Influenza A viruses are divided into subtypes based on antigenic differences between the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). So far, 16 subtypes of HA and 9 subtypes of NA have been described that originate from waterfowl (H1 to H16, N1 to N9) (3). The combination of HA and NA of a virus leads to the common abbreviations such as H1N1, H2N2, H5N1, H7N9, H16N9 to specify virus subtypes. In addition, two influenza A-like viral genomes have been identified in fruit bats, which contain antigenically and functionally distinct surface glycoproteins and have been provisionally designated “HL17NL10” and “HL18NL11”, with HL and NL indicating HA-like and NA-like surface glycoproteins (4, 5). Influenza was first described in 1878 as a distinct avian disease (6). In the 1930s, influenza A viruses were identified to cause disease in human and swine (7). The fact that these viruses from birds, pigs, and humans were closely related was not recognized until much later. Influenza virus isolates from different species have been collected since 1902 to cover the pandemic outbreaks of H2N2 in 1957, H3N2 in 1968, H1N1 in 2009 as well as zoonotic events in humans of H5N1 from 1997 and H7N9 from 2013 onwards respectively.

Influenza A viruses - Genome constellation and virion structure

Influenza A virus particles are pleopmorphic, spherical to filamentous, with a diameter of about 80-120 nm, and a lipid bilayer envelope that is derived from the host cell membrane (Figure 1). The two major glycoproteins HA and NA cover the viral surface and have important functions during virus attachment and release. M1 proteins line the inner part of the viral membrane mainly conferring stability to the virion. M2 proteins are transmembrane proteins acting as ion channels. The genome of influenza A viruses consists of 8 gene segments of negative stranded RNA encoding at least 11 viral proteins. Each RNA segment in the infectious virus particle is associated with nucleoprotein (NP) and the polymerase proteins basic polymerase 1 and 2, and acidic polymerase (PB2, PB1, and PA), together forming the ribonucleoprotein (RNP) complex. The polymerase proteins and NP are required during virus genome replication and transcription of RNA (8).

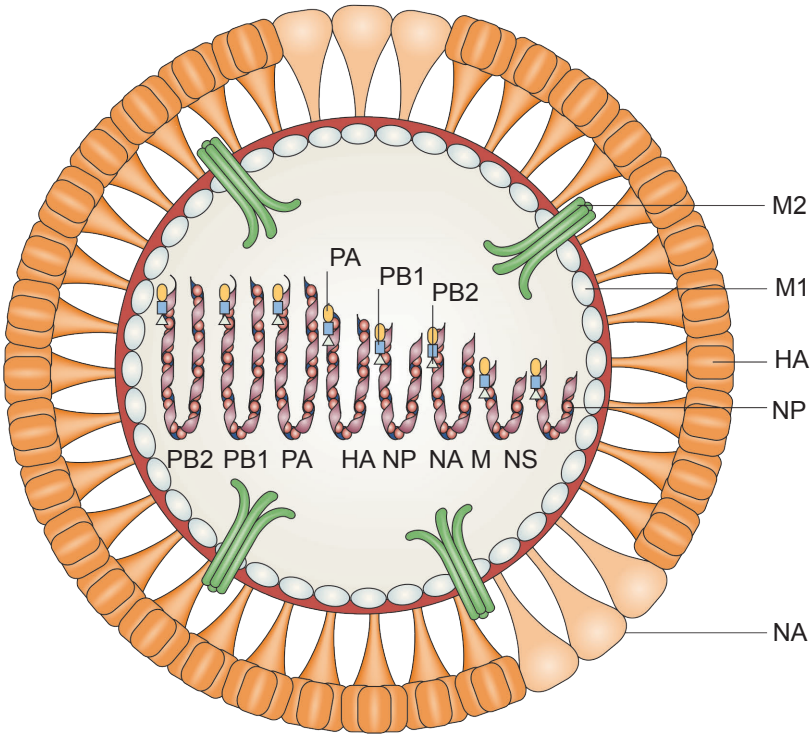


Figure 1: Structure of the influenza A virion
The viral genome consisting of eight viral RNPs is surrounded by a lipid bilayer lined by M1 proteins. HA, NA and M2 are surface molecules anchored in the membrane. Figure taken from Subbarao et al. (9).

Influenza A virus infection and replication

Influenza A viruses attach to cells through interaction of the HA protein with terminal sialic acid containing receptors located on the cell surface. The viruses are subsequently internalized into endosomes by a process called receptor-mediated endocytosis (10). The lowering of the pH in the endosome triggers a conformational change of the HA allowing the viral and endosomal membranes to fuse and the viral RNPs to be released into the cytosol. The RNPs are then transported to the nucleus, leading to the production of three different types of RNA: viral RNA (vRNA) that represents the genome of the virus inside the virus particle, messenger RNA (mRNA) that is translated into new proteins; and copy RNA (cRNA) which is required as a template for transcription of the vRNA. Viral proteins are translated from mRNA in the cytosol or at the endoplasmic reticulum (ER). Surface proteins HA, NA and M2 are transported via the Golgi apparatus to the cellular membrane. This is where new infectious particles are assembled and released through cleavage of remaining linkages between HA and the sialic acid receptor by NA (8).

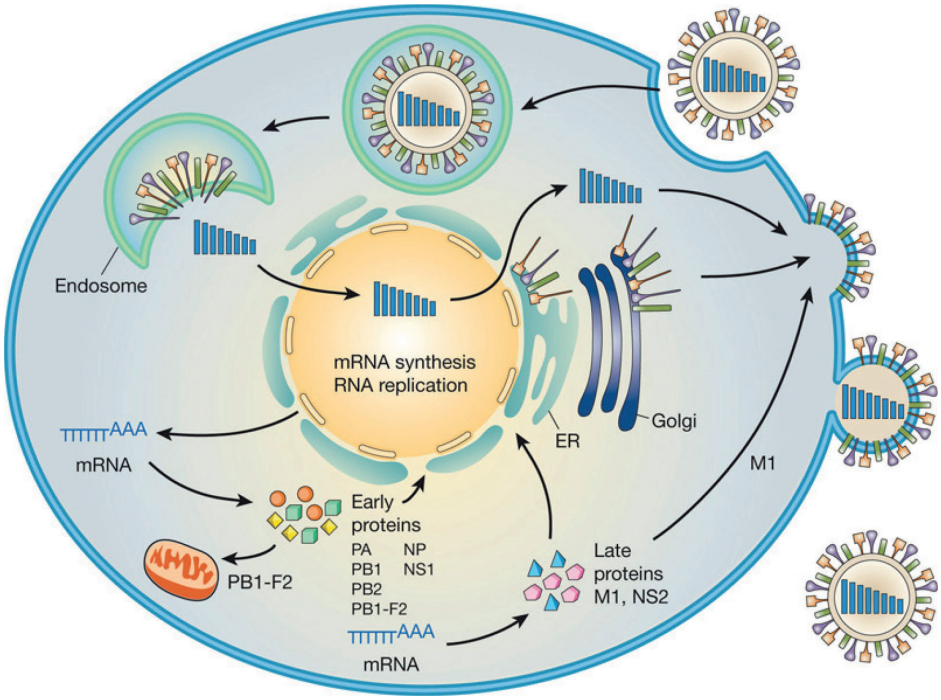


Figure 2: Replication cycle of influenza A viruses
Upon binding to the sialic acid receptor, influenza A virus is internalized by receptor-mediated endocytosis, their genomes are replicated in the nucleus, and new virus particles are assembled at the membrane before they can bud. Figure adapted from Neumann et al. (11).

The viral polymerases and nucleoprotein

The three longest gene segments of influenza A viruses encode the polymerase proteins PB2, PB1, and PA which together form the polymerase complex. Since these viral RNA dependent RNA polymerases lack proofreading activity, the resulting high mutation rate can ultimately lead to rapid adaptation of the virus to a new host. PB2 binds to a cap, the altered nucleotide at the 5' end of some RNAs and this cap-binding step is important for transcription initiation (12). PB2 also harbors the well-studied glutamic acid (E) to lysine (K) substitution at position 627, which is associated with mammalian host-adaptation (13). The PB1 protein is the catalytic subunit of the polymerase complex and allows synthesis of RNA. PA is important for the interaction of all subunits in the complex and exerts endonuclease function. In addition to these 3 polymerase subunits, NP is essential for viral genome replication; it is associated with and physically protects viral RNAs from degradation by host proteins (14). For a full list of influenza A virus gene products, please refer to Table 1.

The hemagglutinin molecule

The HA surface glycoprotein is both a major target for antibodies and a crucial determinant of pathogenicity of influenza A viruses. The HA molecule is a homotrimer (i.e. consisting of three identical subunits or monomers) and harbors the receptor binding site (RBS) (15), a discontinuous 'groove' of amino acids interacting with SA-receptors on host cells. HA is essential for infection, because it induces fusion between the membranes of the virus and the endosome allowing the release of the viral genetic material into the cytosol. This function is dependent on previous cleavage of the HA by host proteases. All influenza A viruses contain a cleavage site, the amino acid position at which the biologically inactive HA0 molecule is split into HA1 and HA2. Low pathogenic avian influenza (LPAI) virus HA can only be cleaved by trypsin-like proteases primarily expressed in the respiratory tract of mammals and intestinal tract of birds, restricting HA0 processing to these organ systems (16). Highly pathogenic avian influenza viruses (HPAI viruses) can emerge after circulation in poultry. They contain a multi basic cleavage site (MBCS) - a stretch of several basic amino acids – that can be cleaved by a range of proteases that are ubiquitously expressed throughout the body of mammals and birds, allowing systemic virus replication and spread. Glycosylation, the addition of sugar-moieties to the HA at specific positions (especially those close to the RBS) can modify binding of antibodies, innate immune sensing and receptor binding, ultimately influencing infectivity, pathogenicity and potentially transmissibility (17). Since glycosylation of the HA depends on the cell machinery, differences in glycosylation pattern exist in different hosts, including cell lines and embryonated chicken eggs (18).

Receptor specificity of HA

Sialic acids at the terminal position of carbohydrate chains on the host cell surface are the target of the HA molecule for attachment. The receptor binding site is located in the head-domain of the HA and individual amino acids within the binding pocket can engage in bonds with the sialic acid molecule. Consequently, single substitutions in the RBS can influence receptor-binding specificity and affinity (19). Avian- and human-adapted influenza A viruses vary in their preference for the type of linkage of the sialic acid molecule. The 'human-type' receptor consists of an oligosaccharide with the terminal sialic acid bound to galactose in an α-2,6 conformation whereas the 'avian type' receptor terminal sugars are arranged in an α-2,3 manner (20). Although human tissue contains both groups of glycans, severity of disease can be at least partially attributed to differential expression of receptors throughout the respiratory tract. In both the ferret and human upper respiratory tract (URT) α-2,6 linked sialic acid receptors are prevailing, whereas in the lower respiratory tract (LRT) both α-2,6 and α-2,3 linked receptors can be found ((21), Figure S2 of chapter 2). In the respiratory and digestive tracts of chickens, α-2,3 linked sialic acids are predominant.

Table 1: Influenza A virus proteins and their major function(s)
Table adapted from Fields Virology, VIth edition (22).

gene number	gene segment	gene product	major function/s
1	PB2	Polymerase Basic 2 (PB2)	internal protein, part of polymerase complex
2	PB1	Polymerase Basic 1 (PB1)	internal protein, part of polymerase complex
		Polymerase Basic 1-F2 (PB1-F2)	targets mitochondria, induction of apoptosis
		Polymerase Basic 1-N40 (PB1-N40)	N-terminally truncated PB1, no polymerase function
3	PA	Polymerase Acidic (PA)	internal protein, part of polymerase complex
		Polymerase Acidic-X (PA-X)	internal protein, host response modulation
		Polymerase Acidic-N155 (PA-N155)	N-terminally truncated PA, no polymerase function
		Polymerase Acidic-N182 (PA-N182)	N-terminally truncated PA, no polymerase function
4	HA	Hemagglutinin (HA)	surface glycoprotein, membrane fusion viral attachment, antigenic determinant
5	NP	Nucleoprotein (NP)	nucleocapsid protein, RNA coating, nuclear targeting
6	NA	Neuraminidase (NA)	surface glycoprotein, virion release, antigenic determinant
7	M	Matrix 1 (M1)	membrane protein, virion stability
		Matrix 2 (M2)	membrane protein ion channel
		Matrix 42 (M42)	M2-like membrane protein, ion channel
8	NS	Nonstructural 1 (NS1)	internal protein multifunctional, immune modulatory
		Nonstructural 2 (NS2 or NEP)	internal protein nuclear export of RNPs

Table 2: Zoonotic influenza virus infections in humans 1974-2019 reported worldwide
Table data adapted from Widdowson et al., 2017 (23) and updated based on WHO monthly risk assessment summaries until May 2nd 2018. Viruses are sorted by first year of detection.

Subtype Group	Year First Detected	Year Last Detected	Countries of Occurrence	Number of Confirmed Cases	Number of Confirmed Fatalities
H1N1v	1958	2018	Canada, China, Czechoslovakia, Italy, Netherlands, Russia, Spain, Switzerland, Thailand, US	43	7
HPAI H7N7	1959	2003	Australia, US, Netherlands	91	1
LPAI H7N7	1979	2013	US, Italy, United Kingdom	5	0
H3N2v	1992	2019	Australia, Canada, Hong Kong SAR, Netherlands, US, Vietnam	435	2
HPAI H5N1	1997	2019	Azerbaijan, Bangladesh, Cambodia, Canada, China, Djibouti, Egypt, Hong Kong, Indonesia, Iraq, Laos, Myanmar, Nepal, Nigeria, Pakistan, Thailand, Turkey, Vietnam	861	455
LPAI H9N2	1998	2015	Bangladesh, China, Egypt, Hong Kong SAR	39	1
LPAI H7N2	2003	2017	United Kingdom, US	7	0
HPAI H7N3	2004	2012	Canada, Mexico	4	0
LPAI H10N7	2004	2012	Australia, Egypt	4	0
LPAI H7N3	2006	2006	United Kingdom	1	1
H1N2v	2007	2017	Brazil, Philippines, US	26	0
LPAI H7N9	2013	2018	Canada, China, Malaysia, Taiwan	1568	616
LPAI H10N8	2013	2014	China	3	2
LPAI H6N1	2013	2013	Taiwan	1	0
HPAI H5N6	2014	2018	China	22	6
HPAI H7N9	2017	2017	China, Taiwan	8	4
LPAI H7N4	2018	2018	China	1	0

Genetic drift and shift

Two distinguishable mechanisms lead to genetic variation of influenza A viruses. The term ‘drift’ denotes the successive accumulation of mutations that become fixed in the virus population either stochastically or due to their fitness advantage. ‘Shift’ refers to the rearrangement of genetic material also termed reassortment and was the main underlying mechanisms that led to the pandemics of 1957, 1968 and 2009 (24, 25). Coinfection of a single cell with two or more different influenza A viruses is required for reassortment to happen. During virion formation, gene segments of influenza A viruses can be redistributed in arrangements different from the two or more parental viruses. Pigs are widely considered to form ‘mixing vessels’, because they can be infected with a range of human and avian adapted influenza A viruses (26). Reassortment is obviously the more drastic change in virus evolution and led to the formation of several - if not all - pandemics of the last century (Figure 3). Individual substitutions seem to ‘fine-tune’ the viral phenotype to yield optimal host adaptation.

Zoonotic influenza A viruses

Incidental infection of mammals with influenza A viruses of various subtypes normally found in avian species can occur. In the past century, influenza A virus subtypes H1, H2, and H3 were capable of causing pandemics (extended outbreaks on multiple continents), while H5, H6, H7, H9, and H10 viruses caused zoonoses (confined infections at the human-animal interface) during the last decades (Table 2). Zoonotic events have been reported frequently recently, partially due to increased awareness, increased surveillance and improved detection methods.

HPAI avian H5N1 virus circulating in South-East Asia since 1996 was rapidly recognized as the causative agent of the first outbreak in humans in 1997 (27, 28). In August 1997, the index human case of HPAI H5N1 virus infection was reported. 18 hospitalized patients in Hong Kong Special Administrative Region were infected with viruses closely resembling those found in chicken (29); six of them succumbed due to the infection. Rigorous control measurements including the temporary closing down of live bird markets and culling of poultry prevented the further spread of H5N1 virus in Hong Kong. H5N1 virus seemed to have disappeared after that but reemerged in 2003 in China. Since then the virus has been detected frequently in various hosts in different parts of the world and continues to be detected to date. In addition to domestic poultry, HPAI H5N1 virus was also found in pigs, cats, dogs, martens, tigers and leopards. Human infections are detected from time to time, so far adding up 861 cases, 455 of which were fatal (30). The case fatality rate in hospitalized cases is 50-60% with the peak age group of infected humans at 10-19 years (31). However, the true case-fatality rate is probably lower as a consequence of underreporting. Epidemiology studies

identified poultry markets as a major source for human infections (32). In 2015, clusters of human infections have been reported from Egypt totaling to 119 infected individuals by the end of March (33). In recent years, the number of H5N1 infections has been decreasing with only four laboratory-confirmed reports in 2017 and a single case in Nepal in 2019.
Eighty-seven human infections of H7N7 virus were confirmed in the Netherlands in 2003, one of which was fatal (34, 35). Human infections with H7N9 viruses were reported first in China in 2013 (36), cumulating to 1568 human infections including 616 fatal cases by May 2019 (37). Sporadic human infections have also been detected in Hong Kong, Taiwan, Canada, and Malaysia. The disease can rapidly progress from general influenza-like symptoms to severe pneumonia (38). Poultry workers in China are at great risk for H7N9 infection (39). Infections seem to appear in waves, with the fifth wave currently ongoing (40). H7N9 displays limited transmission capability in the ferret model by the airborne route, but sustained transmission upon direct contact (39, 41, 42). This H7N9 virus has acquired a number of mutations that are associated with human adaptation and resistance against antiviral drugs and could possibly lead to a future pandemic after further human adaptation (43). In February 2017, Chinese authorities reported the detection of human infections with highly-pathogenic H7N9 viruses in the Guangdong Province (44). These viruses contained a multiple basic cleavage site, but are not associated with increased disease severity in humans so far (45).
Three other H7 virus subtypes (H7N2, H7N3, H7N4) have been associated with sporadic human infection (46, 47). An H6N1 virus was isolated from one patient in Taiwan in 2013 (48). H6 viruses are capable of infecting mice and ferret and are transmissible between guinea pigs (49-51). Avian H9N2 viruses, a subtype that was previously shown to acquire transmissibility between ferrets (52, 53) has been circulating extensively in the Eastern hemisphere for more than a decade. In 2013, H10N8 viruses caused three human infections in China, two of which were fatal (54). Numbers mentioned above are correct as of 8 May 2019.

Pandemic influenza A viruses

In rare cases, zoonotic influenza A viruses can become established in the human population. Upon introduction in humans, the high error rate of the viral polymerase and the capacity of reassortment (gene segment mixing) facilitate rapid adaptation to the new host. To spread efficiently between humans and cause a pandemic, dissemination via the airborne route (virus transmission via aerosol and/or respiratory droplets) and a sufficiently large immunologically naïve population is crucial. It was long assumed that influenza viruses capable of causing pandemics comprised subtypes H1, H2 and H3 only, since pandemics of the past were restricted to these three subtypes. For the pandemics of 1889 (H2N2) and 1900 (H3N8), only seroarcheological

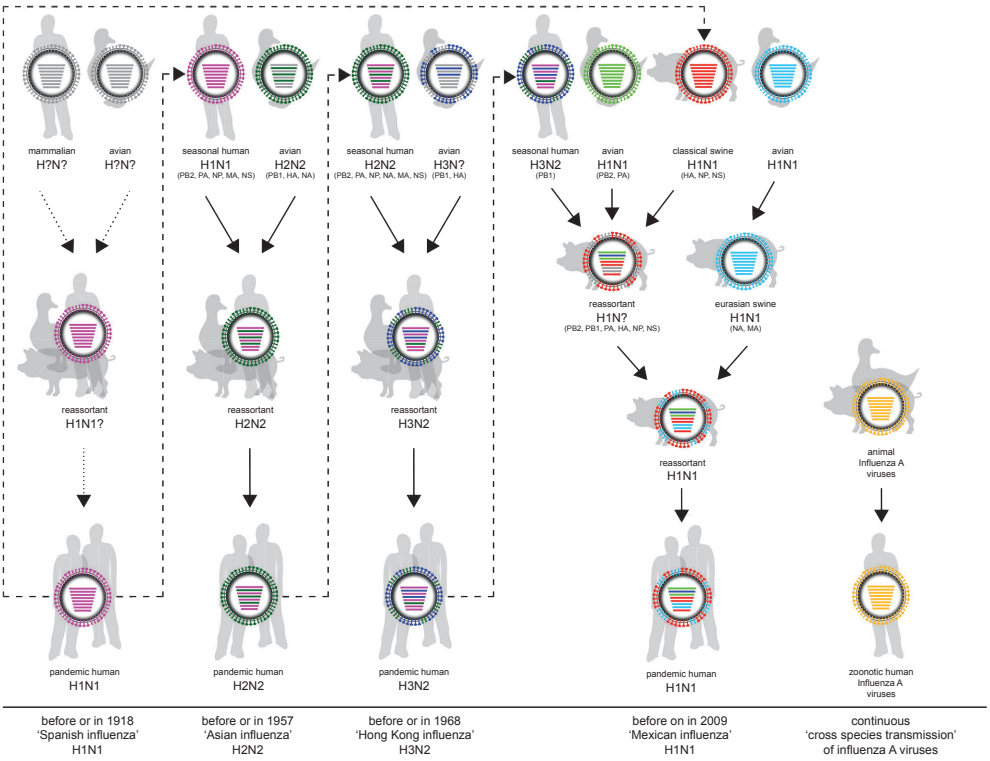


Figure 3: Schematic overview of genetic events leading to past pandemic viruses and interspecies transmission of zoonotic influenza viruses.
Gene segments are indicated in decreasing size
(PB2, PB1, PA, HA, NP, NA, MP, NS). Figure adapted from Sorrell et al. (1).

evidence is available (55). Viruses that caused the pandemic in 1918 (H1N1) were reconstructed from tissues of individuals that succumbed to the infection and were buried in the permafrost and from archived autopsy material (56). For the 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1) pandemics, virus was isolated from infected patients directly.

A century ago, the deadliest influenza pandemic recorded to date was sparked by an H1N1 virus. This virus was most likely directly introduced in the human population from birds (57). An estimated 50 to 100 million individuals succumbed during the 'Spanish flu' pandemic in the winter months of 1918/1919 and remarkably the main death toll was observed in otherwise healthy adults (58).

H2N2 influenza A viruses were first reported in Southeast Asia in 1957 and caused a pandemic with an estimated mortality of 1 to 4 million people worldwide (59). Reassortment between avian and human influenza A viruses led to the formation of H2N2 viruses consisting of the HA, NA and PB1 gene segments from avian influenza viruses, and the other five gene segments from the human H1N1 virus that had been circulating since 1918 (60, 61). In 1968, after 12 years of circulation in the human population, H2N2 was replaced by H3N2, after one or several reassortment events with avian viruses. Since then, influenza A viruses of the H2N2 subtype and other combinations of H2 and N2 keep circulating in waterfowl and pigs globally (62-65), thereby providing a reservoir from which H2N2 may be reintroduced into the human population. Since H2N2 viruses have been absent in the human population from 1968 onwards, many individuals, and certainly those born after 1968, have no significant immunological protection against these viruses.

Subsequently, in 1968, a new H3N2 virus strain appeared in Asia. The virus was a reassortant from the previously circulating H2N2 virus and contained gene segments PB1 and HA from an avian H3 virus (61). The first virus isolate was detected in Hong Kong in July 1968, hence the name 'Hong Kong flu' pandemic (66).

In the years preceding 2009, a number of reassortment events involving human H3N2, avian H1N1 and swine H1N1 viruses led to the generation of a new reassortant H1N1 virus that was at the start of the first pandemic of the 21st century (67). The 'Mexican flu' virus was first detected in South America and had direct precursors in pigs (68). Both H3N2 and the recent pandemic H1N1 virus are circulating as seasonal influenza A viruses to date.

Airborne transmission of influenza A viruses between mammals

Ultimately, all pandemic influenza A viruses were once zoonotic in nature, but not all zoonotic viruses have acquired or will ever acquire the potential to become airborne transmissible and cause a pandemic. Human-adapted viruses usually cause infections that are restricted to the respiratory tract with severe cases leading to pneumonia, but they do generally not infect other organs (69). Mutagenesis of the RBS of pandemic 1918 H1N1, pandemic 1957 H2N2 and seasonal H3N2 HA resulted in a switch of receptor preference, from the human- to avian-type receptor, abrogating airborne transmission between ferrets (70-72). It might be speculated that the scarcity of avian receptors in human upper respiratory epithelium cannot prevent infection with influenza A viruses displaying avian-type receptor preference, but does not allow airborne transmission.

We previously hypothesized, after analyses of the processes leading to the H1N1 (1918), H2N2 (1957), H3N2 (1968), and H1N1 (2009) pandemic viruses, that three different virus properties may be crucial for aerosol transmission between mammals to occur; binding to cells in the upper respiratory tract (URT), replication to sufficiently high viral titers in the cells of the URT and the generation of individual virus particles rather than virus aggregates (1). Virus-containing aerosols or respiratory droplets can be exhaled directly from the URT without settling of substantial portions of the particles on inanimate surfaces after their formation. Respiratory droplets are commonly defined as moisture particles >5 μm in diameter. They are attracted by gravity and settle rather quickly. Aerosols are spherical particles smaller than 5 μm in size that can stay in the air for prolonged periods. Both types of particles travel through the air and are formed by coughing, sneezing, breathing and talking. Since aerosols can travel larger distances in the air, this type of particle is suggested to be at the origin of pandemics when spiked with human-adapted influenza A viruses. To establish infection, a sufficient number of aerosols needs to contain virus. High viral titers and the packaging of individual virions in airborne droplets seem to be required for effective airborne spread.

Vaccines and antivirals

Influenza virus infection can cause symptoms in humans ranging from cough, sore throat, fatigue and nausea to pneumonia and acute respiratory distress syndrome. Currently, licensed vaccines are available for seasonal A(H1N1) pdm09 and H3N2 influenza A viruses, and the two lineages of influenza B virus. Vaccination is recommended for high-risk individuals including the elderly, people with chronic diseases, pregnant women and healthcare workers. Although the effectiveness of current vaccines is far from perfect, they have an important role to reduce influenza associated morbidity and mortality (73). Since influenza viruses mutate continuously, regular updates in vaccine formulation are required and yearly revaccination is advised for

improved protection. Currently, H3N2, H1N1 and influenza B viruses are circulating worldwide. Vaccines comprise strains of the above-mentioned viruses that are selected twice yearly by an expert group under guidance of the World Health Organization (WHO). Recently available tetravalent influenza vaccines include both Victoria and Yamagata lineage influenza B virus seed strains.

Antiviral drugs against influenza A viruses comprise the two neuraminidase inhibitors oseltamivir and zanamivir as well as the two ion channel inhibitors amantadine and rimantadine. Immune compromised patients are likely to profit from treatment with anti-influenza drugs to reduce viral load. Resistance mutations have been described for both classes of antivirals and are of growing concern nowadays (74).

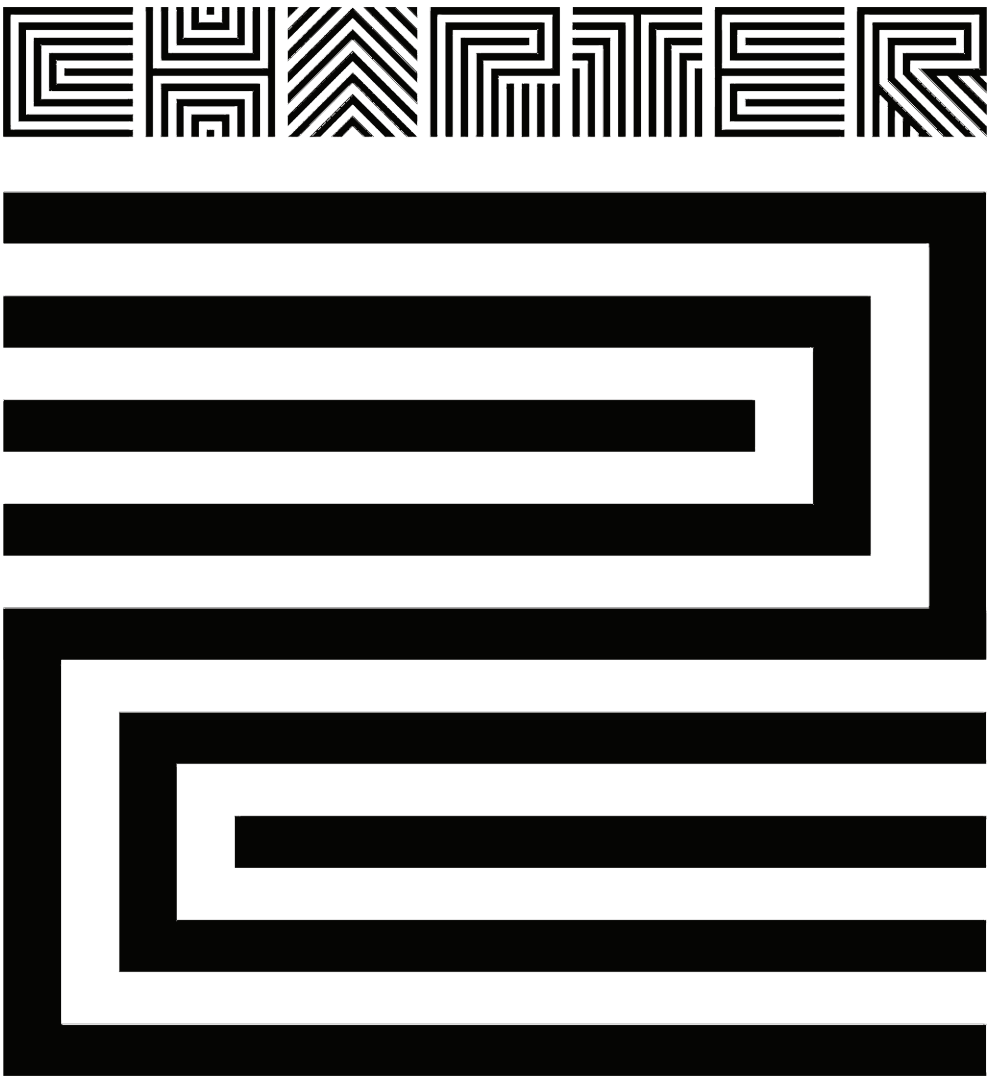
Scope and outline of the thesis

This thesis presents research on influenza A virus subtypes H5N1 and H2N2 to gain knowledge about zoonotic and pandemic viruses and potentially to aid in pandemic risk mitigation in the future. Both influenza A virus subtypes are considered to be possible candidates for a future influenza pandemic. Whereas H5N1 viruses circulate in avian species and cause incidental zoonotic infections, H2N2 virus was at the origin of a pandemic in 1957 and continued to circulate in waterfowl.

Chapter 2 describes the natural selection of an altered H5N1 virus isolate in ferrets by repeated inoculation of naïve animals. The resulting passaged mutant virus was shown to be able to transmit via the air between ferrets. Chapter 3 describes the identification of the mutations essential for this airborne spread and the viral phenotypes associated with each of the five required substitutions. Moreover, this chapter demonstrates that adaptive changes in the H5N1 virus genome accumulated very rapidly during passage in ferrets.

In chapter 4, the diversity of avian and human H2N2 virus isolates is highlighted in phylogenetic analyses, and sites within the viral genome that are under selection pressures are identified. Chapter 5 further describes investigations to map the antigenic evolution of H2N2 viruses that circulated between 1957 and 1968, while chapter 6 depicts the potential to spread via the air for a range of avian and human H2N2 virus isolates.

A summarizing discussion of the studies presented in this thesis in the context of work of others is presented in chapter 7.



Airborne transmission of influenza A/H5N1 virus between ferrets

Science, 2012

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ABSTRACT

Highly pathogenic avian influenza H5N1 virus can cause morbidity and mortality in humans but thus far has not acquired the ability to be transmitted by aerosol or respiratory droplet ("airborne transmission") between humans. To address the concern that the virus could acquire this ability under natural conditions, we genetically modified H5N1 virus by site-directed mutagenesis and subsequent serial passage in ferrets. The genetically modified H5N1 virus acquired mutations during passage in ferrets, ultimately becoming airborne transmissible in ferrets. None of the recipient ferrets died after airborne infection with the mutant H5N1 viruses. Four amino acid substitutions in the host receptor-binding protein hemagglutinin, and one in the polymerase complex protein basic polymerase 2, were consistently present in airborne-transmitted viruses. The transmissible viruses were sensitive to the antiviral drug oseltamivir and reacted well with antisera raised against H5 influenza vaccine strains. Thus, avian H5N1 influenza viruses can acquire the capacity for airborne transmission between mammals without recombination in an intermediate host and therefore constitute a risk for human pandemic influenza.

MAIN TEXT

Influenza A viruses have been isolated from many host species, including humans, pigs, horses, dogs, marine mammals, and a wide range of domestic birds, yet wild birds in the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders) are thought to form the virus reservoir in nature (75). Influenza A viruses belong to the family Orthomyxoviridae; these viruses have an RNA genome consisting of eight gene segments (76, 77). Segments 1 to 3 encode the polymerase proteins: basic polymerase 2 (PB2), basic polymerase 1 (PB1), and acidic polymerase (PA), respectively. These proteins form the RNA-dependent RNA polymerase complex responsible for transcription and replication of the viral genome. Segment 2 also encodes a second small protein, PB1-F2, which has been implicated in the induction of cell death (78, 79). Segments 4 and 6 encode the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), respectively. HA is responsible for binding to sialic acids (SAs), the viral receptors on host cells, and for fusion of the viral and host cell membranes upon endocytosis. NA is a sialidase, responsible for cleaving SAs from host cells and virus particles. Segment 5 codes for the nucleocapsid protein (NP) that binds to viral RNA and, together with the polymerase proteins, forms the ribonucleoprotein complexes (RNPs). Segment 7 codes for the viral matrix structural protein M1 and the ion-channel protein M2 that is incorporated in the viral membrane. Segment 8 encodes the nonstructural protein NS1 and the nucleic export protein (NEP) previously known as NS2. NS1 is an antagonist of host innate immune responses and interferes with host gene

expression, whereas NEP is involved in the nuclear export of RNPs into the cytoplasm before virus assembly (76, 77).

Influenza A viruses show pronounced genetic variation of the surface glycoproteins HA and NA (75). Consequently, the viruses are classified based on the antigenic variation of the HA and NA proteins. To date, 16 major antigenic variants of HA and 9 of NA have been recognized in wild birds and are found in numerous combinations designated as virus subtypes (for instance, H1N1, H5N1, H7N7, and H16N3), which are used in influenza A virus classification and nomenclature (3, 75). This classification system is biologically relevant, as natural host antibodies that recognize one HA or NA subtype will generally not crossreact with other HA and NA subtypes.

On the basis of their virulence in chickens, influenza A viruses of the H5 and H7 subtypes can be further classified into highly pathogenic avian influenza (HPAI) and low-pathogenic avian influenza (LPAI) viruses. Viruses of subtypes H1 to H4, H6, and H8 to H16 are LPAI viruses. The vast majority of H5 and H7 influenza A viruses are also of the LPAI phenotype. HPAI viruses are generally thought to arise in poultry after domestic birds become infected by LPAI H5 and H7 viruses from the wild-bird reservoir (80, 81). The HA protein of influenza A viruses is initially synthesized as a single polypeptide precursor (HA0), which is cleaved into HA1 and HA2 subunits by trypsin-like proteases in the host cell. The switch from LPAI to HPAI virus phenotype occurs upon the introduction of basic amino acid residues into the HA0 cleavage site, also known as the multibasic cleavage site (MBCS). The MBCS in HA can be cleaved by ubiquitously expressed host proteases; this cleavage facilitates systemic virus replication and results in mortality of up to 100% in poultry (16, 82).

Since the late 1990s, HPAI H5N1 viruses have devastated the poultry industry of numerous countries in the Eastern Hemisphere. To date, H5N1 has spread from Asia to Europe, Africa, and the Middle East, resulting in the death of hundreds of millions of domestic birds. In Hong Kong in 1997, the first human deaths directly attributable to avian H5N1 virus were recorded (28). Since 2003, more than 600 laboratory-confirmed cases of HPAI H5N1 virus infections in humans have been reported from 15 countries (83). Although limited H5N1 virus transmission between persons in close contact has been reported, sustained human-to-human transmission of HPAI H5N1 virus has not been detected (84-86). Whether this virus may acquire the ability to be transmitted via aerosols or respiratory droplets among mammals, including humans, to trigger a future pandemic is a key question for pandemic preparedness. Although our knowledge of viral traits necessary for host switching and virulence has increased substantially in recent years (87, 88), the factors that determine airborne transmission of influenza viruses among mammals, a trait necessary for a virus to become pandemic, have remained largely unknown (53, 89-91). Therefore, investigations of routes

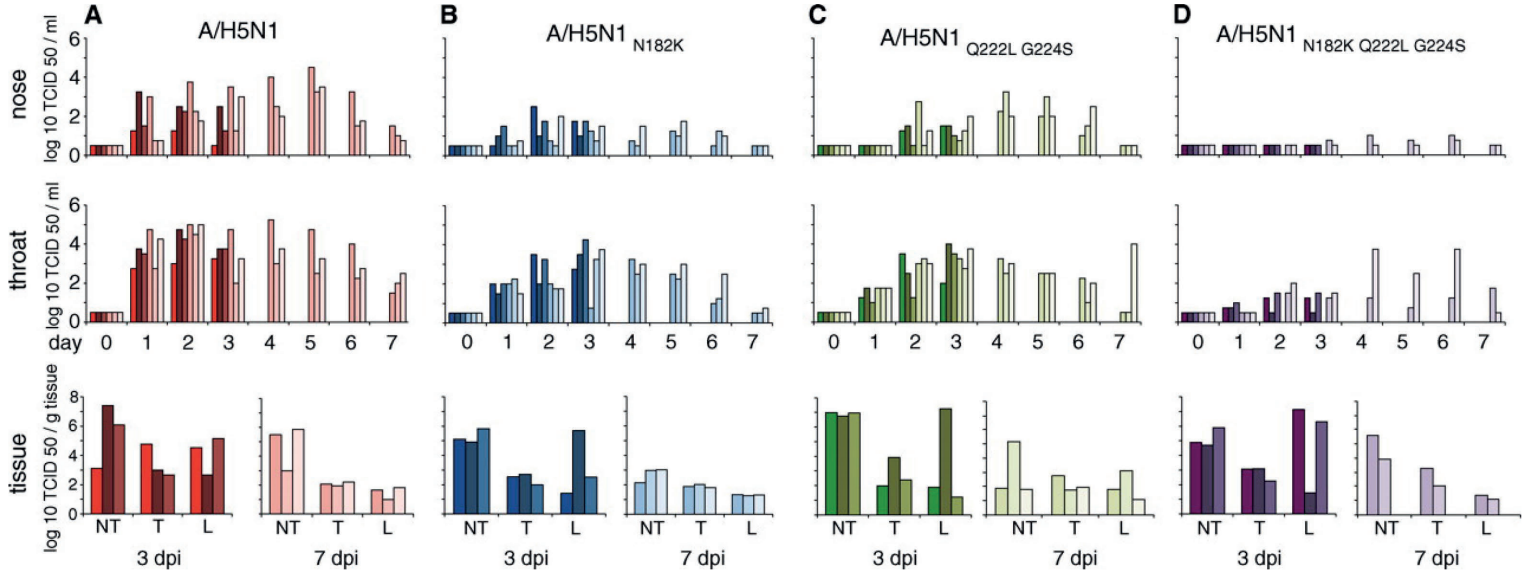

Figure 1:
In experiment 1, we inoculated groups of six ferrets intranasally with 1× 10^6 TCID50 of (A) influenza $H5N1_{wildtype}$ virus and the three mutants (B) $H5N1_{HA\ N182K}$, (C) $H5N1_{HA\ Q222L,G224S}$, and (D) $H5N1_{HA\ N182K,Q222L,G224S}$. Three animals were euthanized at day 3 for tissue sampling and at day 7, when this experiment was stopped. Virus titers were measured daily in nose swabs (top) and throat swabs (middle) and also on 3 and 7 dpi in respiratory tract tissues (bottom) of individual ferrets. Virus titers in swabs and nasal turbinates (NT), trachea (T), and lungs (L) were determined by end-point titration in MDCK cells. [One animal inoculated with $H5N1_{HA\ N182K,Q222L,G224S}$ died at 1 dpi due to circumstances not related to the experiment (D).] (Top two rows) Virus shedding from the URT as determined by virus titers in nasal and throat swabs was highest in $H5N1_{wildtype}$-inoculated animals. The mutant that yielded the highest virus titers during the 7-day period was $H5N1_{HA\ Q222L,G224S}$, but titers were ~1 log lower than for the $H5N1_{wildtype}$-inoculated animals. In the first 3 days, when six animals per group were present, no significant differences were observed between $H5N1_{HA\ N182K}$ and $H5N1_{HA\ Q222L,G224S}$-inoculated animals, as calculated by comparing the viral titer (Mann-Whitney test, P = 0.589 and 0.818 for nose and throat titers, respectively) (Bottom row). No marked differences in virus titers in respiratory tissues were observed between the four groups. Each bar color denotes a single animal.

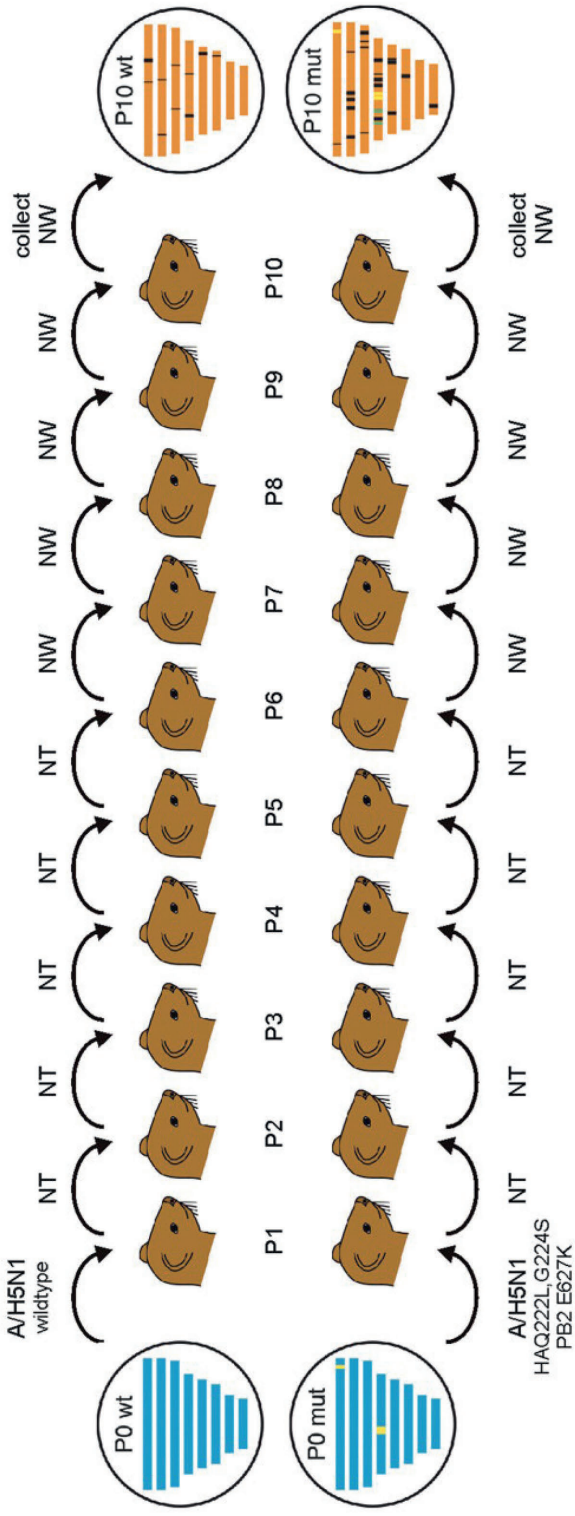

Figure 2:
Experiment 3, virus passaging in ferrets (P1 to P10, passages 1 to 10). Because no airborne transmission was observed in experiment 2, $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$ were serially passaged in ferrets to allow adaptation for efficient replication in mammals. Each virus was inoculated intranasally with 1×10^6 TCID50 in one ferret (2×250 ml, divided over both nostrils). Nose and throat swabs were collected daily. Animals were euthanized at 4 dpi, and nasal turbinates and lungs were collected. Nasal turbinates were homogenized in virus-transport medium, and this homogenate was used to inoculate the next ferret, resulting in passage 2 (Figure S6). Subsequent passages 3 to 6 were performed in the same way. From passage six onward, nasal washes (NW) were collected at 3 dpi in addition to the nasal swabs. To this end, 1 ml of PBS was delivered drop wise into the nostrils of the ferrets, thereby inducing sneezing. Approximately 200 ml of the sneeze was collected in a Petri dish, and PBS was added to a final volume of 2 ml. For passages 7 through 10, the nasal-wash sample was used for the passages in ferrets. The passage-10 nasal washes were subsequently used for sequence analyses and transmission experiments to be described in experiment 4. For details, see the supplementary materials.

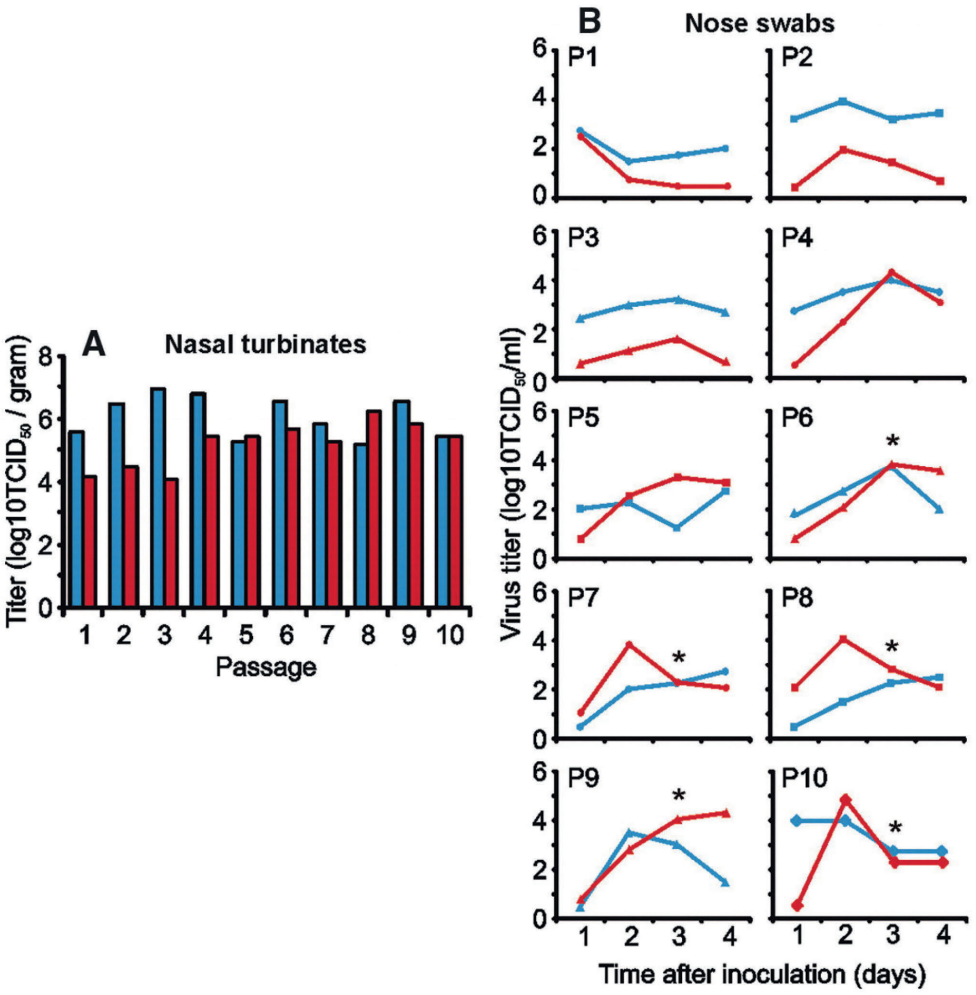

Figure 3:
Virus titers in (A) the nasal turbinates collected at day 4 and (B) nose swabs collected daily until day 4, from ferrets inoculated with H5N1$_{wildtype}$ (blue) and H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ (red) throughout the 10 serial passages described in Figure 2. Virus titers were determined by end-point titration in MDCK cells. After inoculation with H5N1$_{wildtype}$, virus titers in the nasal turbinates were variable but high, ranging from 1.6 × 10^5 to 7.9 × 10^6 TCID50/gram tissue (A), with no further increase observed with repeated passage. After inoculation with H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$, virus titers in nasal turbinates averaged 1.6 × 10^4 in the first three passages, 2.5 × 10^5 in passages four to seven, and 6.3 × 10^5 TCID50/gram tissue in the last three passages, suggestive of improved replication and virus adaptation. A similar pattern of adaptation was observed in the virus titers in the nose swabs of animals inoculated with H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ (B). These titers also increased during the successive passages, with peak virus shedding of 1 × 10^5 TCID50 at 2 dpi after 10 passages. Altogether, these data indicate that H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ adapted to more efficient replication in the ferret URT upon repeated passage, with evidence for such adaptation by passage number 4. In contrast, analyses of the virus titers in the nose swabs of the ferrets collected at 1 to 4 dpi throughout the 10 serial passages with H5N1$_{wildtype}$ revealed no changes in patterns of virus shedding. Asterisks indicate that a nose wash was collected before the nose swab was taken, which may influence the virus titer that was detected.

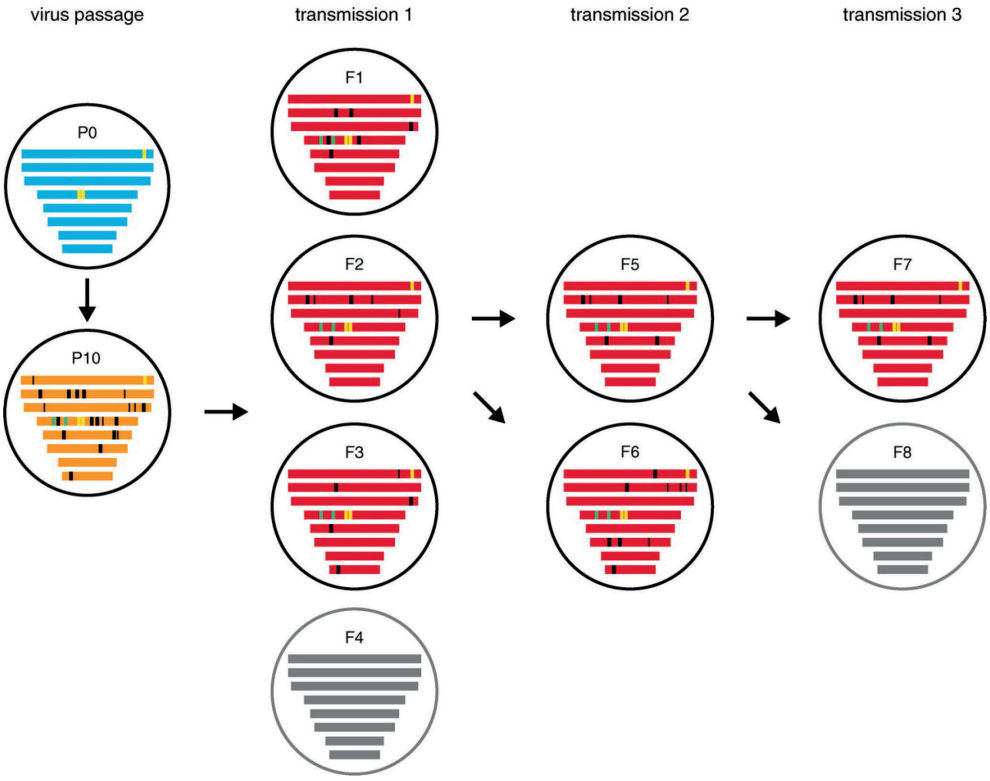

Figure 4:
Summary of the substitutions detected upon serial passage and airborne Transmission of H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ virus in ferrets. The eight influenza virus gene segments and substitutions are drawn approximately to scale (top to bottom: PB2, PB1, PA, HA, NP, NA, M, NS). Viruses shown in blue, orange, and red represent the initial recombinant H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ virus (P0), ferret passage-10 virus (P10), and P10 virus after airborne transmission to recipient ferrets, respectively. Viruses shown in gray indicate that virus was not transmitted to the recipient ferret. First, we tested whether airborne-transmissible viruses were present in the heterogeneous virus population of ferret P10. We inoculated four donor ferrets intranasally, which were then housed in transmission cages and paired with four recipient ferrets. Transmissible viruses were isolated from three out of four recipient ferrets (F1 to F3). Next, we took a throat-swab sample from F2 (this sample contained the highest virus titer among the positive recipient ferrets), and this sample was used to inoculate two more donor ferrets intranasally. In a transmission experiment, these donors infected two recipient ferrets via airborne transmission (F5 and F6). Virus isolated from F5 was passaged once in MDCK cells and was subsequently used in a third transmission experiment in which two intranasally inoculated donor ferrets transmitted the virus to one of two recipient ferrets (F7). The genetic composition of the viral quasi-species present in the nasal wash of ferret P10 was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform. Conventional Sanger sequencing was used to determine the consensus sequence in one high-titer nasal or throat-swab sample for each ferret. Thick and thin black vertical bars indicate amino acid and nucleotide substitutions, respectively; substitutions introduced by reverse genetics are shown in yellow; substitutions detected in passage 10 and all subsequent transmissions are shown in green.

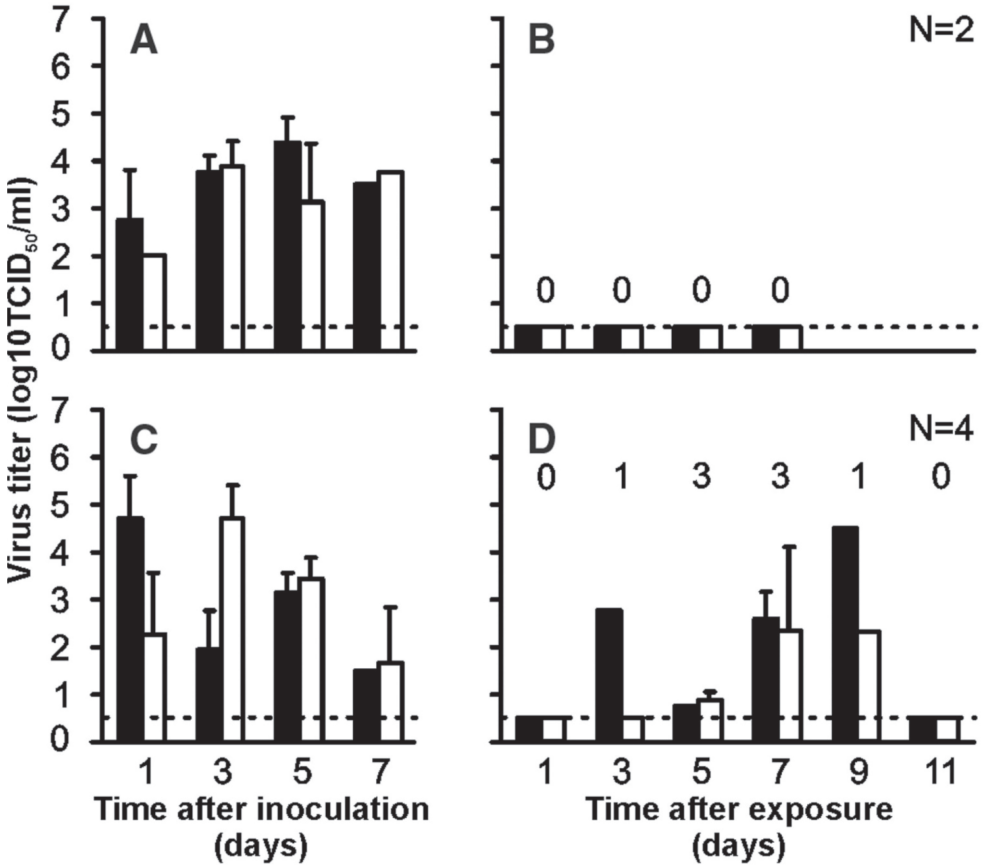

Figure 5:
Airborne transmission of H5N1 viruses in ferrets.
Transmission experiments are shown for $H5N1_{wildtype}$ (A and B) and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ (C and D) after 10 passages (P10) in ferrets. Two or four ferrets were inoculated intranasally with nasal wash samples collected from P10 virus of $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$, respectively, and housed individually in transmission cages (A and C). A naïve recipient ferret was added to each transmission cage adjacent to a donor ferret at 1 dpi (B and D). Virus titers in throat (black bars) and nose swabs (white bars) were determined by end-point titration in MDCK cells. Geometric mean titers and SDs (error bars) of positive samples are shown. The number of animals infected via airborne transmission is indicated in (D) for each time point after exposure; the drop from three animals infected at day 7 to one animal at day 9 and no animals at day 11 is explained by the fact that the animals that became infected via airborne transmission had cleared the virus by the end of the experiment and, therefore, detectable amounts of virus were no longer present. The dotted lines indicate the lower limit of virus detection.

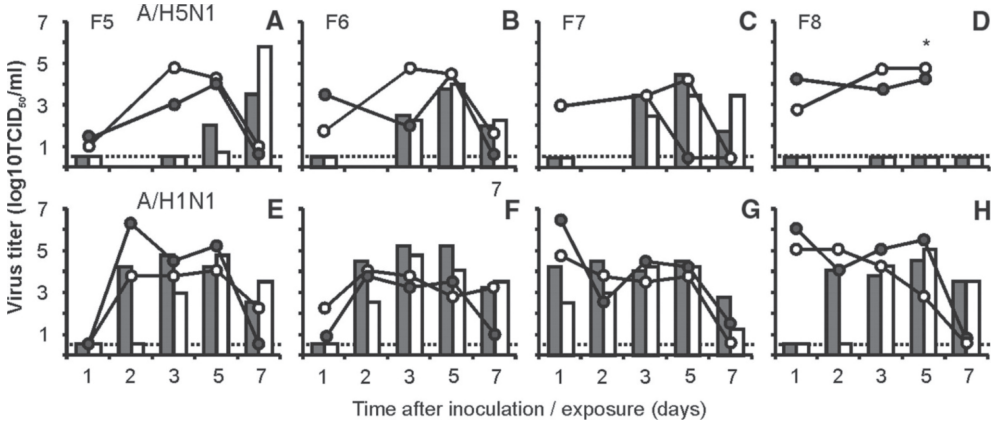

Figure 6:
Comparison of airborne transmission of experimental passaged H5N1 and 2009 pandemic H1N1 viruses in individual ferrets. A throat-swab sample from ferret F2 at 7 days postexposure (dpe) (Figure 5D) was used for the transmission experiments shown in (A) and (B), and a virus isolate obtained from a nose swab collected from ferret F5 at 7 dpi (Figure 6A) was used for the experiments in (C) and (D). For comparison, published data on transmission of 2009 pandemic H1N1 virus between ferrets is shown in (E) to (H) (95). Data for individual transmission experiments is shown in each panel, with virus shedding in inoculated and airborne virus-exposed animals shown as lines and bars, respectively. For the transmission experiments with airborne-transmissible H5N1 (A to D), nose or throat swabs were not collected at 2 dpi and 2 dpe. White circles and bars represent shedding from the nose; black circles and bars represent shedding from the throat. The asterisk indicates the inoculated animal that died 6 days after intranasal inoculation.

of influenza virus transmission between animals and on the determinants of airborne transmission are high on the influenza research agenda.

The viruses that caused the major pandemics of the past century emerged upon reassortment (that is, genetic mixing) of animal and human influenza viruses (1). However, given that viruses from only four pandemics are available for analyses, we cannot exclude the possibility that a future pandemic may be triggered by a wholly avian virus without the requirement of reassortment. Several studies have shown that reassortment events between H5N1 and seasonal human influenza viruses do not yield viruses that are readily transmitted between ferrets (89-92). In our work, we investigated whether H5N1 virus could change its transmissibility characteristics without any requirement for reassortment.

We chose influenza virus A/Indonesia/5/2005 for our study because the incidence of human H5N1 virus infections and fatalities in Indonesia remains fairly high (83), and there are concerns that this virus could acquire molecular characteristics that would allow it to become more readily transmissible between humans and initiate a pandemic. Because no reassortants between H5N1 viruses and seasonal or pandemic human influenza viruses have been detected in nature and because our goal was to understand the biological properties needed for an influenza virus to become airborne transmissible in mammals, we decided to use the complete A/Indonesia/5/2005 virus that was isolated from a human case of HPAI H5N1 infection.

We chose the ferret (Mustela putorius furo) as the animal model for our studies. Ferrets have been used in influenza research since 1933 because they are susceptible to infection with human and avian influenza viruses (93). After infection with human influenza A virus, ferrets develop respiratory disease and lung pathology similar to that observed in humans. Ferrets can also transmit human influenza viruses to other ferrets that serve as sentinels with or without direct contact (Figure S1) (94, 95).

Host restriction of replication and transmission of influenza A viruses is partly determined by specific SA receptors on the surface of susceptible cells. The affinity of influenza viruses for these receptors varies according to the species from which they are isolated. Influenza viruses of avian origin preferentially bind to α-2,3–linked SA receptors, whereas human influenza viruses recognize α-2,6–linked SA receptors. The receptor distribution in ferrets resembles that of humans in that the α-2,6–linked SA receptors are predominantly present in the upper respiratory tract (URT), and the α-2,3– linked SA receptors are mainly present in the lower respiratory tract. In chickens and other birds, α-2,3–linked SAs predominate, but both α-2,3–linked and α-2,6–linked SA are present throughout the respiratory and enteric tracts (Figure S2) (96). The differences in receptor distribution between humans and avian species are thought to determine the host restriction of influenza A viruses. A switch in receptor specificity from avian α-2,3-SA to human α-2,6-SA receptors,

which can be acquired by specific mutations in the receptor binding site (RBS) of the HA, is expected to be necessary for an avian virus to become transmissible and, thus, gain the potential to become pandemic in humans.
Besides a switch in receptor specificity to facilitate infection of cells in the URT, increased virus production in the URT and efficient release of virus particles from the respiratory tract to yield airborne virus may also be required (1). Such traits are likely to be determined by the viral surface glycoproteins and the proteins that form the viral polymerase complex. Amino acid substitutions in the polymerase proteins have already been shown to be major determinants of host range and transmission, including for pandemic influenza viruses (97-99). Whereas avian viruses, in principle, replicate at temperatures around 41°C (the temperature in the intestinal tract of birds), for replication in humans the viruses need to adapt to 33°C (the temperature of the human URT). The amino acid substitution Glu627→Lys627 (E627K) in the polymerase complex protein PB2 has been associated with increased virus replication in mammalian cells at such lower temperatures (13, 88, 100)
In addition, when newly formed virus particles bud from the host cell membrane after virus replication, the NA present on the virus membrane facilitates the release of particles. For H5N1, this process is rather inefficient, and released particles tend to form virus aggregates (1). Therefore, a balance between the properties endowed by HA and NA may be required to generate single particles. These established effects were thus used as the basis for the initial substitutions chosen in the current study.
Human-to-human transmission of influenza viruses can occur through direct contact, indirect contact via fomites (contaminated environmental surfaces), and/or airborne transmission via small aerosols or large respiratory droplets. The pandemic and epidemic influenza viruses that have circulated in humans throughout the past century were all transmitted via the airborne route, in contrast to many other respiratory viruses that are exclusively transmitted via contact. There is no exact particle size cut-off at which transmission changes from exclusively large droplets to aerosols. However, it is generally accepted that for infectious particles with a diameter of 5 mm or less, transmission occurs via aerosols. Because we did not measure particle size during our experiments, we will use the term “airborne transmission” throughout this Report.
Biosafety and biosecurity concerns have remained foremost in our planning for this research program. The details are explained in the supplementary materials and are summarized here: The enhanced Animal Biosafety Laboratory level 3 (ABSL3+) facility at Erasmus Medical Center (MC) Rotterdam, the Netherlands, was constructed for the specific purpose of containing pathogenic and transmissible influenza viruses and other pathogens of concern. The facility consists of a negatively pressurized laboratory with an interlock room. All in vivo and in vitro experimental work

is carried out in negatively pressurized class 3 isolators or class 3 biosafety cabinets, respectively. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at Erasmus MC, as well as Dutch and U.S. government inspectors.
Before and during the research, biosafety officers of Erasmus MC and inspectors from the Dutch government, as well as from the U.S. Centers for Disease Control and Prevention, approved the facilities and procedures. Explicit permits for research on genetically modified airborne-transmissible H5N1 virus were obtained from the Dutch government. The research was performed strictly in accordance with the Dutch Code of Conduct for Biosecurity (101). All personnel were instructed and trained extensively for working in the ABSL3+ facility, handling (highly pathogenic) influenza virus, and controlling incidents (such as spills). To further prevent occupational risks, research personnel used protective equipment and were offered seasonal and H5N1 influenza vaccines. For emergency purposes, Erasmus MC holds supplies of oseltamivir and has quarantine hospital rooms.
Using a combination of targeted mutagenesis followed by serial virus passage in ferrets, we investigated whether H5N1 virus can acquire mutations that would increase the risk of mammalian transmission (102). We have previously shown that several amino acid substitutions in the RBS of the HA surface glycoprotein of A/Indonesia/5/2005 change the binding preference from the avian α-2,3–linked SA receptors to the human α-2,6–linked SA receptors (103). A/Indonesia/5/2005 virus with amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S (numbers refer to amino acid positions in the mature H5 HA protein; N, Asn; Q, Gln; L, Leu; G, Gly; S, Ser) in HA display attachment patterns similar to those of human viruses to cells of the respiratory tract of ferrets and humans (103). Of these changes, we know that together, Q222L and G224S switch the receptor binding specificity of H2 and H3 subtype influenza viruses, as this switch contributed to the emergence of the 1957 and 1968 pandemics (20). N182K has been found in a human case of H5N1 virus infection (104). Our experimental rationale to obtain transmissible H5N1 viruses was to select a mutant H5N1 virus with receptor specificity for α-2,6–linked SA shed at high titers from the URT of ferrets. Therefore, we used the QuickChange multisite-directed mutagenesis kit (Agilent Technologies, Amstelveen, the Netherlands) to introduce amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S in the HA of wildtype (WT) A/Indonesia/5/2005, resulting in $H5N1_{HA\ N182K}$, $H5N1_{HA\ Q222L,G224S}$, and $H5N1_{HA\ N182K,Q222L,G224S}$. Experimental details for experiments 1 to 9 are provided in the supplementary materials. For experiment 1, we inoculated these mutant viruses and the $H5N1_{wildtype}$ virus intranasally into groups of six ferrets for each virus (Figure S3). Throat and nasal swabs were collected daily, and virus titers were determined by end-point dilution in Madin Darby canine kidney (MDCK) cells to quantify virus shedding from the ferret URT. Three

animals were euthanized after day 3 to enable tissue sample collection. All remaining animals were euthanized by day 7 when the same tissue samples were taken. Virus titers were determined in the nasal turbinates, trachea, and lungs collected postmortem from the euthanized ferrets. Throughout the duration of experiment 1, ferrets inoculated intranasally with $H5N1_{wildtype}$ virus produced high titers in nose and throat swabs - up to 10 times more than $H5N1_{HA\ Q222L,G224S}$, which yielded the highest virus titers of all three mutants during the 7-day period (Figure 1). However, no significant difference was observed between the virus shedding of ferrets inoculated with $H5N1_{HA\ Q222L,G224S}$ or $H5N1_{HA\ N182K}$ during the first 3 days when six animals per group were present. Thus, of the viruses with specificity for α-2,6-linked SA, $H5N1_{HA\ Q222L,G224S}$ yielded the highest virus titers in the ferret URT (Figure 1).

As described above, amino acid substitution E627K in PB2 is one of the most consistent hostrange determinants of influenza viruses (97-99). For experiment 2 (Figure S4), we introduced E627K into the PB2 gene of A/Indonesia/5/2005 by site-directed mutagenesis and produced the recombinant virus $H5N1_{HAQ222L,G224S\ PB2E627K}$. The introduction of E627K in PB2 did not significantly affect virus shedding in ferrets, because virus titers in the URT were similar to those seen in $H5N1_{HAQ222L,G224S}$-inoculated animals [up to 1×10^4 50% tissue culture infectious doses (TCID50)] (Mann-Whitney U rank-sum test, $P = 0.476$) (Figure 1 and Figure S5). When four naïve ferrets were housed in cages adjacent to those with four inoculated animals to test for airborne transmission as described previously (95), $H5N1_{HAQ222L,G224S\ PB2E627K}$ was not transmitted (Figure S5).

Because the mutant virus harboring the E627K mutation in PB2 and Q222L and G224S in HA did not transmit in experiment 2, we designed an experiment to force the virus to adapt to replication in the mammalian respiratory tract and to select virus variants by repeated passage (10 passages in total) of the constructed $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ virus and $H5N1_{wildtype}$ virus in the ferret URT (Figure 2 and Figure S6). In experiment 3, one ferret was inoculated intranasally with $H5N1_{wildtype}$ and one ferret with $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$. Throat and nose swabs were collected daily from live animals until 4 days postinoculation (dpi), at which time the animals were euthanized to collect samples from nasal turbinates and lungs. The nasal turbinates were homogenized in 3 ml of virus-transport medium, tissue debris was pelleted by centrifugation, and 0.5 ml of the supernatant was subsequently used to inoculate the next ferret intranasally (passage 2). This procedure was repeated until passage 6.

From passage 6 onward, in addition to the samples described above, a nasal wash was also collected at 3 dpi. To this end, 1 ml of phosphate-buffered saline (PBS) was delivered dropwise to the nostrils of the ferrets to induce sneezing. Approximately 200 ml of the “sneeze” was collected in a Petri dish, and PBS was added to a final volume of 2 ml. The nasal-wash samples were

Table 1:
Lethality of WT and airborne-transmissible H5N1 virus in ferrets upon inoculation via different routes. n, number of animals; N.A., not applicable.

Inoculation route	Virus	Dead or moribund (no. dead/no. tested)	Day of death postinoculation (no.)
Intratracheal	A/H5N1$_{wildtype}$	6/6*	2 (n = 2), 3 (n = 4)
	A/H5N1/F5	6/6	3 (n = 6)
Intranasal	A/H5N1$_{wildtype/P10}$	2/2†	6 (n = 2)
	A/H5N1HA$_{Q222L,G224S\ PB2\ E627K/P10}$	0/4	N.A.
	A/H5N1/F2	0/2	N.A.
	A/H5N1/F5	1/2	6 (n = 1)
Airborne	A/H5N1$_{wildtype}$	N.A.	N.A.
	A/H5N1HA$_{Q222L,G224S\ PB2\ E627K/P10}$	0/3	N.A.
	A/H5N1/F2	0/2	N.A.
	A/H5N1/F5	0/1	N.A.

*These data refer to a published study (112).

†These ferrets were inoculated with P10 H5N1$_{wildtype}$ virus, but data are consistent with previous studies that used larger groups of animals inoculated with the original strain (106, 107).

Table 2:
Receptor specificity of the different mutant H5N1 viruses, as determined by a modified TRBC hemagglutination assay. Introduction of Q222L and G224S in the H5N1 HA resulted in a receptor binding preference switch from the avian α-2,3- to the human α-2,6-linked SA receptor. Subsequent substitution of H103Y and T156A resulted in an increased affinity for α-2,3- and α-2,6-linked SA, in agreement with glycan array studies (118). For details, see supplementary experiment 9. HAU, hemagglutination units.

Virus	Subtype	HA titer (HAU/50 µl)		
		TRBC	α-2,3–linked TRBC	α-2,6–linked TRBC
A/Netherlands/213/03	H3N2	64	0	64
A/Vietnam/1194/04	H5N1	64	64	0
A/H5N1$_{PB2\ E627K}$	H5N1	64	16	0
A/H5N1$_{HA\ H103Y,T156A\ PB2\ E627K}$	H5N1	64	48	0
A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$	H5N1	64	0	24
A/H5N1$_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$	H5N1	64	4	32

used for intranasal inoculation of the ferrets for the subsequent passages 7 through 10. We changed the source of inoculum during the course of the experiment, because passaging nasal washes may facilitate the selection of viruses that were secreted from the URT. Because influenza viruses mutate rapidly, we anticipated that 10 passages would be sufficient for the virus to adapt to efficient replication in mammals.

Virus titers in the nasal turbinates of ferrets inoculated with $H5N1_{wildtype}$ ranged from ~ 1×10^5 to 1×10^7 TCID50/gram tissue throughout 10 serial passages (Figure 3A and Figure S7). In ferrets inoculated with $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ virus, a moderate increase in virus titers in the nasal turbinates was observed as the passage number increased. These titers ranged from 1×10^4 TCID50/gram tissue at the start of the experiment to 3.2×10^5 to 1×10^6 TCID50/gram tissue in the final passages (Figure 3A and Figure S7). Notably, virus titers in the nose swabs of animals inoculated with $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ also increased during the successive passages, with peak virus shedding of 1×10^5 TCID50 at 2 dpi after 10 passages (Figure 3B). These data indicate that $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ was developing greater capacity to replicate in the ferret URT after repeated passage, with evidence for such adaptation becoming apparent by passage number 4. In contrast, virus titers in the nose swabs of the ferrets collected at 1 to 4 dpi throughout 10 serial passages with $H5N1_{wildtype}$ revealed no changes in patterns of virus shedding.

Passaging of influenza viruses in ferrets should result in the natural selection of heterogeneous mixtures of viruses in each animal with a variety of mutations: so-called viral quasispecies (105). The genetic composition of the viral quasi-species present in the nasal washes of ferrets after 10 passages of $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform (Roche, Woerden, the Netherlands) (Tables S1 and S2). The mutations introduced in $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ by reverse genetics remained present in the virus population after 10 consecutive passages at a frequency >99.5% (Figure 4 and Table S1). Numerous additional nucleotide substitutions were detected in all viral gene segments of $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ after passaging, except in segment 7 (Tables S1 and S2). Of the 30 nucleotide substitutions selected during serial passage, 53% resulted in amino acid substitutions. The only amino acid substitution detected upon repeated passage of both $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ was T156A (T, Thr; A, Ala) in HA. This substitution removes a potential N-linked glycosylation site (Asn-X-Thr/Ser; X, any amino acid) in HA and was detected in 99.6% of the $H5N1_{wildtype}$ sequences after 10 passages. T156A was detected in 89% of the $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ sequences after 10 passages, and the other 11% of sequences possessed the substitution N154K, which removes the same potential N-linked glycosylation site in HA.

In experiment 4 (see supplementary materials), we investigated whether

airborne-transmissible viruses were present in the heterogeneous virus population generated during virus passaging in ferrets (Figure S4). Nasal-wash samples, collected at 3 dpi from ferrets at passage 10, were used in transmission experiments to test whether airborne-transmissible virus was present in the virus quasispecies. For this purpose, nasal-wash samples were diluted 1:2 in PBS and subsequently used to inoculate six naïve ferrets intranasally: two for passage 10 $H5N1_{wildtype}$ and four for passage 10 $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ virus.

The following day, a naïve recipient ferret was placed in a cage adjacent to each inoculated donor ferret. These cages are designed to prevent direct contact between animals but allow airflow from a donor ferret to a neighboring recipient ferret (Figure S1) (95). Although mutations had accumulated in the viral genome after passaging of $H5N1_{wildtype}$ in ferrets, we did not detect replicating virus upon inoculation of MDCK cells with swabs collected from naïve recipient ferrets after they were paired with donor ferrets inoculated with passage 10 $H5N1_{wildtype}$ virus (Figure 5, A and B). In contrast, we did detect virus in recipient ferrets paired with those inoculated with passage 10 $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ virus. Three (F1 to F3) out of four (F1 to F4) naïve recipient ferrets became infected as confirmed by the presence of replicating virus in the collected nasal and throat swabs (Figure 5, C and D). A throat-swab sample obtained from recipient ferret F2, which contained the highest virus titer among the ferrets in the first transmission experiment, was subsequently used for intranasal inoculation of two additional donor ferrets. Both of these animals, when placed in the transmission cage setup (Figure S1), again transmitted the virus to the recipient ferrets (F5 and F6) (Figure 6, A and B). A virus isolate was obtained after inoculation of MDCK cells with a nose swab collected from ferret F5 at 7 dpi. The virus from F5 was inoculated intranasally into two more donor ferrets. One day later, these animals were paired with two recipient ferrets (F7 and F8) in transmission cages, one of which (F7) subsequently became infected (Figure 6, C and D).

We used conventional Sanger sequencing to determine the consensus genome sequences of viruses recovered from the six ferrets (F1 to F3 and F5 to F7) that acquired virus via airborne transmission (Figure 4 and Table S3). All six samples still harbored substitutions Q222L, G224S, and E627K that had been introduced by reverse genetics. Surprisingly, only two additional amino acid substitutions, both in HA, were consistently detected in all six airborne-transmissible viruses: (i) H103Y (H, His; Y, Tyr), which forms part of the HA trimer interface, and (ii) T156A, which is proximal but not immediately adjacent to the RBS (Figure S8). Although we observed several other mutations, their occurrence was not consistent among the airborne viruses, indicating that of the heterogeneous virus populations generated by passaging in ferrets, viruses with different genotypes were transmissible. In addition, a single transmission experiment is not sufficient to select for

clonal airborne-transmissible viruses because, for example, the consensus sequence of virus isolated from F6 differed from the sequence of parental virus isolated from F2.

Together, these results suggest that as few as five amino acid substitutions (four in HA and one in PB2) may be sufficient to confer airborne transmission of HPAI H5N1 virus between mammals. The airborne-transmissible virus isolate with the least number of amino acid substitutions, compared with the $H5N1_{wildtype}$, was recovered from ferret F5. This virus isolate had a total of nine amino acid substitutions; in addition to the three mutations that we introduced (Q222L and G224S in HA and E627K in PB2), this virus harbored H103Y and T156A in HA, H99Y and I368V (I, Ile; V, Val) in PB1, and R99K (R, Arg) and S345N in NP (Table S3). Reverse genetics will be needed to identify which of the five to nine amino acid substitutions in this virus are essential to confer airborne transmission.

During the course of the transmission experiments with the airborne-transmissible viruses, ferrets displayed lethargy, loss of appetite, and ruffled fur after intranasal inoculation. One of eight inoculated animals died upon intranasal inoculation (Table 1). In previously published experiments, ferrets inoculated intranasally with WT A/Indonesia/5/2005 virus at a dose of 1×10^6 TCID50 showed neurological disease and/or death (106, 107). It should be noted that inoculation of immunologically naïve ferrets with a dose of 1×10^6 TCID50 of H5N1 virus and the subsequent course of disease is not representative of the natural situation in humans. Importantly, although the six ferrets that became infected via respiratory droplets or aerosol also displayed lethargy, loss of appetite, and ruffled fur, none of these animals died within the course of the experiment. Moreover, previous infections of humans with seasonal influenza viruses are likely to induce heterosubtypic immunity that would offer some protection against the development of severe disease (108, 109). It has been shown that mice and ferrets previously infected with an H3N2 virus are clinically protected against intranasal challenge infection with an H5N1 virus (110, 111).

After intratracheal inoculation (experiment 5; Figure S9), six ferrets inoculated with 1×10^6 TCID50 of airborne-transmissible virus F5 in a 3 ml volume of PBS died or were moribund at day 3. Intratracheal inoculations at such high doses do not represent the natural route of infection and are generally used only to test the ability of viruses to cause pneumonia (112), as is done for vaccination-challenge studies. At necropsy, the six ferrets revealed macroscopic lesions affecting 80 to 100% of the lung parenchyma with average virus titers of 7.9×10^6 TCID50/gram lung (Figure S10). These data are similar to those described previously for $H5N1_{wildtype}$ in ferrets (Table 1). Thus, although the airborne-transmissible virus is lethal to ferrets upon intratracheal inoculation at high doses, the virus was not lethal after airborne transmission.

To test the effect of the mutations in HA in the airborne-transmissible virus on

its sensitivity to antiviral drugs, we used virus isolated from F5 (experiment 6). This airborne-transmissible virus with nine amino acid substitutions displayed sensitivity to the antiviral drug oseltamivir similar to that of $H5N1_{wildtype}$ (Table S4).

In experiment 7, we evaluated the recognition of the airborne-transmissible virus by antisera raised against potential H5N1 vaccine strains. Because only HA recognition by antibodies is evaluated in this assay, chimeric viruses were generated based on six gene segments of the mouse-adapted A/Puerto Rico/8/34 (PR8) virus with the HA and PB2 genes of the transmissible virus harboring amino acid substitutions H103Y, T156A, Q222L, and G224S in HA and E627K in PB2. We replaced the MBCS of the HA by a monobasic cleavage site, allowing us to do these experiments under BSL2 conditions. The chimeric PR8/H5 virus reacted well with ferret antisera raised against A/Indonesia/5/2005 and several other prepandemic vaccine strains (Table S5). In fact, the presence of the four HA mutations increased the reactivity with H5 antisera by twofold or more.

We subsequently used the same PR8/H5 chimeric virus in experiment 8 to evaluate the presence of existing immunity against the airborne-transmissible virus in sera obtained from human volunteers more than 70 years of age. The introduction of receptor-binding site mutations Q222L/G224S and the mutations H103Y and T156A in HA, acquired during ferret passage, did not result in increased cross-reactivity with human antisera (Table S6), indicating that humans do not have antibodies against the HA of the airborne-transmissible H5N1 virus that was selected in our experiments.

Substitutions Q222L and G224S have previously been shown to be sufficient to switch receptor-binding specificity of avian influenza strains (i.e., α-2,3-linked SA) to that of human strains (i.e., α-2,6-linked SA) (91, 103, 113, 114). Amino acid position 103 is distal from the RBS, forms part of the trimer interface, and is unlikely to affect receptor specificity (Figure S8). T156 is part of a N-glycosylation sequon, and T156A (as well as N154K) would delete this potential glycosylation site (Figure S8); amino acid T156 is proximal but not immediately adjacent to the RBS. Loss of N-glycosylation sites at the tip of HA has been shown to affect receptor binding of H1 (115, 116) and the virulence of H5 virus (117). We evaluated the impact of the HA mutations that emerged during passaging in ferrets in a modified turkey red blood cell (TRBC) assay (Table 2). In this assay, the binding of influenza viruses, with a mutated HA, to normal TRBCs (expressing both α-2,3-linked SA and α-2,6-linked SA) and modified TRBCs with either α-2,3-linked SA or α-2,6-linked SA on the cell surface was evaluated and compared to two reference viruses with known receptor binding preference: avian H5N1 and human H3N2 viruses. As expected and shown before, introduction of the Q222L and G224S mutations in the HA of H5N1 changed the receptor binding preference from α-2,3-linked SA to α-2,6-linked SA (103). Furthermore, in our hands, the

introduction of substitutions H103Y and T156A not only enhanced binding of $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ to α-2,6-linked SA, as expected from glycan array studies (118), but also increased the affinity for α-2,3-linked SA. When these two mutations were introduced in the $H5N1_{wildtype}$ HA, the affinity for α-2,3-linked SA also increased.

Substitutions Q222L and G224S have previously emerged in avian H2 and H3 viruses in nature (20, 119), and mutations associated with similar changes in receptor binding specificity have been detected repeatedly in H5 viruses for instance, substitution N182K has been reported nine times (104, 118), which is why we initially selected it for our investigations. The other three substitutions we found consistently in airborne-transmissible viruses have all previously been detected in HPAI H5N1 viruses circulating in the field (120). Only a minor fraction of the H5N1 viruses that have circulated in outbreaks have been sequenced (estimated to be <0.001%) (120, 121). Yet the individual substitutions we obtained, as well as combinations of T156A and H103Y or T156A and E627K, have already been reported in public sequence databases (120); thus, we conclude that these mutations do not appear to have a detrimental effect on virus fitness. Substitution H103Y has only been found once, in combination with T156A in a duck in China (120). Substitution E627K in PB2 has been found in ~27% of avian H5N1 virus sequences and in ~29% of human H5N1 viruses (120). Substitution T156A in HA has been reported in >50% of the viruses sequenced and was detected in 100% of the viruses from human cases in Egypt (120).

Investigations of viral quasi-species during a massive avian influenza H7N7 virus outbreak in the Netherlands indicated that viruses with human adaptation markers, including HA mutations that alter receptor specificity and mutations in polymerase proteins that increase polymerase activity like E627K in PB2, emerged rapidly in poultry (122-124). Given the large numbers of HPAI H5N1 virus-infected hosts globally, the high viral mutation rate, and the apparent lack of detrimental effects on fitness of the mutations that confer airborne transmission, it may simply be a matter of chance and time before a human-to-human transmissible H5N1 virus emerges.

The specific mutations we identified in these experiments that are associated with airborne transmission represent biological traits that may be determined by a set of different amino acid substitutions. For example, amino acid substitutions D701N (D, Asp) or S590G/R591Q in PB2 yield a similar phenotype to E627K (97). N182K and other substitutions in the RBS of HA may yield a similar phenotype to Q222L/G224S (103). Such mutations should be considered for H5N1 surveillance studies in outbreak areas. Imai et al. recently identified different RBS changes (N220K, Q222L) along with N154D (affecting the same N-glycosylation sequon as T156A) and T314I in HA as determinants of airborne transmission of an H5 virus (125). This airborne virus contained seven genes of the 2009 pandemic H1N1 virus

(which has S590G/R591Q in PB2 rather than E627K), with the HA of H5N1 virus A/Vietnam/1203/2004 (125). These data indicate that different lineages of H5N1 virus and different amino acid substitutions that affect particular biological traits (receptor binding, glycosylation, replication) can yield airborne-transmissible H5N1 viruses.

Although our experiments showed that H5N1 virus can acquire a capacity for airborne transmission, the efficiency of this mode remains unclear. Previous data have indicated that the 2009 pandemic H1N1 virus transmits efficiently among ferrets and that naïve animals shed high amounts of virus as early as 1 or 2 days after exposure (95). When we compare the H5N1 transmission data with that of reference (95), keeping in mind that our experimental design for studying transmission is not quantitative, the data shown in Figs. 5 and 6 suggest that H5N1 airborne transmission was less robust, with less and delayed virus shedding compared with pandemic H1N1 virus.

Airborne transmission could be tested in a second mammalian model system such as guinea pigs (126), but this would still not provide conclusive evidence that transmission among humans would occur. The mutations we identified need to be tested for their effect on transmission in other H5N1 virus lineages (127) , and experiments are needed to quantify how they affect viral fitness and virulence in birds and mammals. For pandemic preparedness`, antiviral drugs and vaccine candidates against airborne-transmissible virus should be evaluated in depth. Mechanistic studies on the phenotypic traits associated with each of the identified amino acid substitutions should provide insights into the key determinants of airborne virus transmission. Our findings indicate that HPAI H5N1 viruses have the potential to evolve directly to transmit by aerosol or respiratory droplets between mammals`, without reassortment in any intermediate host`, and thus pose a risk of becoming pandemic in humans. Identification of the minimal requirements for virus transmission between mammals may have prognostic and diagnostic value for improving pandemic preparedness (102).

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SUPPLEMENTAL MATERIAL

Risk assessments prior to start of research

The research on transmission of H5N1 virus has been discussed among staff members of the Department of Virology at Erasmus MC since 1998. The work did not commence until much later because we considered the laboratory facilities available at the Rotterdam site at that time inappropriate for this type of work. Between 1998 and 2007, the H5N1 transmission experiments were discussed explicitly and extensively among the staff members of the Department of Virology, followed by discussions with biosafety officers and facility managers of Erasmus MC, as well as with numerous specialists from the influenza and general infectious disease fields around the globe (128). Throughout these discussions, and upon site- visits of facilities where research with class 3 and class 4 pathogens is conducted routinely, a plan was drawn to develop adequate research facilities, to obtain permits for working with genetically modified organisms (GMO), and for review of research proposals.

Following a Broad Agency Announcement of the National Institute of Allergy and Infectious Diseases and National Institutes of Health (BAA NIH-NIAID-DMID-07-20) in 2005, the Department of Virology, along with partners in the USA, drafted a research proposal to become an NIAID/NIH Center of Excellence for Influenza Research and Surveillance to support the research agenda of the US Department of Health and Human Services (HHS) Pandemic Influenza Plan in the USA. This proposal was reviewed favorably by NIAID/NIH in consultation with HHS and supported by external expert advisors. Upon signing the research contract, a new GMO permit - explicitly for conducting work with airborne-transmissible H5N1 virus and early pandemic viruses - was obtained from the Dutch Ministry for Infrastructure and the Environment (I&M) in 2007. To this end, I&M was advised by the Committee Genetic Modification (COGEM), which is an independent scientific advisory committee for the Dutch government. I&M and COGEM concluded that the proposed work could be performed with negligible risk to humans and the environment under the conditions realized (permit IG-07-038). The COGEM advice is available on-line (129).The COGEM advice is available on-line (129).

In 2007, a “Code of Conduct for Biosecurity” was drafted by the Royal Netherlands Academy of Arts and Sciences (KNAW) upon request of the Dutch Ministry of Education, Culture and Science (OCW) as required by the Biological and Toxin Weapons Convention (BTWC) ratified in 1972 and the Statement on Biosecurity issued by the InterAcademy Panel (IAP) in 2005 (101). As R.A.M. Fouchier was a member of the Biosecurity Focus Group that advised during the conception of this Code of Conduct for Biosecurity,

the department of Virology of Erasmus MC was well aware of the Code prior to its publication in 2007, and adhered strictly to this Code at all times. The principles and procedures outlined in the Dutch Code of Conduct are similar to those agreed upon in the USA.

Biosafety and biosecurity measures

All experiments were conducted within the enhanced animal biosafety level 3 (ABSL3+) facility of Erasmus MC that was completed in 2007. The ABSL3+ facility consists of a negative pressurized (-30 Pa) laboratory in which all in vivo and in vitro experimental work is carried out in class 3 isolators or class 3 biosafety cabinets, which are also negative pressurized (< -200 Pa). Air released from the class 3 units is filtered twice by High Efficiency Particulate Air (HEPA) filters and then leaves via the facility ventilation system, again via double HEPA filters. Only authorized personnel that have received the appropriate training can access the ABSL3+ facility. For animal handling in the facilities, personnel always work in pairs. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at ErasmusMC and Dutch and United States government inspectors.

All facilities, procedures, training records, safety drills, inventory records, and logbooks, are subject to inspection and oversight by the institutional biosafety officers of Erasmus MC in close consultation with the facility management. The facilities, personnel, and procedures are further inspected by the US Centers for Disease Control and Prevention (CDC) every 3 years in agreement with the US select agent regulations for oversees laboratories and by the Dutch government (VROM inspection). The most recent CDC inspections took place in February 2011 and March 2012 at which time no shortcomings in biosafety and biosecurity measures were identified.

Occupational health risk

Although the laboratory is considered ‘clean’ because all experiments are conducted in closed class 3 cabinets and isolators, special personal protective equipment, including laboratory suits, gloves and FFP3 facemasks, is used and all personnel are offered seasonal and prototype H5N1 influenza vaccines with informed consent. Additional immunizations with H5N1 vaccine were administered if seroconversion could not be demonstrated. Consent records are held by the Department of Virology at ErasmusMC.

All personnel are given basic training in laboratory safety under BSL2 conditions. Employees are trained for a further 3 months under standard BSL3 conditions, supervised by highly experienced personnel. Following initial BSL3 training and a period of independent work under BSL3 conditions, employees are trained for a further 3 months in the ABSL3+ facility, again

under the constant supervision of highly experienced personnel with >8 years of research experience. These training programs consist of hands-on work under supervision, following theory components on facilities, procedures and safety drills. Upon completion of the supervised training period, the supervisors judge whether trainees fulfill all requirements for working independently in the facilities. Annual refreshment training sessions on biosafety and biosecurity are provided by the principal investigators, biosafety officers, and facility managers.

All equipment in the facilities is monitored electronically and both acoustic and telephone alarms are employed to ensure that workers do not enter the facilities if equipment is malfunctioning. All personnel have been instructed and trained how to act in case of incidents. All incidents are handled with consultation between a senior staff member of the Virology Department, a clinical microbiologist, the biosafety officers, and the facility management. Antiviral drugs (oseltamivir and zanamivir) are directly available. Erasmus MC has isolation hospital rooms (negative pressure rooms with interlocks) with trained nursing and medical staff to be used in case of serious incidents and to quarantine the infected individual to prevent further dissemination of the pathogen.

MATERIALS AND METHODS

Viruses

Influenza virus A/Indonesia/5/2005 (H5N1) was isolated from a human case of HPAI virus infection and passaged once in embryonated chicken eggs followed by a single passage in Madin-Darby Canine Kidney (MDCK) cells. All eight gene segments were amplified by reverse transcription polymerase chain reaction and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 (100, 130). Mutations of interest (N182K, Q222L, G224S in HA and E627K in PB2) were introduced in reverse genetics vectors using the QuikChange multi-site-directed mutagenesis kit (Agilent, Amstelveen, The Netherlands) according to the instructions of the manufacturer. Recombinant viruses were produced upon transfection of 293T cells and virus stocks were propagated and titrated in MDCK cells as described (100).

Cells

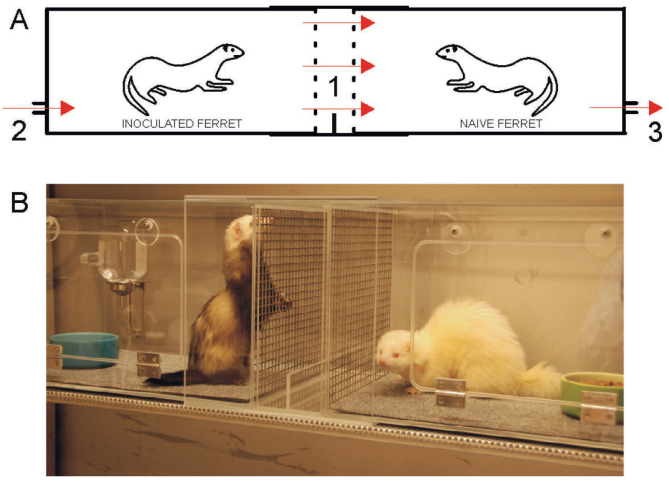
MDCK cells were cultured in Eagle's minimal essential medium (EMEM, Lonza Benelux BV, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM Hepes (Lonza), and non-essential amino acids (MP Biomedicals Europe, Illkirch, France). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM, Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and non-essential amino acids.

Virus titration in MDCK cells

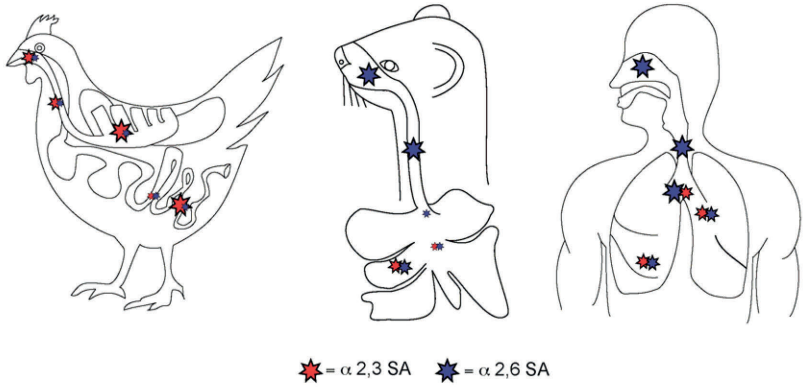
Virus titrations were performed as described previously (95). Briefly, MDCK cells were inoculated with tenfold serial dilutions of virus preparations, homogenized tissues, nose swabs, and throat swabs. Cells were washed with PBS one hour after inoculation and cultured in 200 µl of infection media, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM Hepes, non-essential amino acids, and 20 µg/ml trypsin (Lonza). Three days after inoculation, supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of virus replication in the cells. Infectious virus titers were calculated from four replicates each of the homogenized tissue samples, nose swabs, and throat swabs and for ten replicates of the virus preparations by the method of Spearman-Karber (131).

Ferret experiments

An independent animal experimentation ethical review committee, approved by Dutch Government (Stichting DEC Consult) approved all animal studies. All experiments with ferrets were performed under animal biosafety level 3+ conditions in class 3 isolator cages.



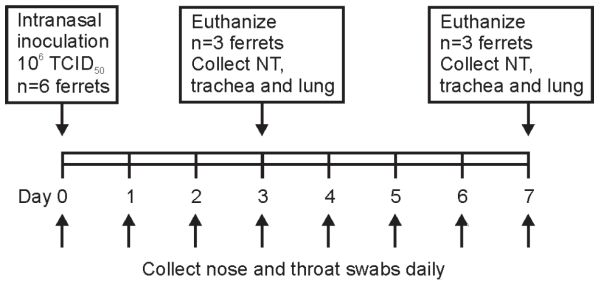
Supplemental Figure 1:
Schematic presentation (A) and a photograph (B) of paired transmission cages. The transmission cages were specifically designed to allow transmission experiments to be conducted in negatively pressurized isolator cages (1.6 x 1 x 1 m) in the ABSL3+ facility. The ferrets are housed in clear Perspex cages, in which each inoculated animal was housed individually next to a naive ferret. Each ferret cage was 30 x 30 x 55 cm (W x H x L) and the two cages were separated by two stainless steel grids (1), with a grid size of 0.5 cm2, 10 cm apart. Negative pressure within the isolator cage is used to direct a modest (< 0.1 m/sec) flow of HEPA-filtered air (2) from the inoculated to the naive ferret. The outlet airflow (3) is HEPA filtered to prevent continuous circulation of infectious influenza A virus particles and to prevent cross-contamination. Animals are housed on solid rubber floor tiles, which do not generate dust and avoid unwanted fomite transmission among animals. Arrows indicate airflow.



Supplemental Figure 2: Influenza virus receptor distribution in the respiratory and enteric tract of chickens, and the respiratory tract of ferrets and humans. Avian and human influenza viruses preferentially bind to α-2,3-linked (red) and α-2,6-linked (blue) sialic acid (SA) receptors, respectively. In chicken, although both α-2,3-linked SA and α-2,6-linked SA are present throughout the respiratory tract and gut, α-2,3-linked SA are expressed more abundantly. In contrast, humans and ferrets predominantly express α-2,6-linked SA receptors in the upper respiratory tract (URT) and trachea, and α-2,3-linked SA receptors in the lower respiratory tract (96). The size of the red and blue symbols correlates with the relative abundance of SA receptors.

Experiment 1

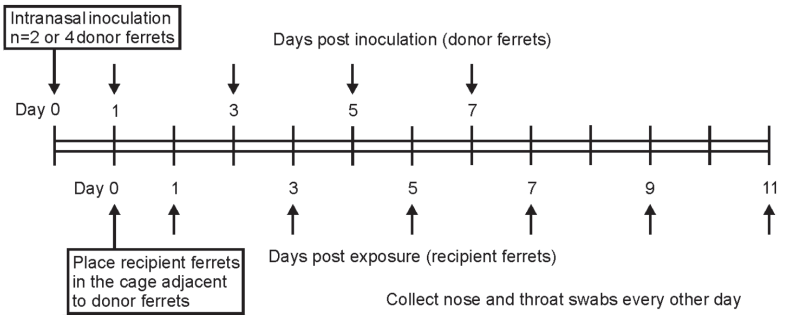
To evaluate the effect of receptor binding site (RBS) mutations on H5N1 virus replication in the ferret respiratory tract, four groups of six influenza virus seronegative female ferrets (Mustela putorius furo) were inoculated intranasally with 1 x 10^6 TCID50 of $H5N1_{wildtype}$, $H5N1_{HA\ N182K}$, $H5N1_{HA\ Q222L,G224S}$, or $H5N1_{HA\ N182K,Q222L,G224S}$ (2 × 250 µL, divided over both nostrils), respectively (Figure S3). Nose and throat swabs were collected daily and immediately suspended in 1 ml of virus transport medium (VTM) containing glycerol and antibiotics. Three animals of each group were euthanized at 3 and 7 days post inoculation (dpi), and nasal turbinates (narrow and curled bone shelves that stick out into the breathing passage of the nose), trachea and lungs were collected, homogenized in 3 ml of VTM, after which the supernatant was collected and stored at -80°C. Virus titers in swabs and respiratory tissues were determined by end-point titration in MDCK cells as described above.



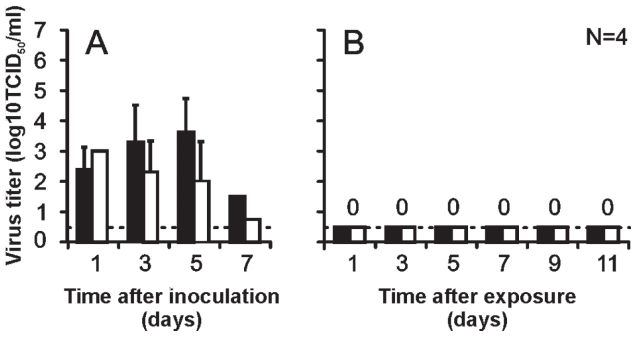
Supplemental Figure 3:
Schematic overview of experiment 1 to evaluate replication of $H5N1_{wildtype}$, $H5N1_{HA\ N182K,\ H5N1HA\ Q222L,G224S}$, or $H5N1_{HA\ N182K,Q222L,G224S}$ in ferrets. NT; nasal turbinates.

Experiment 2

Amino acid substitution E672K in PB2 is one of the most consistent host-range determinants of influenza viruses. To test its effect on airborne-transmission of an H5N1 virus with specificity for the human α-2,6-linked SA receptor, we introduced this E627K mutations in virus $H5N1_{HA\ Q222L,G224S}$ that was used in experiment 1. Airborne transmission experiments were performed as described previously (95). In short, four female adult ferrets were inoculated intranasally with 1 x 10^6 TCID50 of $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ by applying 250 µL of virus suspension to each nostril. Each donor ferret was then placed in a transmission cage (Figure S1). One day after inoculation, one naïve recipient ferret was placed opposite each inoculated ferret. Each transmission pair was housed in a separate transmission cage designed to prevent direct contact between the inoculated and naïve ferrets but allowing airflow from the donor to the recipient ferret. Nose and throat swabs were collected on 1, 3, 5, and 7 dpi for donor ferrets and on 1, 3, 5, 7, 9 and 11 days post exposure (dpe) for the recipient ferrets as described for experiment 1. Virus titers in swabs were determined by end-point titration in MDCK cells. If virus shedding was detected in the recipient ferrets upon exposure to the donor ferrets, this was judged as evidence for airborne transmission.



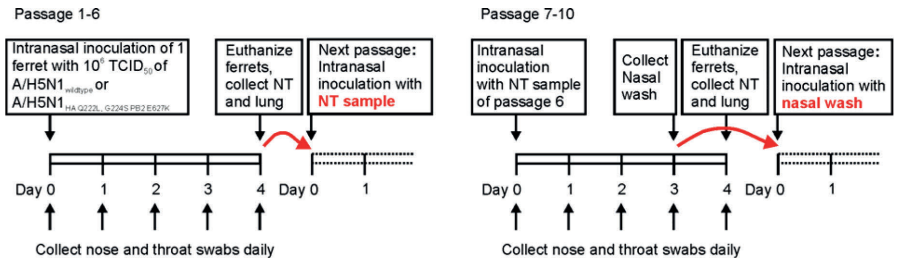
Supplemental Figure 4:
Schematic overview of experiments to test airborne transmission of influenza virus in experiments 2 and 4.



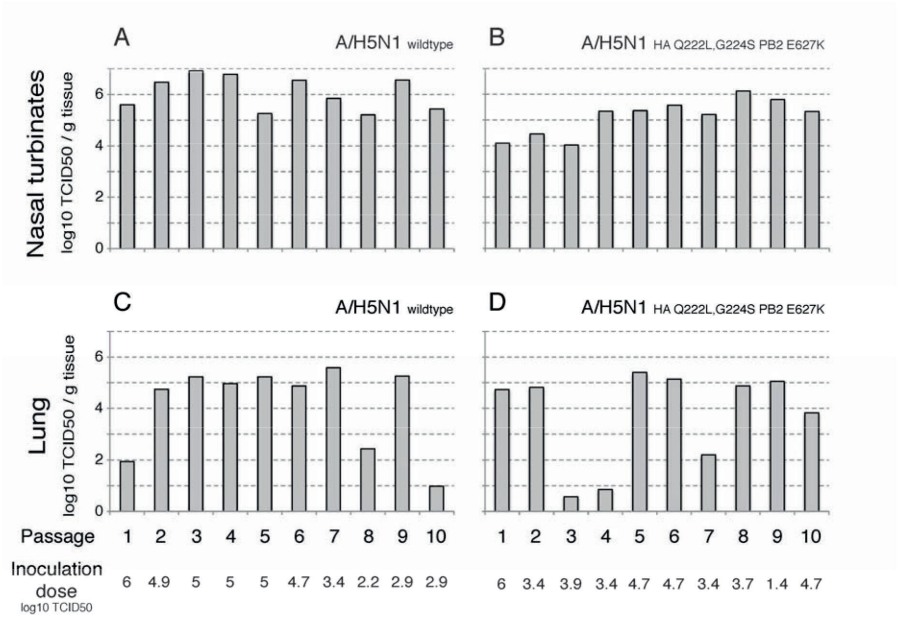
Supplemental Figure 5:
Shedding of influenza virus H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ in ferrets in transmission experiments (experiment 2). (A) Nose (white bars) and throat swabs (black bars) were collected on days 1, 3, 5, and 7 after inoculation from ferrets inoculated intranasally and (B) on days 1, 3, 5, 7, 9, and 11 after exposure for naïve ferrets in an adjoining cage. Virus titers were determined by end-point titration in MDCK cells. The geometric mean titers of positive samples are displayed, with error bars indicating standard deviations. Numbers in panel B indicate the number of ferrets infected via airborne transmission.

Experiment 3

Since no airborne transmission was observed in experiment 2, $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ were serially passaged in ferrets to allow adaptation for efficient replication in mammals. Each virus was inoculated intranasally with 1 x 10^6 TCID50 in one ferret (2 x 250 µl, divided over both nostrils). Nose and throat swabs were collected daily, as described for experiment 1. Animals were euthanized at 4 dpi and nasal turbinates and lungs were collected as described for experiment 1. Nasal turbinates were homogenized in VTM, and this homogenate was used to inoculate the next ferret intranasally, resulting in passage 2 (Figure 2, Figure S6). Subsequent passages 3 - 6 were performed in the same way. From passage six onwards, nasal washes were collected at 3 dpi in addition to the nasal swabs. To this end, one milliliter of PBS was delivered drop wise to the nostrils of the ferrets, thereby inducing sneezing. Approximately 200 µl of the 'sneeze' was collected in a Petri dish, and PBS was added to a final volume of 2 ml. For passage 7 through 10, the nasal wash sample was used for the following passages in ferrets. The passage-10 nasal washes of the ferrets inoculated with $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ were used for sequence analyses using the 454/Roche GS-FLX sequencing platform, as well as for transmission experiments (experiment 4).



Supplemental Figure 6:
Schematic overview of experiment 3 to allow adaptation for efficient replication in the upper respiratory tract by serial passaging in ferrets. NT; nasal turbinates.



Supplemental Figure 7:
Virus titers in the nasal turbinates (A, B) and lungs (C, D) collected on day 4 from ferrets inoculated with $H5N1_{wildtype}$ (A, C) and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ (B, D) throughout the ten serial passages as numbered on the x axes. Virus titers were determined by end- point titration in MDCK cells. The total virus dose for each inoculation is shown below panels C and D; these numbers also correspond to parts A and B, respectively. This virus dose was given in a 0.5 ml volume (0.25 ml/nostril). After inoculation with $H5N1_{wildtype}$, virus titers in the nasal turbinates were variable but high, ranging from 1.6×10^5 to 7.9×10^6 TCID50/gram tissue (panel A), with no further increase observed with repeated passage. After inoculation with $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$, virus titers in nasal turbinates averaged 1.6×10^4 in the first three passages, 2.5×10^5 in passage four to seven and 6.3×10^5 TCID50/gram tissue in the last three passages, suggestive of improved replication and virus adaptation. In the lungs, no apparent adaptation was observed for animals inoculated with either virus. Virus titers in lungs were highly variable; presumably it was a matter of chance whether the virus reached the lower airways.

Sequencing of virus quasispecies using a 454 sequencing platform

Viral RNA was extracted from nasal washes of ferrets after 10 passages with influenza viruses $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ using the High Pure RNA Isolation Kit (Roche). RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR), using 32 primer sets that cover the full viral genome (132). These fragments, approximately 400-600 nucleotides in length, were sequenced using the 454/Roche GS-FLX sequencing platform. The fragment library was created for each sample according to the manufacturer's protocol without DNA fragmentation (GS FLX Titanium Rapid Library Preparation, Roche). The emulsion PCR (Amplification Method Lib-L) and GS junior sequencing runs were performed according to instructions of the manufacturer (Roche). Sequence reads from the GS-FLX sequencing data were sorted by bar code and aligned to reference sequence A/Indonesia/5/2005 using CLC Genomics software 4.6.1. The sequence reads were trimmed at 30 nucleotides from the 3' and 5' ends to remove all primer sequences. The threshold for the detection of single nucleotide polymorphisms was manually set at 10% (Table S1, Table S2).

Supplemental Table 1:
Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ virus, using the 454/Roche GS-FLX sequencing platform (ferret P10 in figure 4 of the main text). The viral gene segment is indicated, along with the nucleotide substitutions, the frequency (percent) of sequence reads with the detected mutations, the coverage (total number of sequence reads covering the position), and the nucleotide (nt) and amino acid (aa) positions. All changes from $H5N1_{wildtype}$ are shown that have a frequency > 10; the introduced mutations are underlined.

Segment	Reference	Mutation	Frequency	Coverage	nt position	aa position	aa	aa subst
PB2	G	A	26.4	416	252	75	Gln	S[1]
PB2	G	A	99.6	260	1906	627	Glu	Lys
PB1	C	T	45.0	464	319	99	His	Tyr
PB1	G	T	34.3	542	885	287	Arg	Ser
PB1	G	A	39.2	102	982	320	Ala	Thr
PB1	A	G	50.8	177	1126	368	Ile	Val
PB1	C	T	18.2	390	1500	492	Phe	S
PA	G	A	10.6	416	579	185	Arg	S
PA	C	T	15.0	360	1569	515	Thr	S
PA	T	C	54.6	646	1653	543	Leu	S
PA	A	G	27.7	913	1864	614	Asn	Asp
HA	C	T	85.3	225	383	103	His	Tyr
HA	C	A	11.1	814	538	154	Asn	Lys
HA	A	G	89.9	975	542	156	Thr	Ala
HA	A	T	99.9	932	741	222	Gln	Leu
HA	G	A	99.7	998	746	224	Gly	Ser
HA	A	C	99.9	1033	748	224	Gly	Ser
HA	G	A	15.1	436	977	301	Glu	Lys
HA	C	T	12.9	708	1020	315	Thr	Ile
HA	G	A	17.4	619	1156	360	Gln	S
HA	G	A	11.8	977	1611	512	Gly	Glu
NP	C	T	11.1	36	29	NCR[2]		
NP	G	A	18.1	1189	341	99	Arg	Lys
NP	G	A	22.2	1083	1430	462	Gly	Glu
NP	A	G	12.0	1296	1431	462	Gly	S
NA	A	G	21.7	650	981	321	Asn	Asp
NS	G	A	41.4	596	219	65	Val	Met

1. S; silent substitution, 2. NCR; non-coding region.

Supplemental Table 2:
Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with $H5N1_{wildtype}$ virus, using the 454/Roche GS-FLX sequencing platform. The viral gene segment is indicated, along with the nucleotide substitutions, the frequency (percent) of sequence reads with the detected mutations, the coverage (total number of sequence reads covering the position), and the nucleotide (nt) and amino acid (aa) positions. All changes from $H5N1_{wildtype}$ are shown that have a frequency > 10.

Segment	Reference	Mutation	Frequency	Coverage	nt position	aa position	aa	aa subst
PB2	A	G	100.0	415	1296	423	Arg	S[1]
PB2	G	A	100.0	37	2008	661	Ala	Thr
PB1	T	C	99.8	567	303	93	Ala	S
PB1	A	G	12.1	174	1398	458	Gly	S
PA	G	A	100.0	522	816	264	Thr	S
PA	G	A	99.8	515	1821	599	Glu	S
HA	A	G	99.6	926	542	156	Thr	Ala
HA	T	C	99.5	605	1123	349	Asp	S
NP	G	A	17.9	1497	1430	462	Gly	Glu
NA	C	T	100.0	561	1337	439	Asp	S

1. S; silent substitution

Experiment 4

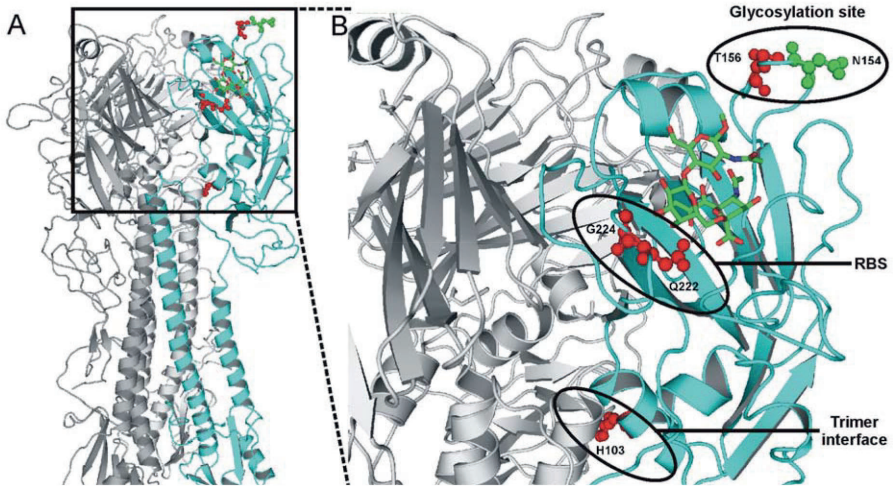
Nasal wash samples collected at 3 dpi from ferrets at passage ten (experiment 3) were used in transmission experiments, to test if airborne-transmissible virus was present in the virus quasispecies. Transmission experiments were done as described above for experiment 2 (Figure S4). Four and two donor ferrets were inoculated intranasally with nasal wash samples collected from passage 10 (P10) of the $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ and $H5N1_{wildtype}$ virus, respectively. As shown in figure 5 of the main text, three out of four recipients ferrets became infected upon airborne transmission of the P10 $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$ virus (F1, F2 and F3). As shown in Figure 6A and 6B of the main text, virus isolated from a throat swab collected at 3 dpi from ferret F2 was subsequently used to inoculate two additional donor ferrets and both of these animals again transmitted the virus to two other recipient ferrets (F5, F6). As shown in figure 6C and 6D of the main text, a virus isolate from F5 that was passaged once in MDCK cells, was next inoculated intranasally into two more donor ferrets. When these ferrets were paired with two recipient ferrets (F7, F8) the next day, one of them (F7) became infected. See Figure 4 of the main text for overall summary.

Sanger sequencing to determine the consensus genome sequences of airborne-transmissible viruses

To determine the consensus sequence of airborne-transmissible viruses in throat and nose swabs obtained from recipient ferrets in experiment 4, viral RNA was extracted using the High Pure RNA Isolation Kit (Roche). All eight influenza virus gene segments were amplified by RT-PCR (133) using 32 primer sets that cover the full viral genome (132) and sequenced using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) and a 3130XL genetic analyzer (Applied Biosystems), according to the instructions of the manufacturer (Table S3, Figure 4).

Supplemental Table 3:
Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with $H5N1_{wildtype}$ virus, using the 454/Roche GS-FLX sequencing platform. The viral gene segment is indicated, along with the nucleotide substitutions, the frequency (percent) of sequence reads with the detected mutations, the coverage (total number of sequence reads covering the position), and the nucleotide (nt) and amino acid (aa) positions. All changes from $H5N1_{wildtype}$ are shown that have a frequency > 10.

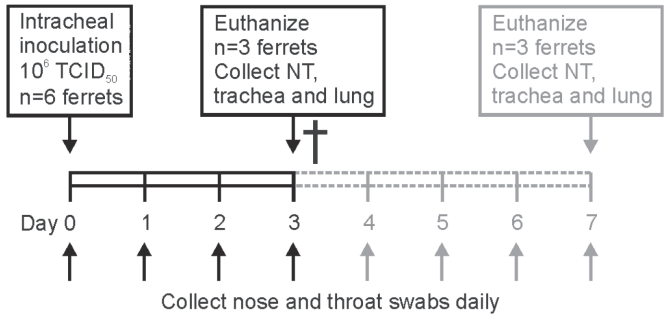
Segment	nt pos	nt wildtype	nt mutant	aa pos	aa wildtype	aa mutant	Transmission 1			Transm 2		Transm 3
							F1	F2	F3	F5	F6	F7
PB2	1298	C	T	424	Ala	Val					x	
PB2	1884	A	G	619	Leu	S[1]			x			
PB2	1906	G	A	627	Glu	Lys	x	x	x	x	x	x
PB1	319	C	T	99	His	Tyr		x		x		x
PB1	591	G	A	189	Arg	S		x		x		x
PB1	885	G	T	287	Arg	Ser	x		x			
PB1	982	G	A	320	Ala	Thr					x	
PB1	1113	G	A	363	Lys	S					x	
PB1	1126	A	G	368	Ile	Val	x	x		x		x
PB1	1500	C	T	492	Phe	S					x	
PB1	1672	C	T	550	Leu	S		x		x	x	x
PA	1719	G	A	565	Val	S		x				
PA	1864	A	G	614	Asn	Asp			x			
HA	383	C	T	103	His	Tyr	x	x	x	x	x	x
HA	538	C	A	154	Asn	Lys	x					
HA	542	A	G	156	Thr	Ala	x	x	x	x	x	x
HA	741	A	T	222	Gln	Leu	x	x	x	x	x	x
HA	746	G	A	224	Gly	Ser	x	x	x	x	x	x
HA	748	A	C	224	Gly	Ser	x	x	x	x	x	x
HA	1020	C	T	315	Thr	Ile	x					
NP	29	C	T	NCR[2]	C	T			x			
NP	341	G	A	99	Arg	Lys	x	x	x	x		x
NP	1079	G	A	345	Ser	Asn				x		x
NA	324	A	G	102	Ile	Val					x	
NA	447	G	A	143	Val	Met					x	
NA	995	G	A	325	Gly	S					x	
NS1	219	G	A	65	Val	Met			x		x	



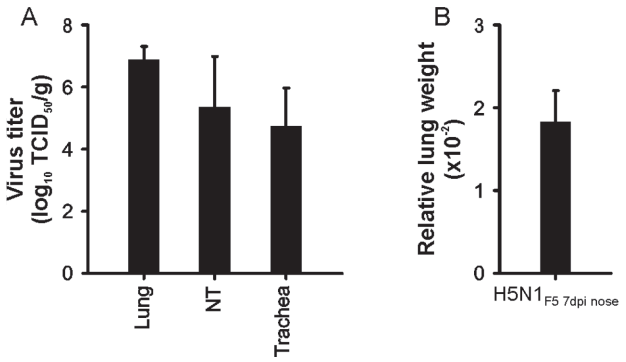
Supplemental Figure 8:
Cartoon representation of a model of the trimer structure HA of A/Indonesia/5/2005 (134) (PDB ID: 1JSM). One monomer is colored cyan for clarity. The structure of the three- sugar glycan NeuAcα2,6Galβ1-4GlcNAc is docked into the receptor binding site (RBS). The substitutions discussed in the text are shown as sticks and balls and colored red (H103, T156, Q222 and G224). N154 of the 154-156 potential N-glycosylation site at the tip of HA is also shown as sticks and balls and colored green.
Amino acid T156 is proximal but not immediately adjacent to the RBS and does not interact directly with the three-sugar glycan that is depicted in the figure. Thus, T156A is unlikely to affect binding to three sugar glycans. Longer glycans may interact with positions as distal from the RBS as amino acid position 156, but little is currently known for such binding. Residue T156 is part of a N-glycosylation sequon (Asn-X-Thr/Ser) and mutation at T156A (as well as N154K) would delete this potential glycosylation site. Wang et al. have shown that this N-glycosylation site is used in H5N1 viruses (135). Loss of N-glycosylation sites at the tip of HA has been shown to enhance virulence of strains of H1 (115, 116) and H5 (117). Mir-Shekari et al. (116) postulated that large, complex N-linked glycans at a site so close to the RBS may occlude binding to the cells, and loss of such a site may improve binding to sialic acids. The T156A substitution was detected in 99.6% of the $H5N1_{wildtype}$ sequences after ten passages. While T156A was detected in only 89% of the $H5N1_{HAQ222L,G224S\ PB2\ E627K}$ sequences after ten passages, the other 11% of sequences had N154K, which removes the same potential N-linked glycosylation site in HA.
Substitutions Q222L and G224S have previously been shown to be sufficient to switch receptor-binding specificity of avian influenza strains to that of human strains (91, 103, 113, 114) G224S can form an additional polar interaction with atom O9 of the NeuAc sugar and Q222L forms favorable van der Waals interactions with the C8 atom of NeuAc as well as the C6 atom of the α-2,6-linkage (113). Amino acid position 103 is distal from the RBS and thus H103Y is unlikely to affect receptor specificity directly. This amino acid forms part of the trimer interface, making both hydrophobic interactions and charge interactions with neighboring amino acids. H103Y maintains the aromatic characteristic at this position but with a slight increase in size. Thus, we expect H103Y would largely maintain its ability to form polar interactions.

Experiment 5

To test whether the airborne-transmissible H5N1 virus could cause pneumonia upon high- dose intra-tracheal challenge, six ferrets were inoculated with 1 x 10^6 TCID50 of virus in a 3 ml volume (Figure S9). This virus was obtained from a nose swab collected from ferret F5 at 7 dpi (experiment 4) and passaged once in MDCK cells. After inoculation, animals were monitored daily for clinical signs. We initially planned to euthanize 3 ferrets each at 3 and 7 dpi, however two animals were found dead at 3 dpi and all other animals were moribund, requiring euthanasia at 3 dpi. Necropsies were performed and nasal turbinates, trachea and lungs were collected for virological examination (Fig S9).



Supplemental Figure 9:
Schematic overview of experiment 5, to test whether airborne-transmissible H5N1 virus caused pneumonia upon high dose intratracheal inoculation. The experiment was halted at day 3.



Supplemental Figure 10:
(A) Virus titers and (B) relative lung weight in six ferrets inoculated intratracheally with 1 x 10^6 TCID50 (in 3 ml) airborne-transmissible H5N1 virus obtained from the nose swab of transmission ferret F5 ($H5N1_{F5\ 7dpi\ nose}$), which was passaged once in MDCK cells and used as inoculum. At 3 dpi, lungs, nasal turbinates (NT) and trachea were collected and virus titers were determined by means of end-point titration in MDCK cells. Geometric mean titer ± SD is indicated. At 3 dpi, two out of six ferrets had succumbed to the infection. The four remaining animals were euthanized at this time point since they were moribund. Intratracheal inoculation with the wildtype H5N1 A/Indonesia/5/2005 virus had a similar effect (112). In this previous work two and four animals were euthanized when they were moribund at 2 and 3 dpi respectively. Mean virus titers in the lungs of animals inoculated with the airborne-transmissible H5N1 virus obtained in this passage experiment (6.9 ± 0.4 $\log_{10}$ TCID50/gram) were slightly but not significantly higher than titers found in $H5N1_{wildtype}$-inoculated animals (6.2 ± 0.6 $\log_{10}$ TCID50/gram) (p=0.093, Mann-Whitney). The relative lung weights of animals inoculated with $H5N1_{wildtype}$ were slightly higher compared to those of the $H5N1_{F5\ 7dpi\ nose}$-inoculated animals (0,024 ± 0,006 and 0,018 ± 0,004 respectively, p=0.180, Mann-Whitney) (112).

Experiment 6: Antiviral resistance

MDCK cells were seeded at 3 x 10^4 cells per well in 96-well plates and cultured overnight. Virus stocks of interest were diluted to 1 x 10^3 TCID50/ml, and added to cells in a volume of 100 ml per well. After incubation for one hour at 37°C, 100 ml of serially diluted oseltamivir carboxylate was added (range 170 pM – 50 μM) in four replicates. After three days of incubation at 37°C, the presence of virus was determined in the supernatant of cell cultures using the agglutinating activity of turkey erythrocytes as an indicator of virus replication in the cells to calculate the 50% inhibitory concentration (IC50).

Supplemental Table 3:
Evaluation of the 50% inhibitory concentration (IC50) of oseltamivir for $H5N1_{wildtype}$, $H5N1_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$ and $H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$. The 2009 pandemic H1N1 virus A/Netherlands/602/2009 was used as a control in this assay. Introduction of the well-known H275Y mutation or the I223R mutation in the A/Netherlands/602/09 NA resulted in a decreased susceptibility for oseltamivir as described previously (136). Introduction of mutations found in airborne-transmissible H5N1 viruses did not affect the sensitivity of H5N1 virus to oseltamivir.

Virus	IC50 (nM)
$A/H5N1_{wildtype}$	5000
$A/H5N1_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$	1100
$A/H5N1_{HA\ Q222L,G224S\ PB2\ E627K}$	1700
A/Netherlands/602/2009	5000
$A/Netherlands/602/2009_{NA\ H275Y}$	≥ 50000
$A/Netherlands/602/2009_{NA\ I223R}$	≥ 50000

Experiment 7: Hemagglutination inhibition assay with ferret sera

The hemagglutination inhibition (HI) assay is based on the ability of the influenza virus HA protein to cause agglutination of red blood cells (RBCs) and of specific antisera to block this reaction. HI assays were performed as described previously (137). Briefly, serum samples were first pre-treated with Vibrio cholerae neuraminidase to remove non-specific inhibitors of hemagglutination activity. Two-fold serial dilutions of the antisera (in 50 ml) were incubated with four hemagglutinating units of virus (in 25 ml) and incubated at 37°C for 30 minutes. Next, 25 ml of turkey RBCs were added, followed by one-hour incubation at 4°C. The highest serum dilution able to block the agglutination of RBCs was recorded as the HI-titer. For these experiments, ferret antisera raised against potential H5N1 vaccine strains were used to evaluate if pre-pandemic H5 vaccine candidates sufficiently matched the airborne-transmissible virus (Table S5). For all HI assays, we used recombinant viruses with six gene segments of influenza virus A/PR/8/34 and the HA and PB2 gene segments of H5N1 virus with or without substitutions Q222L, G224S, H103Y, T156A, and E627K. The multi basic cleavage site in HA was removed by changing the cleavage site PQRERRRKKR↓G in the H5 HA to PQIETR↓G by RT-PCR with specific primers as described (103). Due to this genetic modification, the HI assays could be performed under BSL2 conditions.

Supplemental Table S5:
Hemagglutination inhibition (HI) assay with ferret antisera raised against a panel of candidate H5N1 vaccine viruses for pandemic preparedness selected by the WHO network. Reactivity of ferret post-infection sera is shown for PR8/H5 recombinant influenza viruses with HA and PB2 of $H5N1_{wildtype}$, $H5N1_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ 627K}$ and $H5N1_{HA\ H103Y,T156A\ PB2\ E627K}$. Numbers in bold indicate HI-titers to wildtype A/Indonesia/5/2005 virus antiserum. The antiserum to A/Indonesia/5/2005 reacted with high titers with the HA of airborne-transmissible virus. HA of airborne-transmissible virus cross-reacted better with sera raised against other candidate vaccine viruses representing other clades of H5N1 virus, compared with $H5N1_{wildtype}$ (indicated in italic), presumably owing to changes in the receptor binding site and loss of the potential glycosylation site (135).

	Ferret antisera raised against:								
Viruses	A/Mallard/Netherlands/3/1999 (LPAI H5)	A/HongKong/156/1997 (clade 0)	A/Vietnam/1194/2004 (clade 1)	A/Indonesia/5/2005 (clade 2.1)	A/Turkey/Turkey/1/2005 (clade 2.2)	A/Anhui/1/2005 (clade 2.3)	IVR-148, A/Brisbane/059/2007 (sH1N1)	X175C, A/Uruguay/716/2007 (H3N2)	A/Netherlands/602/2009 (pH1N1)
$A/H5N1_{wildtype}$	<10	<20	10	**1280**	<10	10	<10	<10	<10
$A/H5N1_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$	*20*	*80*	*80*	***2560***	*480*	*120*	<10	<10	<10
$A/H5N1_{HA\ H103Y,T156A,\ PB2\ E627K}$	<10	<20	10	**1280**	160	10	<10	<10	<10
Serum control	<10	10	<10	**<10**	<10	<10	<10	<10	<10

Experiment 8: Hemagglutination inhibition assay with human sera

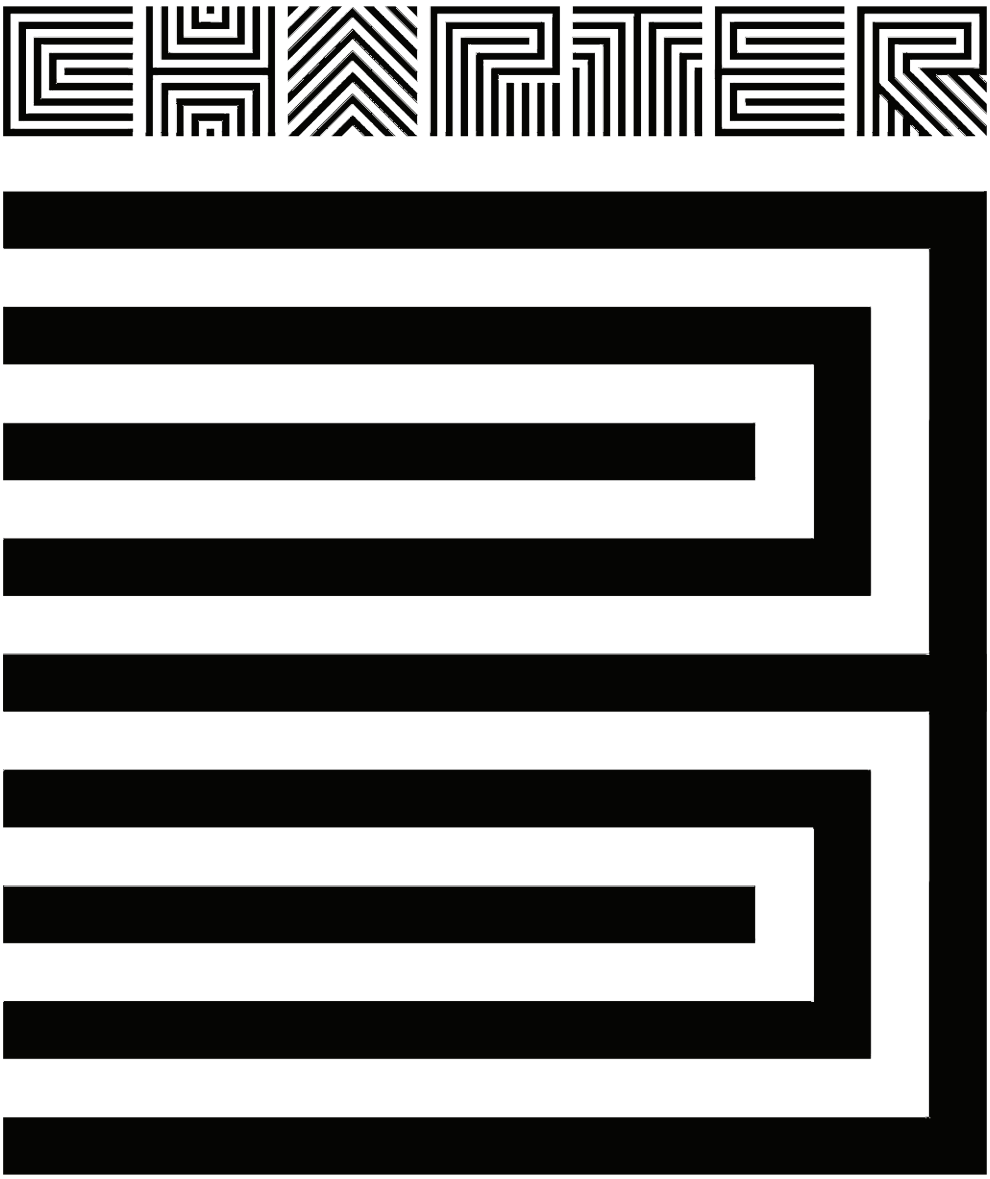
The effect of the introduced RBS mutations Q222L/G224S and co-mutations H103Y and T156A in HA on cross-reactivity with human sera was tested using HI assays as described for experiment 7. For this purpose, the reactivity of 24 human sera obtained from individuals over 70 years of age (chosen because these persons are more likely to have had a history of exposure to pandemic influenza viruses), to the HA of the airborne-transmissible H5N1 and to the currently circulating 2009 pandemic H1N1, seasonal H1N1 and seasonal H3N2 viruses was investigated (Table S6).

Supplemental Table S6:
Hemagglutination inhibition assay using sera from human volunteers older than 70 years of age. The virus tested was a PR8/H5 recombinant influenza virus with HA and PB2 of $H5N1_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$. Titers of human sera against the vaccine strains NYMC-IVR-148 (seasonal H1N1, A/Brisbane/059/2007), NYMC X-181 (pandemic H1N1, A/California/7/2009) and NYMC X-187 (seasonal H3N2, A/Victoria/210/2009) were included as controls. Human sera did not react with the HA of airborne-transmissible H5N1 virus. In contrast, 22 of 24 human sera reacted with one or more H1N1 and H3N2 viruses (numbers in boldface).

Serum#	Serum control	H5N1	sH1N1	pH1N1	H3N2
1	<10	<10	**80**	**80**	**240**
2	<10	<10	<10	**20**	**320**
3	<10	<10	**30**	**20**	**10**
4	<10	<10	**30**	**40**	**60**
5	<10	<10	**10**	<10	<10
6	<10	<10	**30**	<10	**10**
7	20	<30	**80**	<30	<30
8	<10	<10	**80**	**40**	**160**
9	40	<60	**80**	<60	<60
10	30	<40	**40**	<40	<40
11	120	<160	<160	<160	<160
12	<10	<10	**40**	<10	**640**
13	<10	<10	<10	**30**	**1920**
14	20	<30	**30**	**240**	**640**
15	<10	<10	<10	**10**	**40**
16	<10	<10	**20**	**40**	**40**
17	<10	<10	<10	**20**	**240**
18	<10	<10	**640**	**40**	<10
19	<10	<10	**20**	**40**	**160**
20	80	<120	<120	<120	<120
21	<10	<10	**20**	<10	<10
22	<10	<10	**40**	**120**	**160**
23	<10	<10	**80**	**320**	**320**
24	<10	<10	**20**	**160**	**80**

Experiment 9: Modified TRBC hemagglutination assay

For the experiment shown in Table 2 of the main text, modified turkey red blood cells (TRBC) were prepared as described previously (138). Briefly, all α-2,3-, α-2,6-, α-2,8-, and α-2,9- linked sialic acids (SA) were removed from the surface of TRBC by incubating 62.5 ml of 1% TRBC in PBS with 50 mU Vibrio cholerae neuraminidase (VCNA; Roche, Almere, Netherlands) in 8 mM calcium chloride at 37°C for 1 hour. Removal of sialic acids was confirmed by observation of complete loss of hemagglutination of the TRBC by control influenza A viruses. Subsequently, resialylation was performed using 0.5 mU of α-2,3-(N)-sialyltransferase (Calbiochem, San Diego, CA) or 2 mU of α-2,6-(N)-sialyltransferase (Japan Tobacco, Inc., Shizuoka, Japan) and 1.5 mM CMP-sialic acid (Sigma-Aldrich, Zwijndrecht, Netherlands) at 37°C in 75 ml for 2 h to produce α-2,3-TRBC and α-2,6-TRBC, respectively. After a washing step, the TRBC were resuspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5% TRBC. Resialylation was confirmed by hemagglutination of viruses with known receptor specificity; chimeric PR8 viruses with the HA of A/Vietnam/1194/2004 (H5N1, no MBCS; affinity for α-2,3-TRBC) or A/Netherlands/213/2003 (H3N2; affinity for α-2,6-TRBC). The receptor specificity of recombinant viruses with six gene segments of PR8 and the HA and PB2 gene segments of H5N1 virus with or without substitutions Q222L, G224S, H103Y, T156A, and E627K, was tested by performing a standard hemagglutination assay with the modified TRBC. Serial two-fold dilutions of virus in PBS were made in a 50 ml volume; 50 ml of 0.5% TRBC was added, followed by incubation for 1 h at 4°C before determining the hemagglutination titer.



Characterization, and Natural Selection of Mutations Driving Airborne Transmission of A/H5N1 virus

Cell, 2014

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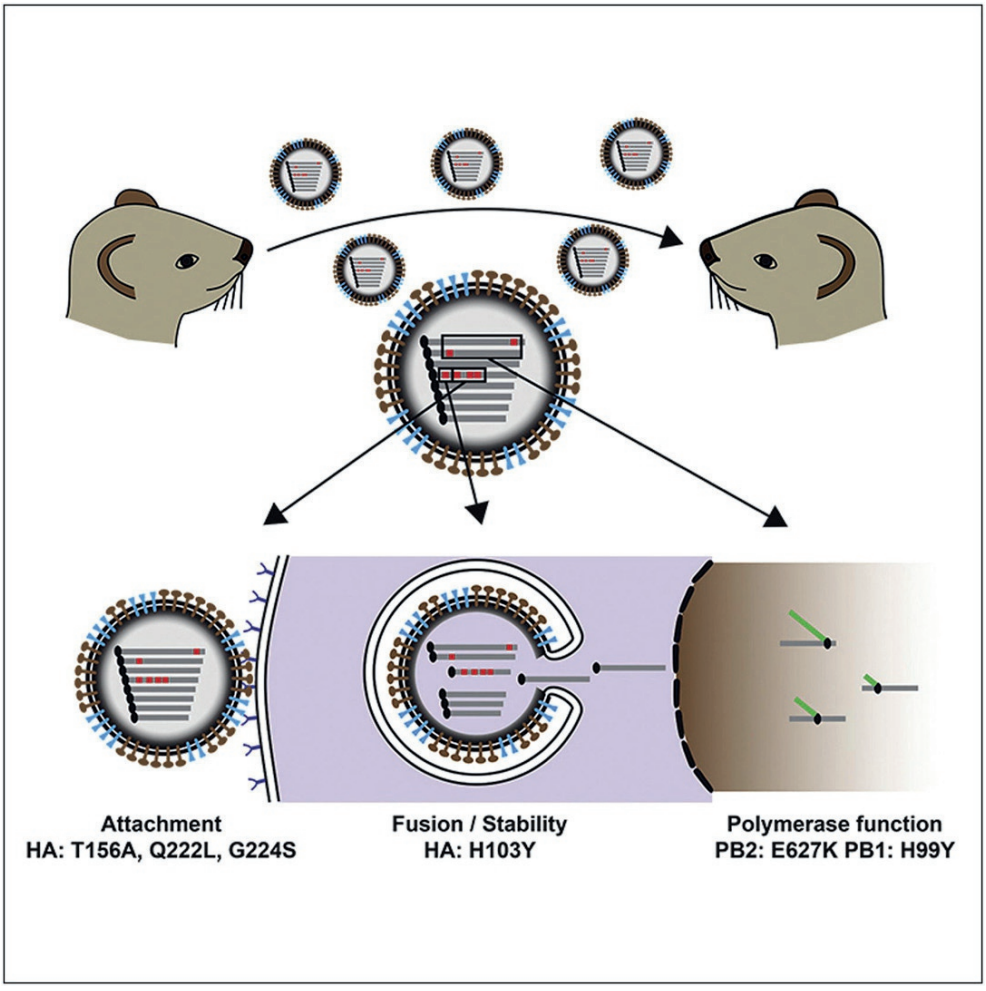


Graphical Abstract

SUMMARY

Recently, H5N1 influenza viruses were shown to acquire airborne transmissibility between ferrets upon targeted mutagenesis and virus passage. The critical genetic changes in airborne A/Indonesia/5/05 were not yet identified. Here, five substitutions proved to be sufficient to determine this airborne transmission phenotype. Substitutions in PB1 and PB2 collectively caused enhanced transcription and virus replication. One substitution increased HA thermostability and lowered the pH of membrane fusion. Two substitutions independently changed HA binding preference from α-2,3-linked to α-2,6-linked sialic acid receptors. The loss of a glycosylation site in HA enhanced overall binding to receptors. The acquired substitutions emerged early during ferret passage as minor variants and became dominant rapidly. Identification of substitutions that are essential for airborne transmission of avian influenza viruses between ferrets and their associated phenotypes advances our fundamental understanding of virus transmission and will increase the value of future surveillance programs and public health risk assessments.

INTRODUCTION

Since the first detection in the late 1990s (139), highly pathogenic avian influenza (HPAI) H5N1 viruses continue to circulate in poultry in Asia and the Middle East. Hundreds of millions of domestic birds have died as a result of infection and during culling activities to control the spread of the H5N1 virus. Occasional cross-species transmission events have been reported for several species of wild birds, pigs, felids, dogs, and mustelids. To date, 650 laboratory-confirmed cases of H5N1 virus infection in humans have been reported to the World Health Organization from 16 countries, of which ~60% had fatal outcome. Sustained human-to-human transmission has not yet been described. However, the enzootic nature of H5N1 virus, its broad host range, the large number of infected hosts, and the observed accumulation of mammalian adaptive substitutions in the virus could potentially increase the risk of a future H5N1 virus pandemic.

For 15 years, one of the key questions for pandemic preparedness has been whether the H5N1 virus might acquire the ability to transmit via aerosols or respiratory droplets (‘‘airborne transmission’’) among humans, a trait necessary for the virus to become pandemic. It was recently shown that a fully avian H5N1 virus can become airborne transmissible between ferrets (140). Three other groups demonstrated that reassortant viruses between H5N1 and 2009 pandemic H1N1 viruses that contain the H5 hemagglutinin (HA) were also transmitted between ferrets or guinea pigs via the airborne route (125, 141, 142).

Herfst et al. (140) introduced the well-known glutamic acid to lysine

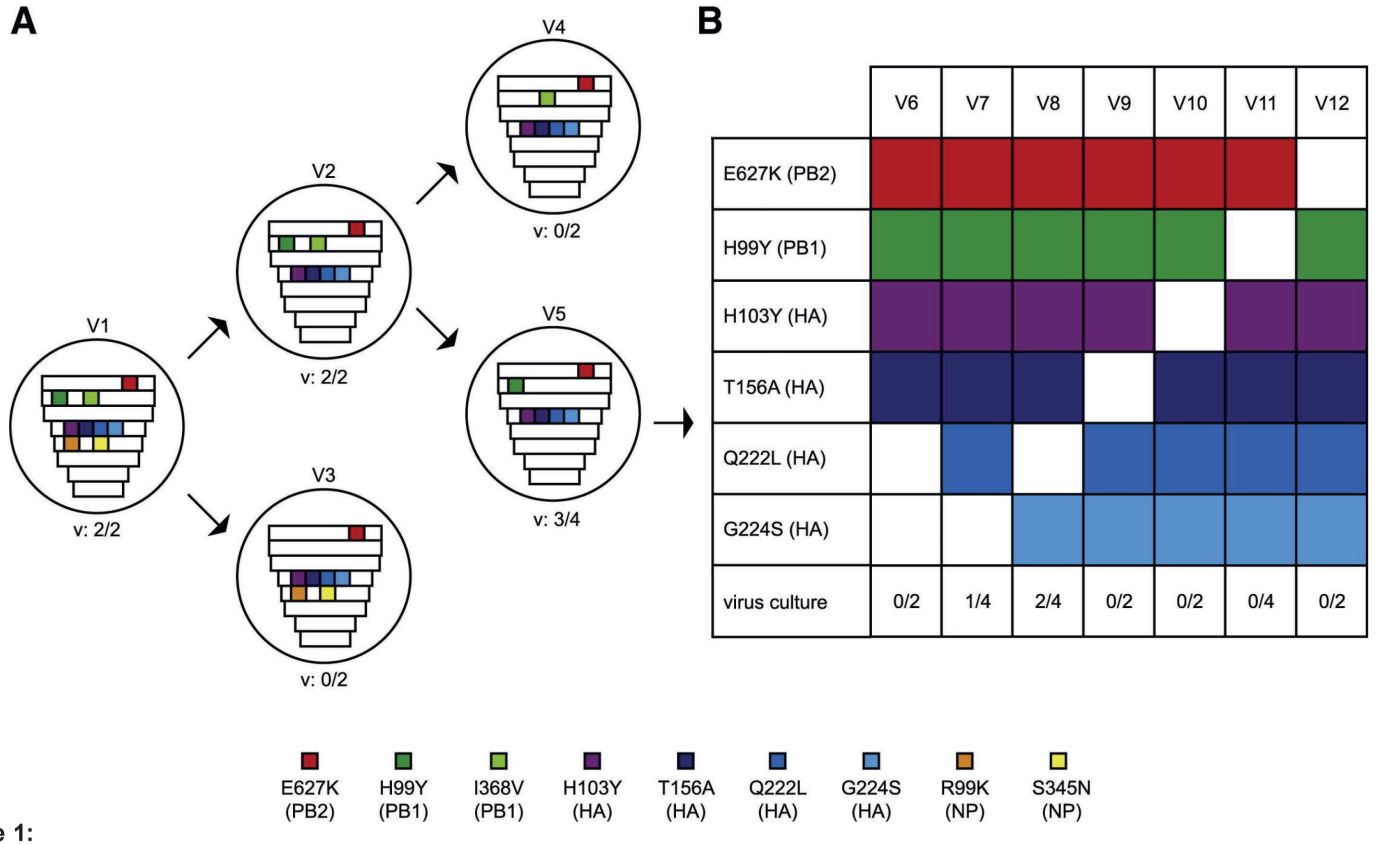


Figure 1:
Summary of Results to Determine a Minimal Set of Substitutions Required for Airborne H5N1 Virus Transmission between Ferrets
(A) Starting with virus V1 that represents the airborne-transmitted virus with the lowest number of amino acid substitutions (nine) as compared to wildtype H5N1 (140), the requirement of substitutions in the PB1 and NP segments was investigated. Recombinant viruses V1-V5 are shown with eight gene segments, in which colored squares represent the presence or absence of indicated substitutions. The proportion of animals positive by virus isolation is indicated with "v."
(B) All substitutions of virus V5 were omitted individually, and transmission was again tested in ferrets for viruses V6 - V12. Virus-shedding patterns in donor and recipient ferrets for V1 - V12 are provided in Figure S1.

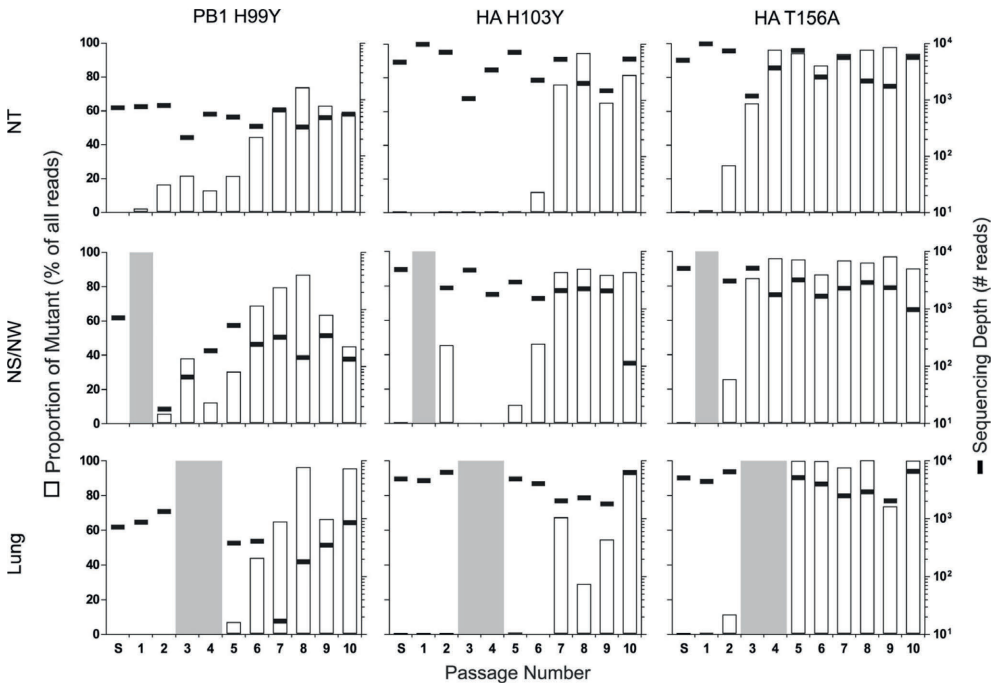


Figure 2:
Single-Nucleotide Variations that Emerged upon Repeated Passaging of Influenza Virus $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$ in Ferrets, as Detected by Deep Sequencing
Passage number is indicated on the x-axis. Left and right y-axes indicated proportion of the mutant among all reads (white bars) and the sequencing depth in number of reads (black lines), respectively. The gray areas indicate that no virus sequences were amplified from these samples. S, virus stock used to inoculate the first ferret (P1); NT, nasal turbinate; NS, nasal swab; NW, nasal wash.

substitution at position 627 (E627K) of the basic polymerase 2 protein (PB2) that is associated with increased replication in mammalian cells at relatively low temperatures (13, 56, 143) and was shown to be important for airborne transmission of 1918 H1N1 and 1999 H3N2 viruses between ferrets and guinea pigs (98, 99). In addition, two substitutions were introduced in the receptor binding site (RBS) of HA that are known to switch receptor specificity from ‘‘avian’’ α-2,3-linked sialic acids (α-2,3-SA) to ‘‘human’’ α-2,6-SA (119), glutamine to leucine at position 222, and glycine to serine at position 224 (Q222L, G224S in H5 HA numbering). These three substitutions or other polymerase and RBS mutations with similar phenotypes were found in all pandemic influenza viruses of the last century and were therefore postulated to represent minimal requirements for adaptation of animal influenza viruses to humans to yield pandemic strains (1, 103). This ‘‘triple mutant’’ virus was passaged ten times in the upper respiratory tract of ferrets to yield mutant H5N1 viruses that were able to transmit via the airborne route between ferrets. In addition to the three substitutions introduced by reverse genetics, two substitutions in HA (H103Y and T156A) were consistently found in all transmitted H5N1 viruses. However, all airborne-transmitted viruses had accumulated additional substitutions. The transmissible virus with the lowest number of amino acid substitutions compared to the $H5N1_{wildtype}$ virus had a total of nine substitutions.

Here, we describe the identification of a minimal set of substitutions required for airborne transmission of influenza virus A/Indonesia/5/05 between ferrets and provide a detailed characterization of the phenotypic changes caused by each of these substitutions. We show that the substitutions acquired upon ferret passage of the triple mutant A/Indonesia/5/05 virus emerged rapidly, which is suggestive for strong natural selection. The identification of previously unrecognized substitutions and phenotypic traits responsible for influenza virus transmission is key to increasing our fundamental understanding of airborne spread of influenza virus and may ultimately increase prognostic capabilities and diagnostic value of surveillance studies necessary for pandemic preparedness.

RESULTS

Airborne Transmission of H5N1 Virus between Ferrets Is Determined by a Minimum of Five Amino Acid Substitutions

To define a minimal number of substitutions in A/Indonesia/5/05 that confer airborne transmission between ferrets, transmission experiments were performed as described previously (95). First, a recombinant virus was produced based on the consensus sequence of a previously identified virus that was airborne transmissible and that contained the lowest number of

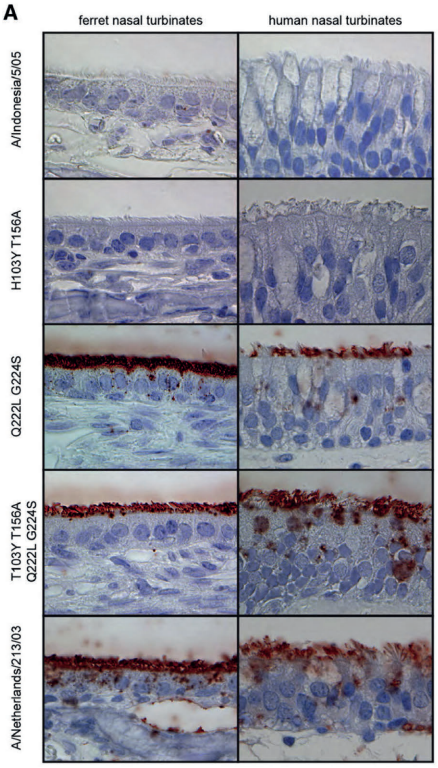
substitutions (n = 9) compared to the wildtype virus (PB2-E627K, PB1-H99Y, and PB1-I368V; HA-H103Y, HA-T156A, HA-Q222L, and HA-G224S; and NP-R99K and NP-S345N) (140). This virus was transmitted to two out of two recipient ferrets, thus reproducing with a recombinant clonal virus our earlier results with a virus isolate (Figure 1A, V1). Next, we omitted either the two substitutions in nucleoprotein (NP) (V2) or the two substitutions in PB1 (V3). Whereas the recombinant virus missing two substitutions in NP was transmitted to two out of two animals, the virus missing two substitutions in PB1 was not transmitted (Figure 1A, V2 and V3). To investigate this further, the two substitutions in PB1 (H99Y and I368V) were tested individually. The virus harboring I368V in addition to the set of five substitutions consistently found in the airborne transmitted viruses (PB2-E627K, HA-H103Y, HA-T156A, HA-Q222L, HA-G224S) was not transmitted to recipient ferrets (Figure 1A, V4), whereas the addition of PB1-H99Y yielded a recombinant virus that was detected in three out of four exposed ferrets (Figure 1A, V5).

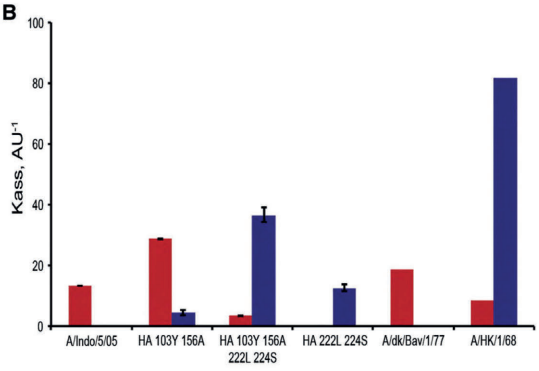
Starting with virus V5 that had six substitutions compared to wildtype A/Indonesia/5/05 (HA-Q222L, HA-G224S, HA-H103Y, HA-T156A, PB2-E627K, and PB1-H99Y), all individual substitutions were omitted one by one (V7–V12). Viruses lacking either the receptor-binding substitution HA-G224S or HA-Q222L were detected in one and two out of four naive ferrets, respectively, upon exposure to inoculated ferrets (Figure 1B, V7 and V8). In contrast, when both HA-Q222L and HA-G224S were omitted, the virus was not transmitted between ferrets (Figure 1B, V6). Viruses lacking HA-T156A, HA-H103Y, PB1-H99Y, or PB2-E627K were not detected in recipient ferrets upon exposure either (Figure 1B, V9 - V12). From this set of experiments, we conclude that PB2-E627K, PB1-H99Y, HA-H103Y, HA-T156A, and either HA-Q222L or HA-G224S in HA represent minimal sets of substitutions required for airborne transmission of A/Indonesia/5/05 between ferrets. Individual virus titers of nose and throat samples obtained from all donor-recipient pairs are shown in Figure S1.

Rapid Emergence of Substitutions Required for Airborne Transmission of H5N1 in Ferrets

We next determined at which passage substitutions H99Y in PB1 and H103Y and T156A in HA emerged during the ten repeated passages of the triple mutant A/Indonesia/5/05. To this end, primers were designed to amplify virus genome fragments covering these amino acid positions, and RT-PCR was performed on RNA isolated from nasal turbinates, lungs, and nasal swabs or washes collected from the ferrets after each passage. Amplicons were sequenced using the Roche 454 GS Junior platform.

PB1-99Y was already detected as a minor variant in the nasal turbinates in passage 1 and increased during subsequent passages to become the





C

virus	untreated	VCNA	α2,3	α2,6
A/Indonesia/5/05	32	0	32	0
156A	32	0	32	4
103Y	32	0	32	0
103Y 156A	32	0	32	2
222L 224S	32	0	0	16
156A 222L 224S	32	0	4	32
103Y 222L 224S	32	0	0	32
103Y 156A 222L 224S	32	0	4	32
103Y 156A 222L	32	0	0	8
103Y 156A 224S	32	0	4	16
A/Netherlands/213/03	32	0	0	32

Figure 3:
Receptor Binding Properties of Wildtype and Mutant H5N1 HA Proteins
(A) Attachment patterns of viruses expressing wildtype or mutant H5 HA to tissue sections of ferret and human nasal turbinates. Red color represents binding of influenza viruses. Images were chosen to reflect representative attachment patterns.
(B) Direct binding of viruses expressing wildtype or mutant H5 HA to fetuin containing either α-2,3-SA (red bars) or α-2,6-SA (blue bars). A/dk/Bav/1/77 and HK/1/68 represent avian and human prototype strains A/duck/Bavaria/1/1977 (H1N1) and Hong Kong/1/1968 (H3N2), respectively. Error bars represent the SD of the mean values (n = 2). See also Figure S2.
(C) Agglutination of TRBCs by viruses expressing wildtype or mutant H5 HA. TRBCs were left untreated, stripped from SA using Vibrio cholerae neuraminidase (VCNA), or modified to contain either α-2,3-SA or α-2,6-SA. Numbers show the HA titers determined with the indicated TRBCs using various mutant viruses. A/Netherlands/213/03 served as a typical human virus with α-2,6 SA preference.

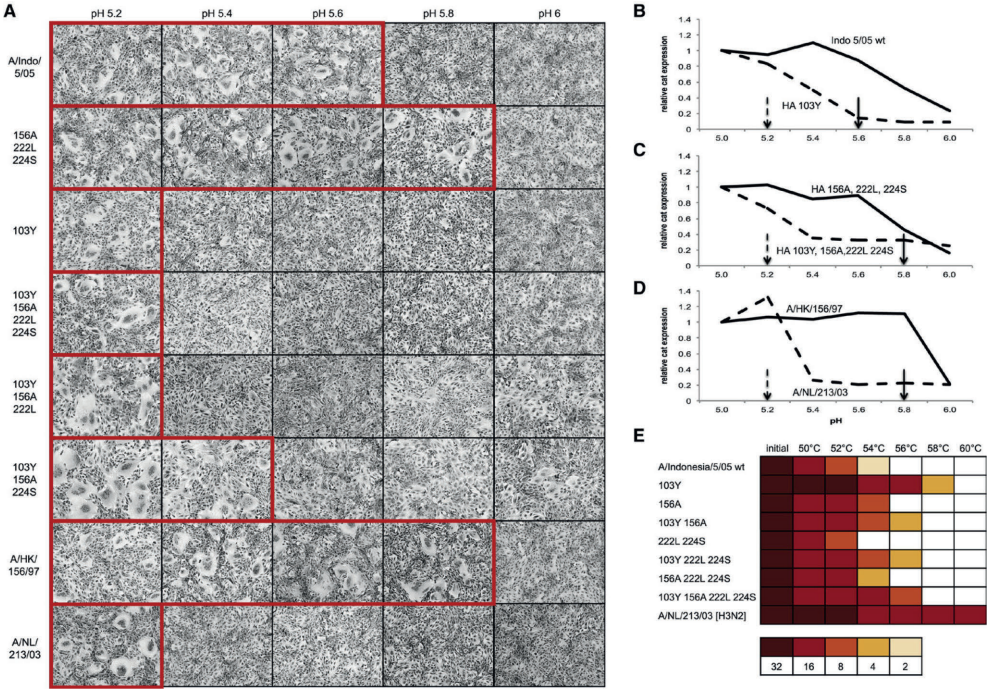


Figure 4:
Analysis of pH Threshold for Fusion and Thermostability of Wildtype and Mutant H5N1 HA Proteins

(A) Syncytium formation in MDCK cells upon expression of wildtype or mutant HA proteins after exposure to different pH. The red line marks the range of pH values at which fusion was detected microscopically. HA of Hong Kong/156/97 (H5N1) and A/Netherlands/213/03 (H3N2) were included as typical avian and human control viruses.

(B–D) Quantification of fusion as measured by the expression of a CAT reporter gene in a cell content mixing assay for (B) influenza virus A/Indonesia/5/05 $HA_{wildtype}$ (solid line) and HA_{H103Y} (dotted line); (C) $HA_{T156A,\ Q222L,\ G224S}$ (solid line) and $HA_{H103Y,\ T156A,\ Q222L,\ G224S}$ (dotted line); and (D) Hong Kong/156/97 (H5N1, solid line) and A/Netherlands/213/03 (H3N2, dotted line). Arrows indicate the pH threshold value at which syncytia were detected visually in (A).

(E) HA protein stability as measured by the ability of viruses to agglutinate TRBCs after incubation at indicated temperatures for 30 min. Colors indicate the HA titers upon treatment at various temperatures for 30 min as shown in the legend.

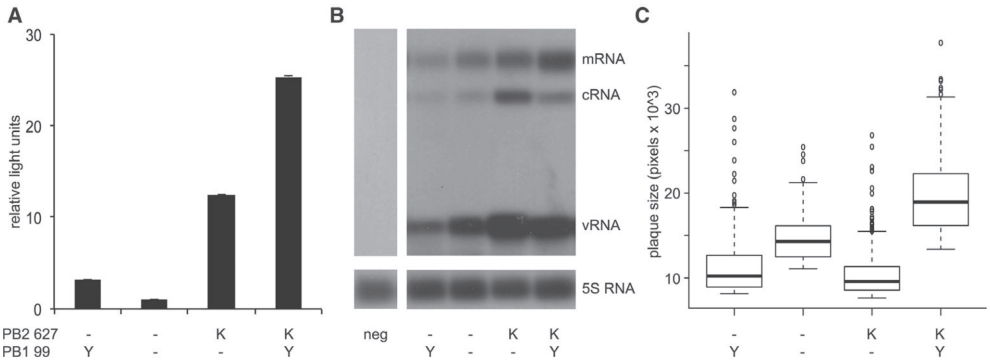


Figure 4:
Effect of H99Y in PB1 and E627K in PB2 on Polymerase Activity and Virus Replication
(A) Minigenome reporter assay. Plasmids encoding PB2, PB1, PA, and NP were cotransfected with a vRNA reporter encoding firefly luciferase. Luminescence of firefly luciferase was standardized using a plasmid constitutively expressing Renilla luciferase. Results are calculated as relative light units (firefly luciferase/ Renilla luciferase) and plotted as fold increase over wildtype. Error bars indicate the SD from the average of two independent experiments.
(B) Primer extension assay. MDCK cells were inoculated at an MOI of 1 with wildtype or mutant A/Indonesia/5/05 viruses. At 3 h postinoculation, cells were lysed, and viral RNA levels were determined by primer extension analysis using primers specific for mRNA, cRNA, or vRNA of PB1. Determination of the 5S RNA levels served as an internal loading control.
(C) Plaque assay. MDCK cells were inoculated with A/Indonesia/5/05 viruses containing the indicated substitutions. After 48 h, plaque formation was visualized by influenza NP-specific staining. Digital images were analyzed using ImageQuant TL software to determine plaque size. The surface of individual plaques in pixels is plotted using boxplots indicating the median value and quartiles. Differences between individual groups were significant ($p < 0.01$) in Student's t test. In all panels, the mutations in PB1 and PB2 are indicated, with dashes representing the wildtype sequences.

dominant variant in all tissues from passage 7 onward (Figure 2). HA-103Y was first detected in the nasal swabs of passage 2, was detected again in passages 5 and 6, and became the major variant from passage 7 onward. Of note, nasal washes instead of nasal turbinates were used to inoculate the ferrets from passage 6 onward, which may have contributed to rapid increase in the proportion of mutants containing HA-103Y after passage 7 (and perhaps also PB1-99Y). HA-156A was detected as a minor variant in passage 1 to rapidly become the dominant variant from passage 3 onward. Thus, the three substitutions that emerged during ferret passage and that contributed to aerosol transmissibility of H5N1 virus arose as early as after one or two passages and became dominant by passage 7.

Nucleotide Variations in the Full Genomes of $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$ during Ferret Passaging and Transmission

We next compared the full genome sequences of viruses described in Herfst et al. (140) present in passage 4, 8, and 10 nasal turbinates of ferrets inoculated with $H5N1_{wildtype}$ or $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$ (Table S1). These samples were selected because they contained high copy numbers of viral RNA as determined by real-time PCR. Substitutions PB2-T23P, PA-T363P, PA-P211T, PA-L498F, NP-G462E, and NA-E239G emerged upon passage with both $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$ but did not become dominant variants. In contrast, T156A became a major variant upon passage of both viruses. Thus, whereas substitutions H99Y in PB1 and H103Y in HA emerged only upon passage of $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$, HA-T156A emergence was independent of PB2-E627K, HA-Q222L, and HA-G224S, which were introduced by reverse genetics. The latter three substitutions remained dominant (>96.2%) throughout all passages tested and were not detected throughout passage of $H5N1_{wildtype}$ (Table S1).

To study the effect of airborne transmission on the viral intra-host nucleotide sequence variation, we compared the full virus genome sequences present in nasal wash samples of ferrets inoculated with $H5N1_{HA\ Q222L,\ G224S\ PB2\ E627K}$ after ten passages in ferrets to nose swab samples of ferrets after two consecutive airborne transmission events (ferrets F5 and F6, Table S2). The number of nucleotide variations present in specimens from F5 (n = 12) and F6 (n = 16) was substantially less as compared to those in the passage 10 nasal wash sample (n = 28). Moreover, substitution PB1-99Y increased in frequency from 44.9% in passage 10 nasal wash to 100% and 73.3% in F5 and F6 after the two consecutive airborne transmission events. Similarly, HA-103Y increased from 87.6% to 100% and 100%, and HA-156A increased from 89.9% to 99% and 98.8%. These collective data are indicative of a strong selection bottleneck occurring on intra-host nucleotide sequence variation.

HA Substitutions Q222L, G224S, and T156A Affect Receptor Binding

To study the impact of the HA substitutions associated with airborne transmission on receptor preference, the attachment patterns of A/Puerto Rico/8/1934 (PR8) viruses harboring wildtype or mutant H5N1 HA proteins were first characterized using formalin-fixed paraffin-embedded tissue sections of ferret and human nasal turbinates, known to express ‘‘human-like’’ α-2,6-SA receptors abundantly. PR8 virus with the wildtype H5 HA did not attach to nasal turbinate sections, whereas the same virus with a control human H3 HA showed abundant attachment, as expected (Figure 3A). Introduction of Q222L and G224S in wildtype H5 HA resulted in abundant virus attachment to the nasal turbinate sections, comparable to the H3 HA control, as shown previously (103). Introduction of H103Y and T156A in either the wildtype H5 HA or $HA_{Q222L,\ G224S}$ did not result in obvious changes in these patterns of virus attachment. The attachment patterns of HAs to the ferret and human nasal turbinate tissue sections were indistinguishable.

We used two solid-phase enzyme-linked receptor-binding assays to determine HA binding of virus immobilized on a 96-well plate to receptor analogs in solution: a direct binding assay and a binding inhibition assay (144). PR8 viruses that harbor wildtype or mutant H5 HAs were tested in direct binding assays with receptor analogs 3’-fetuin and 6’-fetuin (Figure 3B). Introduction of the Q222L and G224S in $HA_{wildtype}$ resulted in a switch in receptor binding specificity from α-2,3-SA to α-2,6-SA as expected, with no residual α-2,3-SA binding. Introduction of H103Y and T156A in $HA_{Q222L,\ G224S}$ resulted in increased binding to α-2,6-SA receptors but also low binding to α-2,3-SA. Introduction of H103Y and T156A in $HA_{wildtype}$ resulted in increased binding to α-2,3-SA and low binding to α-2,6-SA. Thus, in both the context of $HA_{wildtype}$ and $HA_{Q222L,\ G224S}$, substitutions H103Y and T156A resulted in increased binding avidity and limited dual receptor specificity. A direct binding assay performed with 3’-sialyl-N-acetyllactosamine and 6’-sialyl-N-acetyllactosamine-containing synthetic sialylglycopolymers rather than 3’-fetuin and 6’-fetuin yielded similar results (Figure S2A). In a fetuin-binding inhibition assay, we compared binding of the viruses to a panel of sialylglycopolymers containing several different sialyloligosaccharide moieties (Figure S2B). In this assay, the wildtype and mutant H5 viruses in general bound less avidly as compared to the control avian H1N1 virus A/Duck/Bavaria/1/1977 and human H3N2 virus HongKong/1/1968. $HA_{wildtype}$ and $HA_{H103Y,\ T156A}$ bound stronger to various α-2,3-SA analogs than to α-2,6-SA, whereas $HA_{Q222L,\ G224S}$ and $HA_{Q222L,\ G224S,\ H103Y,\ T156A}$ showed the opposite binding preference (Figure S2B), thus yielding similar results as the direct binding assays.

In a third approach, we assessed the binding of PR8 viruses expressing wildtype or mutant H5 HA in a HA assay using normal turkey red blood

cells (TRBC) that contain both α-2,3-SA and α-2,6-SA or modified TRBC that contain either α-2,3-SA or α-2,6-SA alone or no SA (Figure 3C). As shown previously, introduction of Q222L and G224S in H5 HA resulted in a switch in receptor binding preference from α-2,3-SA to α-2,6-SA (103). Additional introduction of the H103Y and T156A substitutions required for airborne transmission increased the HA titers to both α-2,3-SA and α-2,6-SA containing TRBC. Each of the individual receptor-binding site substitutions Q222L and G224S in the context of changes H103Y and T156A displayed binding to α-2,6-SA containing TRBC, which is in agreement with the fact that single RBS substitutions were sufficient for airborne transmission (Figure 1B).

Introduction of H103Y in $HA_{wildtype}$, HA_{T156A}, $HA_{Q222L, G224S}$, and $HA_{T156A, Q222L, G224S}$ did not result in consistent changes in HA titers. In contrast, introduction of T156A in $HA_{wildtype}$, HA_{H103Y}, $HA_{Q222L, G224S}$, and $HA_{H103Y, Q222L, G224S}$ resulted in dual receptor specificity as indicated by a consistent ≥2-fold increase in HA titers, irrespective of the HA used.

Collectively, we conclude from these studies that H103Y had no discernable effect on receptor binding preference, whereas T156A increased overall virus binding to both α-2,3-SA and α-2,6-SA, thus resulting in dual receptor specificity.

HA Substitution H103Y Affects Acid and Temperature Stability

Upon virus attachment to SA receptors on the cell surface and internalization into endosomes, a low-pH-triggered conformational change of HA mediates fusion of the viral and endosomal membranes to release the virus genome in the cytoplasm (76). We measured the pH threshold required for fusion of wildtype and mutant HAs. Vero cells were transfected with HA-expression plasmids and exposed to trypsin to cleave and activate the HA, followed by acidification of the cell culture at a pH range of 5.2 to 6.0. Visual inspection of the cell cultures for the presence of syncytia (multinucleated cells) was used to determine the pH threshold required for fusion (Figure 4A). Fusion of $HA_{wildtype}$ was triggered at pH ≤ 5.6, similar to the threshold pH of the control H5 HA of Hongkong/156/97 (pH ≤ 5.8). HA of the control human H3N2 virus A/Netherlands/213/03 required a lower pH (≤ 5.2) for fusion to occur. These data are in agreement with the observation that avian influenza virus HAs generally trigger fusion at a higher pH than human virus HAs (145). The three substitutions that affect receptor binding (T156A, Q222L, G224S; see above) did not result in a reduction of the threshold pH for fusion as compared to $HA_{wildtype}$. In contrast, upon introduction of H103Y in $HA_{wildtype}$ and $HA_{T156A, Q222L, G224S}$, fusion was triggered only at pH 5.2 and lower. Moreover, $HA_{H103Y, T156A, Q222L}$ and $HA_{H103Y, T156A, G224S}$ also triggered fusion at relatively low pH (pH ≤ 5.2 and pH ≤ 5.4, respectively).

As a second readout for fusion, we used a ‘‘cell content mixing assay,’’ in which two populations of Vero cells were transfected with HA and either a chloramphenicol-acetyl-transferase construct under control of the HIV-1 promoter (LTR-CAT) or an HIV-1 transactivator construct (pTat). Upon mixing of the two cell populations and after acidification of the cell culture at a pH range of 5.2 to 6.0, fusion was quantified by measuring CAT expression that is dependent on the expression of Tat. Also in this assay, introduction of H103Y in $HA_{wildtype}$ and $HA_{T156A, Q222L, G224S}$ resulted in a lower threshold pH for fusion, as indicated by the dotted lines in Figures 4B and 4C, respectively. The difference in pH required for fusion between HA with and without H103Y was similar to that observed for the reference H3N2 and H5N1 HAs (Figure 4D).

The switch of influenza virus HA from a metastable nonfusogenic to a stable fusogenic conformation can also be triggered at neutral pH when the HA is exposed to increasing temperature. This conformational change of HA is biochemically indistinguishable from the change triggered by low pH (146) and results in a loss in the ability to bind receptor. To further investigate HA stability, PR8 viruses harboring wildtype or mutant HAs were incubated at increasing temperatures, after which the ability of the viruses to agglutinate TRBCs was quantified. The PR8 virus with the H3 HA of A/Netherlands/213/03 retained HA activity even upon treatment for 30 min at 60°C. In contrast, PR8 with H5 HA of A/Indonesia/5/05 lost HA activity upon treatment at 56°C for 30 min. Irrespective of the presence or absence of substitutions affecting receptor binding ($HA_{wildtype}$, HA_{T156A}, $HA_{Q222L, G224S}$, and $HA_{T156A, Q222L, G224S}$ were tested), H103Y resulted in increased temperature stability as measured in the HA assay (Figure 4E).

Collectively, these data indicate that H103Y has a stabilizing effect on the HA of A/Indonesia/5/05 - with respect to both low pH and high temperature treatment - irrespective of the presence or absence of substitutions that affect receptor binding.

PB2-E627K and PB1-H99Y Affect Polymerase Activity

The influenza virus polymerase complex—consisting of the polymerase proteins PA, PB1, and PB2—transcribes the negative sense viral RNA ((-)vRNA) in mRNA and positive sense copy RNA ((+)cRNA), the latter of which is used as the template to yield newly synthesized (-)vRNA. To read out polymerase complex function, we used a (-)vRNA reporter construct consisting of the firefly luciferase open reading frame flanked by the noncoding regions of segment 8 of influenza A virus (123). Upon cotransfection of the reporter with expression plasmids encoding PB1, PB2, PA, and NP, the (-)vRNA reporter is transcribed and the firefly luciferase protein is expressed. A plasmid that constitutively expresses Renilla luciferase was cotransfected as an internal control to standardize transfection efficiency and sample processing. Substitution E627K in PB2 resulted in a 12-fold increase in firefly luciferase expression as compared to the wildtype PB2. Substitution H99Y in PB1 resulted in a 3-fold increase. The polymerase complex with both PB2-E627K and PB1-H99Y yielded a 25-fold increase in firefly luciferase expression as compared to the polymerase complex of the wildtype virus (Figure 5A).

To study the levels of transcription of (-)vRNA, (+)cRNA, and mRNA during the course of a viral infection, we performed primer extension assays using total cellular RNA isolated upon virus inoculation of MDCK cells. Three hours after inoculation with $H5N1_{wildtype}$, all three RNA species were detected (Figure 5B). Introduction of E627K in PB2 resulted in elevated levels of the viral RNAs, predominantly increasing the amount of (-)vRNA and (+)cRNA, while marginally changing mRNA. Substitution H99Y in PB1 resulted in slightly reduced levels of all detected RNA species as compared with $H5N1_{wildtype}$. Upon inoculation with a virus containing both PB2-E627K and PB1-H99Y, the ratio between (-)vRNA, (+)cRNA, and mRNA was similar as observed for $H5N1_{wildtype}$ but with higher overall levels of all three RNA species.

As a third test of polymerase function, we studied in vitro replication of the recombinant H5N1 viruses with and without polymerase substitutions by measuring plaque sizes upon inoculation of MDCK cells. At 48 h after inoculation, cells were fixed and stained with an anti-NP antibody, and the number of pixels representing ~100 to 500 plaques on digital images was quantified. As compared to $H5N1_{wildtype}$, $H5N1_{PB2\ E627K}$ and $H5N1_{PB1\ H99Y}$ displayed a slight reduction in plaque size. However, combination of these two substitutions ($H5N1_{PB2\ E627K,\ PB1\ H99Y}$) yielded plaques that were significantly larger than those observed for $H5N1_{wildtype}$ (Figure 5C). From these assays, we conclude that PB2-E627K and PB1-H99Y collectively resulted in increased levels of (-)vRNA, (+)cRNA, and mRNA transcription and increased virus replication in MDCK cells.

DISCUSSION

Here, we show that, of the nine substitutions observed in an airborne transmissible H5N1 virus (140), two alternative sets of five mutations are identified (E627K in PB2; H99Y in PB1; H103Y, T156A, and either Q222L or G224S in HA), either of which is sufficient to confer ferret transmissibility on A/Indonesia/5/05. Keeping in mind that the design of this ferret transmission model is qualitative rather than quantitative and that such studies need to take the principles of replacement, reduction, and refinement in animal experiments into account (147, 148), it should be noted that the present study is limited by the number of animals that were used and was purposely designed to define a minimal set of substitutions rather than the definitive minimal set of substitutions required for airborne transmission in ferrets. Starting with the recombinant H5N1 virus harboring nine substitutions, omission of two substitutions in NP still yielded virus transmission to five out of six airborne-exposed ferrets (V2 and V5). Subsequently, a virus lacking PB1-H99Y (V4) was not detected by virus isolation and serology in two out of two airborne-exposed ferrets, whereas a virus lacking PB1-I386V was detected by virus isolation in three out of four airborne exposed ferrets, thus providing evidence that H99Y and not I386V was required for airborne transmission. In the subsequent experiment, all individual substitutions from the remaining set of six (PB2-E627K, PB1-H99Y, HA-H103Y, HA-T156A, HA-Q222L, and HA-G224S; Figure 1B) were eliminated one by one. Viruses lacking either HA-Q222L or HA-G224S still resulted in airborne transmission in two and one of four ferrets tested, indicating that a single receptor-binding site substitution was sufficient for transmission. In contrast, in transmission experiments using viruses from which each of the other single substitutions were eliminated, virus was not detected in exposed ferrets. However, some of these exposed animals seroconverted despite a lack of virus detection. Apparently, although transmission may occur for some viruses with five substitutions as measured by serology or single time points of virus detection (Figure S1), the viruses were insufficiently replication competent to cause robust infection and seroconversion consistently in the exposed ferrets. As a consequence, refining the minimal set of substitutions required for airborne transmission would require substantial numbers of ferret pairs, given the statistical considerations for this type of experiment (147, 149).

The substitutions required for airborne transmission in ferrets were determined in in vitro assays to either affect HA binding to receptors, HA stability, or activity of the polymerase complex. Two substitutions introduced by reverse genetics (Q222L and G224S) are known to change the receptor binding preference of the H5 HA from avian-like α-2,3-SA to human-like α-2,6-SA (103). This change in receptor binding preference, either through single or double substitutions, was required for airborne transmissibility, which is in agreement with loss-of-function transmission studies using 1918 H1N1 and 1957 H2N2 viruses

(70, 72). Although HAs with single Q222L or G224S substitutions were less efficient in binding α-2,6-SA-containing TRBC as compared to the double mutant, viruses with the single RBS substitutions were still transmissible. HA substitution T156A increased virus binding to both α-2,6-SA and α-2,3-SA in quantitative binding assays. Imai et al. (125) showed that substitution N154D in HA also affected transmission of a reassortant H5 virus. T156A and N154D result in the loss of the same glycosylation site in HA, suggesting that loss of this glycosylation site rather than the specific amino acid substitutions were important for the change in phenotype. Loss of glycosylation in H5 HA in combination with substitutions Q222L and G224S was previously shown to enhance virus replication in ferrets (135). The airborne-transmissible H5 virus of Imai et al. (125) also contained Q222L in HA but had N220K as a second substitution in the RBS. In a third study, Q222L/G224S, along with Q192R in the context of A/Vietnam/1203/04 HA, also resulted in slightly increased transmission in ferrets (141). These studies thus indicate that changes in receptor specificity critically contribute to airborne transmission of H5 viruses in ferrets. Affinity measurements using A/Indonesia/5/05, A/Vietnam/1203/04 and A/Vietnam/1194/04 HAs with substitutions associated with airborne transmission revealed a binding preference for human receptors (Figure 3) (150-153).

Structural studies of HA further showed that human and avian receptor analogs were bound in the RBS in the same "folded-back" conformation as seen for HA from H1, H2, and H3 pandemic viruses, which is distinct from typical avian virus HAs, including H5 (151-153) . In these studies`, the affinity of the mutant H5 HA was relatively low as compared to HA of human H2 and H3 viruses (152-155). Although it was speculated that an N-linked glycan at the tip of HA might sterically hinder SA binding (152), direct evidence from structural studies on HA of the airborne transmissible viruses is still lacking.

Arguably, one of the more intriguing findings of the Imai et al. (125) and Herfst et al. (140) studies is the requirement of HA mutations that affect stability in terms of temperature and pH. H103Y in A/Indonesia/5/05 HA resulted in increased temperature stability and requirement of lower pH treatment to trigger membrane fusion, similar as described for T315I by Imai et al. (125). H103Y was recently shown to increase the thermostability of HA (150), and temperature-dependent circular dichroism spectroscopic experiments revealed hydrogen bond formation between 103Y and 413N in adjacent monomers that stabilized the trimeric protein (153). In contrast, T315I stabilized the position of the fusion peptide within the HA monomer (152), indicating that multiple mechanisms can lead to increased HA stability. Galloway et al. (145) suggested that an altered pH of fusion may be associated with virus adaptation to new hosts. Furthermore, substitutions that decrease the pH of fusion increased virus replication in the upper respiratory tract of ferrets and in mice (156-158). Although the contribution of H103Y and T315I to increase

airborne transmission between ferrets may be related to the pH of fusion or thermostability, these properties may merely be a surrogate for another - as yet unknown - phenotype, such as stability of HA in aerosols, resistance to drought, stability in mucus, or altered pH in the host environment.
The requirement of increased polymerase activity to yield an airborne transmissible H5 virus was also expected (1). PB2 E627K has been identified as a major determinant of host adaptation of pandemic influenza viruses (13, 56, 98, 99, 143). Here, E627K also resulted in increased polymerase activity. More importantly however, we identified a substitution acting in concert with E627K to increase polymerase activity. Like PB2 E627K, PB1 H99Y alone resulted in increased polymerase activity in minigenome assays but decreased virus replication. When combined, these two substitutions had a synergistic effect in minigenome assays and increased virus replication. In primer extension assays, PB2 E627K predominantly caused an increase in vRNA and cRNA, changing the ratio between RNA replication and mRNA transcription. Addition of PB1 H99Y lowered vRNA and cRNA while further increasing mRNA levels, yielding a similar ratio as observed for $H5N1_{wildtype}$ but at overall increased levels. Here, we thus describe an ‘‘adaptive’’ substitution in PB1 that potentially improved the levels of, and balance among, vRNA, mRNA, and cRNA in concert with PB2 E627K in A/Indonesia/5/05. Beyond HA, only these two amino acid substitutions were required to generate airborne-transmissible H5N1 virus. Such conclusion cannot be obtained from the H5 virus transmission experiments that use reassortant viruses. Zhang et al. (142) showed that the PA or NS1 genes of a pH1N1 virus contributed to airborne transmission of reassortant H5N1 viruses in guinea pigs. At present, it is unclear how the guinea pig and ferret models compare with respect to H5 virus transmission studies.
Although some substitutions leading to an airborne phenotype have been observed in nature, the required combination of substitutions has not yet been detected (140, 148, 159). Keeping in mind that the ferret model may not be predictive for airborne H5N1 virus emergence in humans, it is of interest to note that, upon acquisition of PB2 E627K and the receptor binding changes Q222L/G224S, the additional substitutions associated with the airborne phenotype of H5N1 emerged within only one or two passages in ferrets, became dominant after six passages, and appeared to be selected for during transmission events. Other sets of mutations than those identified here that similarly result in altered receptor specificity and stability of HA and polymerase function in mammalian cells may also be sufficient to increase airborne transmission. The specific mutations to increase transmission may be dependent on virus strain or subtype (160). Given that some of the required substitutions (PB2-E627K and HA-T156A) are commonly found in field isolates and that functionally equivalent substitutions exist for most of the identified substitutions, emergence of transmissible H5N1 influenza

A viruses in nature cannot be excluded (148). In this light, the H7N9 virus outbreak in China is a cause of concern, as some H7N9 field strains contain E627K in PB2 and Q222L in HA, and this H7 lineage lacks a glycosylation site at the tip of HA (161). These viruses were shown to have increased preference for α-2,6-SA and decreased binding to α-2,3-SA (41, 162, 163). Although airborne H7N9 virus transmission in ferrets was shown to be limited (41, 164-166), it cannot be excluded that fully avian viruses adapt upon repeated passage in mammals to gain transmissibility. In analogy to H5, it can be speculated that the H7N9 virus needs to acquire increased binding preference for α-2,6-SA over α-2,3-SA, increased HA stability, and increased polymerase activity. For both H5N1 and H7N9, appropriate surveillance for emergence of mutations that affect HA receptor binding, HA stability, polymerase activity, and transmission is thus warranted. It has been argued that different sets of substitutions may lead to similar virus phenotypes, and hence, sequence-based virus surveillance may be misleading. However, such surveillance may be improved by including phenotyping assays - using relatively simple methods as described here - in the future. Such surveillance may be further improved by deep sequencing, in addition to sequencing consensus virus genomes.

Although it is clear that studies in ferrets may not be predictive for influenza virus outbreaks in humans, the ferret transmission model is one of the best models for influenza available today (94). Experiments like the ones presented here are crucial to increase our basic understanding of airborne virus transmission, as such knowledge is currently very limited. The results of this study do not imply that an H5 influenza pandemic is imminent but warrant an intensified and broadened approach to detect emerging influenza viruses early and take immediate action once viruses naturally gain functions that might enable them to become a pandemic threat.

EXPERIMENTAL PROCEDURES

Viruses and Cells

Madin-Darby canine kidney (MDCK) cells, 293T (human kidney epithelial cells), and subclone 118 of Vero-WHO cells (African green monkey kidney epithelial cells) were used for virus propagation, plasmid transfections, and fusion assays, respectively.

Influenza virus A/Indonesia/5/05 (H5N1) was isolated from a human case of HPAI virus infection. All viruses used in this study were generated upon transfection of 293T cells with reverse genetics plasmids. Recombinant viruses were propagated in MDCK cells, and viral titers were determined by end-point titration. For binding and stability assays, recombinant viruses with seven gene segments of A/PR/8/34 and the wildtype or mutant HA segment of A/Indonesia/5/05 were used.

Ferret Model for Airborne Transmission

Ethical, biosafety, and biosecurity considerations related to the experiments are described in detail in the Supplemental Information.

One to 2-year-old ferrets (mustela putorius furo), free of antibodies against H5 HA, were inoculated with virus. The next day, a second ferret was housed in the same cage, which was divided into two separate sections by a pair of metal grids, allowing the transfer of air between cage mates but preventing direct contact. Nose and throat swabs of inoculated and naive animals were taken every other day to test for virus presence. Blood from naive contact animals was collected 14 days after first exposure to inoculated animals and tested for the presence of antibodies against H5 HA.

Given that airborne transmission in ferrets has never been observed for A/Indonesia/5/05 or any other avian H5N1 virus (Table S3), we define every single event of virus detection in naïve ferrets as ‘‘airborne transmission.’’ The use of small group sizes could result in an underestimation of airborne transmission. Thus, although ‘‘not transmissible’’ does not mean ‘‘will never transmit,’’ ‘‘transmissible’’ is a clearly defined virus phenotype.

Deep Sequencing

Viral RNA was extracted from ferret samples upon virus passaging, converted to cDNA, and amplified by PCR using primers covering the full viral genome. PCR fragments for each virus were pooled in equal concentrations, and libraries were created for each virus. Emulsion PCR and GS Junior sequencing runs were performed according to instructions of the manufacturer (Roche). Sequence reads were sorted by bar code, trimmed at 30 nucleotides from the 30 and 50 ends to remove primer sequences, and the 30 ends were further trimmed to improve quality using a Phred score of 20. Reads were aligned to reference sequence A/Indonesia/5/05 using CLC Genomics software 4.6.1. The threshold for mutation detection was manually set at 1%.

Virus Binding Assays

Formalin-fixed, paraffin-embedded tissues from ferrets and humans were used for virus histochemistry. Sucrose-purified viruses were labeled with fluorescein isothiocyanate (FITC) and incubated with the tissue sections overnight. The FITC label was detected with a peroxidase-labeled anti-FITC antibody, and the signal was amplified using a Tyramide Signal Amplification System. Peroxidase was revealed with 3-amino-9-ethyl-cabazole, and tissues were counterstained with hematoxylin and embedded in glycerol-gelatin.

For direct fetuin binding assays, 96 well plates were first coated with bovine fetuin and subsequently incubated with BPL-inactivated viruses, washed, and blocked with desialylated bovine serum albumin. The virus-coated wells were incubated with 2-fold dilutions of receptor analogs (resialylated fetuin preparations containing either α-2,3-linked SAs [3'-fetuin] or α-2,6-linked SAs [6'-fetuin] labeled with horseradish peroxidase). Plates were washed, and tetramethylbenzidine substrate was added, after which the absorbance at 450 nm was determined. The association constants (Kass) of virus complexes with analogs were determined from the slopes of Scatchard plots.

For modified red blood cell assays, all SAs were removed from the surface of TRBC by incubation with Vibrio cholerae neuraminidase. Complete removal of SAs was confirmed by loss of HA using control viruses. Resialylation was done using α-2,3-(N)-sialyltransferase or α-2,6-(N)-sialyltransferase to produce α-2,3-TRBC and α-2,6-TRBC, respectively. Resialylation of either α-2,3 or α-2,6 was confirmed using viruses with known receptor specificity. Viruses were tested in standard HA assay using native and resialylated TRBCs.

Fusion Assays

HA-mediated membrane fusion was tested by transfecting populations of Vero-118 cells with plasmids expressing HA and β-galactosidase (β-gal) along with either pLTR-CAT (a chloramphenicol acetyltransferase [CAT] gene under the control of the human immunodeficiency virus type 1 long terminal repeat) or pTat (expressing the HIV-1 transactivator of transcription Tat). One day after transfection, both cell populations were harvested, pooled, and replated. The next morning, cells were exposed to PBS at different pH for 10 min. Cell lysates were harvested 24 h after the pH pulse, and CAT and β-gal expression were quantified by enzyme-linked immunosorbent assays. As an alternative to CAT quantification, cells were fixed, washed, and stained with Giemsa for microscopy.

Stability Assay

Viruses were incubated for 30 min at different temperatures before performing an HA assay using TRBCs. Two-fold dilutions of virus in PBS containing 0.25% red blood cells were prepared in a U-shaped 96 well plate and were incubated for 1 h at 4°C, and agglutination was recorded.

Minigenome Assay

A model vRNA, consisting of the firefly luciferase flanked by the noncoding regions of segment 8 of influenza A virus, under the control of a T7 RNA polymerase promoter, was transfected into 293T cells, along with plasmids expressing T7 RNA polymerase, PB2, PB1, PA, and NP, and a constitutive Renilla luciferase expression plasmid. 24 h after transfection, firefly and Renilla luminescence was measured. Relative light units were calculated as the ratio of firefly and Renilla luciferase.

Plaque Assay

MDCK cells were inoculated with virus and grown with an Avicel (FMC biopolymers, Brussels) overlay. Two days later, cells were washed, fixed, permeabilized, and stained with anti-NP monoclonal antibody and goat-anti-mouse-HRP. True blue reagent (KPL) was added to the cells, and digital images were taken to quantify plaque size using ImageQuant TL software (GE Healthcare Life Sciences).

Primer Extension Assay

MDCK cells were inoculated with virus, and RNA was isolated 3 h later using Trizol reagent (Invitrogen). Radioactive-labeled primers specific for mRNA/ cRNA, vRNA of PB1, and 5S rRNA were annealed to prime a reverse transcription reaction. The reaction was stopped, and transcription products were separated on 6% polyacrylamide gels containing 7 M urea in trisborate-EDTA buffer and detected using autoradiography films.

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EXTENDED EXPERIMENTAL PROCEDURES

Biocontainment

All experiments involving highly pathogenic H5N1 viruses were conducted at enhanced animal biosafety level 3 (ABSL3+). The ABSL3+ facility of Erasmus MC consists of a negative pressurized (-30 Pa) laboratory in which all in vivo and in vitro experimental work is carried out in class 3 isolators or class 3 biosafety cabinets, which are also negative pressurized (<-200 Pa). Although the laboratory is considered ''clean'' because all experiments are conducted in closed class 3 cabinets and isolators, special personal protective equipment, including laboratory suits, gloves and FFP3 facemasks is used. Air released from the class 3 units is filtered by High Efficiency Particulate Air (HEPA) filters and then leaves the facility via a second set of HEPA filters. Only authorized personnel that received the appropriate training can access the ABSL3+ facility. All personnel working in the facility is vaccinated against seasonal and H5N1 influenza viruses. For animal handling in the facilities, personnel always work in pairs. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at Erasmus MC and Dutch and United States government inspectors. Antiviral drugs (oseltamivir and zanamivir) and personnel isolation facilities are directly available to further mitigate risks upon incidents.

Publication Considerations

Prior to submission of the manuscript, all authors read and commented on all aspects of the study (design, realization, scientific value, ethical considerations, integrity of data, presentation of experimental outcomes, biosafety and biosecurity aspects etc.). We concluded that research involving airborne transmissible H5N1 influenza viruses must be considered DUR (dual-use research), but that the present study does not qualify as DURC (dual-use research of concern), since no increase in virulence or transmissibility compared to previously published studies is described. Furthermore, we also cannot foresee any direct misapplication of the presented work resulting in a threat to public health. In contrast, the gain of knowledge and future applications of this work will be beneficial for science and society.

The manuscript was sent for clearance to the Biosafety Office of Erasmus MC, representing the institutional Biosafety Advisory Board. Subsequently, we requested (under formal protest) an export license issued by the Dutch government for communication of the manuscript to NIAID (National Institutes of Allergy and Infectious Diseases) and the publisher. Next, members of the Biosecurity Committee and the Ethical Advisory Board of the EU FP7 program ''ANTIGONE'' endorsed publication of the manuscript and delivered comments that were carefully incorporated in the manuscript. NIAID as

another funding agency requested an appendix 5 procedure to account for possible biosafety and biosecurity risks and to develop a communication plan. The final version of the manuscript was sent to the funders and all individuals involved in the evaluation process, after which the manuscript was submitted for publication.

Cells

Madin-Darby Canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (EMEM, Lonza, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate (Lonza), 10 mM HEPES (Lonza), and non-essential amino acids (MP Biomedicals Europe, Illkirch, France). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM, Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and non-essential amino acids. Subclone 118 of Vero-WHO cells (Vero-118) was cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker) supplemented with 10 % fetal calf serum (FCS), 100 IU/ml penicillin, 100 mg streptomycin and 2 mM glutamine (167).

Viruses

Influenza virus A/Indonesia/5/05 (H5N1) was isolated from a human case of HPAI virus infection and passaged once in embryonated chicken eggs followed by one passage in MDCK cells. All eight gene segments were amplified by reverse transcription polymerase chain reaction and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 (100, 168). Substitutions of interest (H103Y, T156A, Q222L and G224S in HA, E627K in PB2, R99K and S345N in NP and H99Y and I368V in PB1) were introduced by reverse genetics using the QuikChange multi-site-directed mutagenesis kit (Stratagene, Leusden, Netherlands) according to the instructions of the manufacturer. Recombinant viruses were produced upon transfection of 293T cells and virus stocks were propagated and titrated in MDCK cells. For binding assays, and stability assays, reassortant viruses consisting of seven gene segments of influenza virus A/PR/8/34 and the HA segment of H5N1 were produced using a previously described reverse genetics system for influenza virus A/PR/8/34 (100). For binding studies, viruses were inactivated by treatment with β-propiolactone (BPL, Ferak Berlin) (169), concentrated by pelleting through a 20% sucrose cushion and stored in 0.02% sodium azide (Sigma-Aldrich).

Virus Titration in MDCK Cells

Virus titers were determined by end-point titration in MDCK cells. MDCK cells were inoculated with tenfold serial dilutions of virus stocks, nose swabs, or throat swabs. Cells were washed with PBS one hour after inoculation and cultured in 200 ml of infection media, consisting of EMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM HEPES, non-essential amino acids, and 20 mg/ml trypsin (Lonza). Three days after inoculation, supernatants of infected cell cultures were tested for agglutinating activity using TRBCs as an indicator of virus replication in the cells. Infectious virus titers were calculated from 4 replicates (nose swabs, and throat swabs) or 10 replicates (virus stocks) by the method of Spearman-Karber.

Airborne Transmission of H5N1 Virus in the Ferret Model

An independent animal experimentation ethical review committee (Dutch Stichting Dier Experimenten Commissie Consult) approved all animal studies. 64 female ferrets between 1 and 2 years of age were obtained from an accredited ferret breeder. All animals were tested for the presence of antibodies against H5, H3 and H1 prototype influenza A viruses and Aleutian Disease Virus, were microchipped and received hormonal treatment to prevent estrus.

Aerosol or respiratory droplet transmission experiments were performed as described previously (95). In short, 2 or 4 seronegative female adult ferrets (Mustela putorius furo) were inoculated intranasally with 10^6 TCID50 of virus by applying 250 ml of virus suspension to each nostril. Each ferret was then placed in a transmission cage. One day after inoculation, one naive ferret was placed opposite to each inoculated ferret. Each transmission pair was housed in a separate transmission cage designed to prevent direct contact between the inoculated and naive ferrets but allowing airflow from the inoculated to the naive ferret. Nose and throat swabs were collected on 1, 3, 5, and 7 days post inoculation (dpi) for inoculated ferrets and on 1, 3, 5, 7, and 9 days post exposure (dpe) for the naive ferrets. Virus titers in swabs were determined by end-point titration in MDCK cells.

Clinical manifestations observed in animals upon inoculation of a virus suspension or infection via aerosols or respiratory droplets included ruffled fur, loss of appetite, and lethargy. Two animals that died in the course of the experiments were examined for the presence of influenza virus in respiratory organs and were found to be free of detectable levels of viral protein. We concluded that these ferrets died due to reasons unrelated to the effect of virus infection. No animals required withdrawal from the study on animal welfare grounds. All animals were humanely killed at the end of the in-vivo phase of the study.

Statistical Considerations for Ferret Transmission Experiments

Given that airborne transmission in ferrets has never been observed for A/Indonesia/5/05 or any other avian H5N1 virus (Table S3), we define every single event of airborne transmission of mutant A/Indonesia/5/05 virus as detected by the presence of virus in naive ferrets as ‘‘airborne transmission.’’ The use of small group sizes could result in an underestimation of airborne transmission, e.g., when 0 of 2 animals show airborne transmission, it could be possible that upon testing of another 2 or 4 animals, airborne transmission would be detected. Thus, while ‘‘not transmissible’’ does not mean ‘‘will never transmit,’’ ‘‘transmissible’’ is a clearly defined virus phenotype. In support of this and other statistical considerations, we performed an extensive analysis of influenza A virus airborne transmission studies in ferrets, extending beyond the analysis by Belser et al. (147) and discussions therein. A search for published literature via http://www.ncbi.nlm.nih.gov/pubmed/ using search terms ’’influenza,’’ ‘‘transmission,’’ and ‘‘ferret’’ was performed on 27-12-2013. Studies that did not report airborne transmission between ferrets based on virus replication data or that tested swine viruses exclusively were discarded from the set. We added two articles manually that were not retrieved automatically in this search and that we were aware of (170, 171). From the total of 48 full-text articles that were selected, we report the wildtype viruses that were tested, the group size of the experiment and the number of airborne transmission events that were reported (Table S3). From this analysis, it was clear that wildtype H5N1 viruses have never been shown to transmit via the airborne route between ferrets (0/28 ferret pairs in 7 published studies) and that the same holds true for various A/Indonesia/5/05 strains (0/8 pairs). Moreover, other avian influenza viruses (with the exception of 2013 H7N9 viruses) were shown to be not transmitted between 73 ferret pairs tested in 10 studies, with the exception of 1 study reporting transmission of a single avian H1N1 virus. In sharp contrast, human influenza viruses were transmitted in 132/149 ferret pairs in 32 published studies. Despite the small group sizes in most published studies, human influenza viruses were transmitted in 32/32 studies, while avian H5N1 viruses were transmitted in 0/7 studies. The 2013 H7N9 virus (which has several mammalian adaptation markers) was transmissible in 19 of 44 ferret pairs in 6 published studies, clearly intermediate in transmissibility between avian and human influenza viruses. Despite small group sizes, also the results with H7N9 virus were comparable (transmission in 1/3, 4/8, 9/18, 3/9, 1/3, 1/3 ferret pairs in the individual studies). Thus, although the statistical power of transmission studies as performed here is insufficient for a quantitative analysis of transmission efficiency due to small group size, sufficiently robust data are generated to draw qualitative conclusions. We can therefore interpret any event of airborne transmission of mutant H5N1 virus as an indicator of increased transmission over wildtype H5N1 (never found to be transmitted),

while we cannot draw strong conclusions about mutant H5N1 viruses that did not transmit between small numbers of ferret pairs.

Serology

The presence of antibodies elicited against the tested viruses was confirmed by HA inhibition (HI) assay using standard procedures (172) and A/Indonesia/5/05 $HA_{H103Y, T156A, Q222L, G224S}$ as a test antigen. Briefly, ferret antisera were prepared upon intranasal inoculation and collecting blood 14 days later. Antisera were pre-treated overnight with receptor destroying enzyme Vibrio cholerae neuraminidase (VCNA) at 37°C, and incubated at 56°C for 1h the next day. Twofold serial dilutions of the antisera, starting at a 1:20 dilution, were mixed with 25 ml of a virus stock containing 4 hemagglutinating units and were incubated at 37°C for 30 min. Subsequently, 25 ml 1% TRBCs was added and the mixture was incubated at 4°C for 1h. HI was read and was expressed as the reciprocal value of the highest dilution of the serum that completely inhibited agglutination of virus and erythrocytes.

Analysis of Virus Nucleotide Sequence Variation Using a 454 Sequencing Platform

Viral RNA was extracted from ferret tracheal swabs, nasal swabs or nasal washes upon passaging with influenza viruses $H5N1_{wildtype}$ and $H5N1_{HA\ Q222L, G224S\ PB2\ E627K}$, using the High Pure RNA Isolation Kit (Roche). A SuperScript III One-Step reverse transcription kit (Invitrogen) was used to synthesize cDNA from extracted RNA. The RT mixture contained 46 ml of RNA extract, 4 ml (2 pmol/ml) primer AGCRAAAGCAGG, 1 ml (40 U/ml) Ribonuclease Inhibitor (Promega), and 4 ml (10 mM each) deoxynucleoside triphosphates (Roche) in a 55 ml volume. After a 5 min incubation at 65°C for optimal primer hybridization to template, 16 ml (10x) First-Strand buffer, 14 ml (0.1 M) DTT, 1 ml (40 U/ml) Ribonuclease Inhibitor (Promega) and 4 ml (200 U/ml) SuperScript III Reverse Transcriptase was added to the mixture in a 80 ml volume. The RT mixture was sequentially incubated at 25°C for 5 min and 50°C for 1 h to obtain cDNA. cDNA was subjected to polymerase chain reaction (PCR), using 32 primer sets (Table S4) that cover the full viral genome (132).

The PCR mixtures contained 10 pmol of each forward and reverse primer, 3 ml of cDNA, 1 ml (10 mM each) deoxynucleoside triphosphate, 5 ml PCR Gold buffer, 5 ml (25 mM) $MgCl_2$, and 1 ml (2.5 U/ml) AmpliTaq Gold DNA Polymerase (Applied Biosystems, Bleiswijk, The Netherlands). Water was then added to achieve a final volume of 50 ml. The PCR mixture was incubated at 95°C for 6 min, followed by 40 cycles at 95°C for 30 s, 45°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR fragments were visualized by blue light after electrophoresis on a 1% agarose gel

containing 1 x SYBR® Safe DNA Gel Stain (Life Technologies, Bleiswijk, The Netherlands) in 1 x Tris-borate buffer (pH 8.0). SmartLadder (Eurogentec) was used to estimate amplicon size. Fragments ranged from 426 to 627 nucleotides in length.
Fragments with the correct size, were extracted from the gel using the MinElute Gel Extraction Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturers protocol, and subsequently DNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).
Fragments for each virus were pooled in equal concentrations and libraries were created for each virus according to the manufacturer's protocol without DNA fragmentation (GS FLX Titanium Rapid Library Preparation, Roche). The emPCR (Amplification Method Lib-L) and GS Junior sequencing runs were performed according to instructions of the manufacturer (Roche). Sequence reads from the GS Junior sequencing data were sorted by barcode and aligned to reference sequence A/Indonesia/5/05 using CLC Genomics software 4.6.1. The sequence reads were trimmed at 30 nucleotides from the 30 and 50 ends to remove all primer sequences and the 30 end of the reads were trimmed to improve quality, using a Phred score of 20. The threshold for detection was manually set at 1%.

Virus Histochemistry

Viruses were purified on a sucrose gradient and labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich) as described previously (173). Briefly, MDCK cells were inoculated and virus-containing supernatant was concentrated using a sucrose cushion and purified on sucrose gradients before inactivation by dialysis against 0.1% formalin and labeling with an equal volume of 0.1 mg/ml FITC.
All selected tissues were free of histological evidence for infection. Virus histochemistry was performed as described previously (173). Briefly, formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and rehydrated with graded alcohol. 50-100 hemagglutinating units of FITC-labeled influenza viruses were incubated with the respective tissue sections overnight at 4°C. The FITC label was detected with a peroxidase-labeled rabbit anti-FITC antibody (Dako, Heverlee, Belgium) and the signal was amplified using a Tyramide Signal Amplification System (Perkin Elmer, Groningen, The Netherlands) according to the instructions of the manufacturer. Peroxidase was revealed with 3-amino-9-ethyl-cabazole (Sigma-Aldrich) and tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Darmstadt, Germany). Attachment of influenza virus to tissues was visible as granular to diffuse red staining of the epithelium.

Direct Receptor Binding Assay

For direct receptor binding assays and fetuin binding inhibition assay, virus working dilutions were prepared as described previously (144). In addition, equal absorption of the wildtype and mutant H5N1 viruses in the wells of microplates was confirmed using a direct enzyme-linked immunosorbent assay with PR/8 neuraminidase-specific antibodies (BEI resources, Manassas, USA). Biotinylated synthetic poly-N-(2-hydroxyethyl)acrylamide-based sialylglycopolymers (SGPs) containing 20 mol% of Neu5Acα2,3Galβ1-4GlcNAcβ (3'SLN) or Neu5Acα2,6Galβ1-4GlcNAcβ (6'SLN) (Lectinity Holding, Inc., Moscow, Russia) were used as receptor analogs. Also re-sialylated fetuin preparations, containing either α-2,3-linked SAs (3'Fetuin) or α-2,6-linked SAs (6'Fetuin) labeled with horseradish peroxidase (HRP) were tested for their binding to the viruses. Ninety-six well plates were coated overnight at 4°C with bovine fetuin (5 mg/ml) in PBS, washed with distilled water and dried at room temperature. Fetuin-coated plates were incubated overnight at 4°C with BPL-inactivated viruses in PBS, washed 3 times with PBS and blocked with 0.1% solution of desialylated bovine serum albumin (BSA-NA, Sigma-Aldrich) in PBS. After washing 3 times with ice-cold washing buffer (PBS with 0.05% Tween-80), the replicate virus-coated wells were incubated with two-fold dilutions of the receptor analogs in incubation buffer (1 mM oseltamivir, 0.02% tween-80, 0.1% BSA) for 1 h at 4°C. To quantify binding of HRP-labeled fetuin, plates were washed 3 times, after which substrate (tetramethylbenzidine) was added to detect HRP activity. To detect binding of biotinylated SGPs, plates were washed 3 times with ice-cold washing buffer, followed by incubation with streptavidin-HRP. After subsequent washing, tetramethylbenzidine was added to quantify binding. The absorbencies at 450 nm were determined, transferred to a PC and processed with Microsoft Excel software. The data were converted to Scatchard plots (A450/C versus A450), where C is the concentration of the receptor analog in solution and A450 is the absorbency in the corresponding well. Concentrations were expressed in arbitrary units (AU) for 3' and 6' fetuin and in mM of sialic acid for SGPs. The apparent association constants (K_{ass}) of virus complexes with analogs were determined from the slopes of the Scatchard plots.

Fetuin Inhibition Assay

In the fetuin binding-inhibition assay, the association constant (Kass) for the virus complex with receptor analog is calculated based on the level of its competition with fetuin-HRP (144). As receptor analogs, several SGPs were used (Lectinity Holding, Inc., Moscow, Russia), which contained the following sialyloligosaccharide ligands: Neu5Acα2,3Galβ1-4GlcNAcβ (3'SLN), Neu5Acα2,6Galβ1-4GlcNAcβ (6'SLN), Neu5Acα2,3Galβ1-4(6-

O-HSO_3)GlcNAcβ (Su-3'SLN), Neu5Acα2,3Galβ1-4(Fucα1-3)GlcNAcβ (SLe^x), Neu5Acα2,3Galβ1-4(Fucα1-3)(6-O-HSO_3)GlcNAcβ (Su-SLe^x), Neu5Acα2,3Galβ1-3GlcNAcβ (SLe^c), Neu5Acα2,3Galβ1-3(6-O-HSO_3) GlcNAcβ (Su-SLe^c) and Neu5Acα2,3Galβ1-3(Fucα1-4)GlcNAcβ (SLe^a). Viruses were first absorbed in fetuin-coated plates as described above. Subsequently, two-fold dilutions of SGPs in HRP-labeled fetuin-HRP-containing buffer were added to the wells, followed by incubation for 1 h at 4°C. After washing, tetramethylbenzidine was added and peroxidase activity was measured. Association constants were calculated from the binding inhibition data as described previously (144).

Modified Red Blood Cell Assay

Modified TRBC's were prepared as described (138) with slight modifications. Briefly, all SAs were removed from the surface of TRBC by incubation of 62.5 ml of 20% TRBC in PBS with 50 mU of VCNA (Roche, Almere, The Netherlands) in 8 mM calcium chloride at 37°C for one hour. Complete removal of SAs was confirmed by loss of HA of treated TRBC using control viruses. Resialylation was done using 0.25 mU α-2,3-(N)-sialyltraferase (Calbiochem, California, USA) or 12 mU α-2,6-(N)-sialyltransferase (Calbiochem, California, USA) and 1.5 mM CMP-SA (Sigma-Aldrich, Zwijndrecht, the Netherlands) at 37°C in 75 ml for 2 h to produce α-2,3-TRBC and α-2,6-TRBC respectively. After washing, the TRBCs were resuspended in PBS containing 1% BSA to a final concentration of 0.5%. Resialylation of either α-2,3 or α-2,6 was confirmed by HA using viruses with known receptor specificity. Viruses were tested in standard HA assay using native and resialylated TRBCs. In brief, twofold dilutions of virus were made in PBS. An equal volume of 0.5% TRBCs was added and incubated at 4°C for one hour before reading the HA titer.

Fusion Assay

Fusion was tested as previously described (174) in a cell content mixing (CM) assay in which two 10 cm dishes containing Vero-118 cells were each transfected with 5 mg of pCAGGS-HA and 1 mg of pTS27 plasmid, a constitutive β-galactosidase (β-Gal) expression vector, using Xtremegene transfection reagent (Roche). The cells in one dish were cotransfected with 4 mg of pLTR-CAT (containing the chloramphenicol acetyltransferase [CAT] gene under the control of the human immunodeficiency virus type 1 [HIV-1] long terminal repeat [LTR]), and the cells in the other dish were cotransfected with 4 mg of pTat (expressing the HIV-1 transactivator of transcription Tat). One day after transfection, both cell populations were harvested using trypsin-EDTA, pooled, and plated in a six-well plate format. The next morning, cells were exposed to PBS at different pH for 10 min. Cell lysates were harvested 24 h after the pH pulse, and CAT and β-Gal expression were quantified

by enzyme-linked immunosorbent assays (Roche). HA-mediated fusion of cellular membranes resulted in polykaryon formation and Tat-mediated transactivation of the HIV-1 LTR resulted in induction of CAT expression. Alternatively to CAT quantification, cells were fixed using 70% ice-cold acetone, washed and stained using a 20% Giemsa mixture for microscopy (Merck Millipore, Darmstadt, Germany).

HA Stability Assay

Viruses were incubated for 30 min at different temperatures before performing an HA assay using TRBCs. Two-fold dilutions of virus in PBS containing 0.25% red blood cells were prepared in a U-shaped 96 well plate and were incubated for one hour at 4°C and agglutination was recorded.

Minigenome Assay

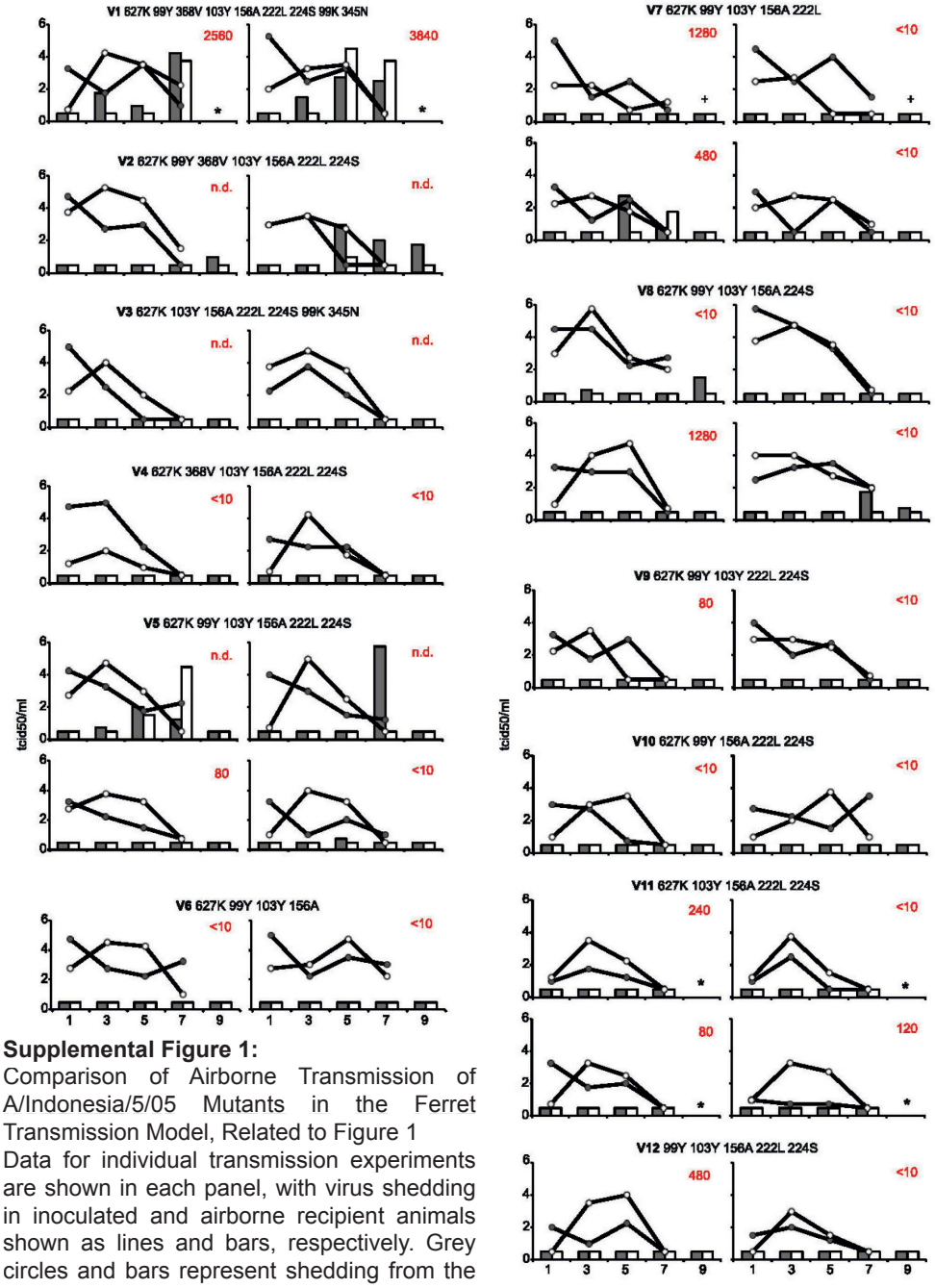
A model vRNA, consisting of the firefly luciferase open reading frame flanked by the noncoding regions (NCRs) of segment 8 of influenza A virus, under the control of a T7 RNA polymerase promoter was used for minigenome assays (123). The reporter plasmid (0.5 mg) was transfected into 293T cells in 6-well plates, along with 0.5 mg of each of the pHW2000 plasmids encoding PB2, PB1, PA, and NP; 1 mg of pAR3132 expressing T7 RNA polymerase (175); and 0.02 mg of the Renilla luciferase expression plasmid pRL (Promega, Leiden, Netherlands) as an internal control. 24 h after transfection, luminescence was measured using a Dual-Glo Luciferase Assay System (Promega) according to the instructions of the manufacturer in a TECAN Infinite F200 machine (Tecan Benelux bv, Giessen, Netherlands). Relative light units (RLU) were calculated as the ratio of firefly and Renilla luciferase luminescence.

Plaque Assay

The assay was performed as described (176). In brief, MDCK cells (10^6 per well) were seeded in a 6 well plate to reach 90% confluency the next day. One hour after inoculation with 10^3 TCID50/ml, the inoculum was replaced with 1:1 mixture of 2.4% Avicel (FMC biopolymers, Brussels, Belgium) with 2xEMEM infection medium. After 48 h, cells were washed 3 times with PBS and 4% formalin solution was added. After two hours of incubation at room temperature (RT) cells were washed twice and then permeabilized using 0.1% Triton X-100 (Sigma Aldrich, Zwijndrecht, the Netherlands). Fixed and permeabilized cells were washed 3 times with PBS/Tween 0.05%, and then incubated for 1 h at RT with mouse-anti-NP monoclonal antibody (1 mg/ml HB65, ATCC) diluted 1:1000 in PBS with 2% milk powder. Following three washes with PBS/Tween 0.05%, cells were incubated for 1 h at RT with goat-anti-mouse-HRP (Invitrogen) 1:10.000 diluted in PBS/milk powder 2%. True blue reagent (KPL Inc., Maryland, USA) was added to the cells and incubated for 5 min or until full development of staining of the plaques. Cells were washed twice with H_2O and allowed to air dry. Digital images were taken and were analyzed using ImageQuant TL software (GE Healthcare Life Sciences). Plaque size was plotted as pixels in the original image.

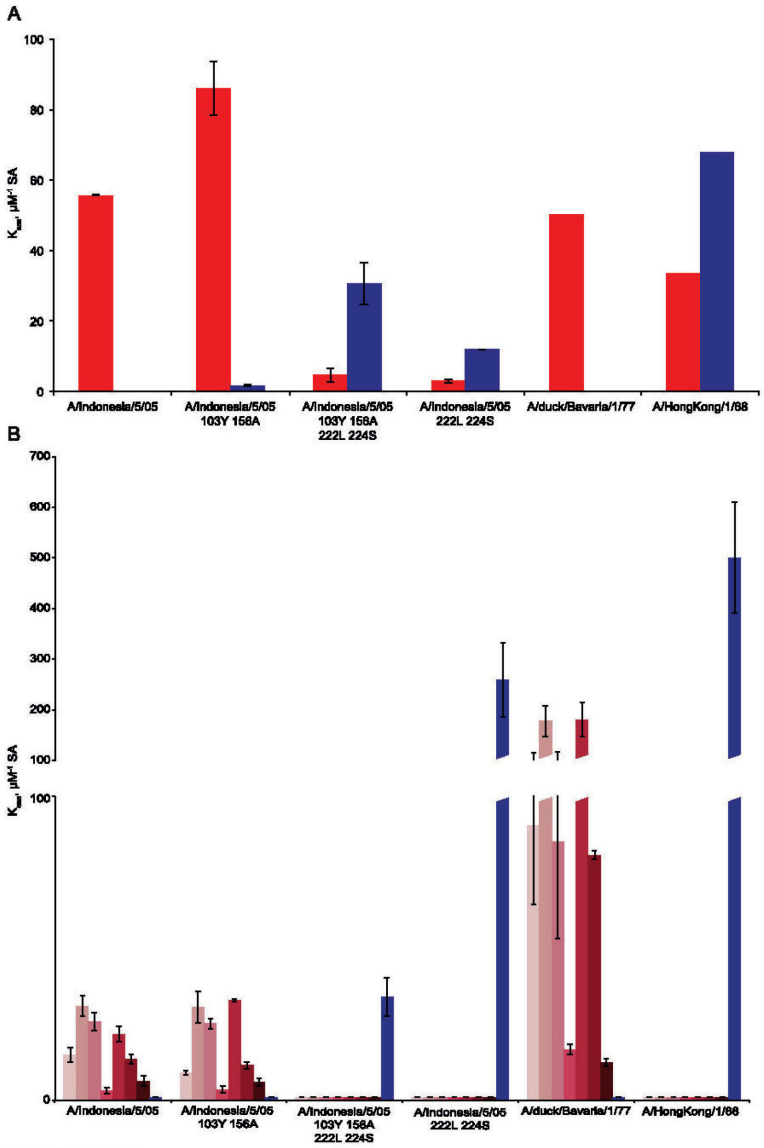
Primer Extension Assay

For quantification of viral transcript levels in virus-infected MDCK cells, primer extension assays were performed as described previously (177) with slight modifications. Cells were seeded in 6-well plates and inoculated with a multiplicity of infection (MOI) of 1 in infection media. At 3 h postinoculation, cells were collected in 1 ml of Trizol and RNA was purified according to the manufacturer's protocol (Invitrogen). 8.5 ml of RNA was incubated for 3 min at 95°C with 1.5 ml primer master mix containing 32P labeled specific primers for the PB1 segment (mRNA and cRNA: TCTGTTGACTGTGTCCATGG, vRNA: TCGAGTCTGGAAGGAT TAAG) and cellular ribosomal RNA (5S rRNA: TCCCAGGCGGTCTCCCATCC). The mixture was cooled on ice and transferred to 50°C. After 5 min, 10 ml of 2x SuperScript III transcription mix (Invitrogen) was added and incubated for 1 h. The reaction was stopped by addition of 10 ml of 90% formamide and heating to 95°C. Transcription products were separated on 6% polyacrylamide gels containing 7 M urea in trisborate-EDTA buffer and detected using autoradiography films.



Supplemental Figure 1:
Comparison of Airborne Transmission of A/Indonesia/5/05 Mutants in the Ferret Transmission Model, Related to Figure 1

Data for individual transmission experiments are shown in each panel, with virus shedding in inoculated and airborne recipient animals shown as lines and bars, respectively. Grey circles and bars represent shedding from the throat, white circles and bars represent shedding from the nose. The indicated virus numbers (V1 - V12) correspond to those used in Figure 1, an asterisk indicates that no samples for that day were collected. Plus signs denote the death of two animals on day 11 post exposure. These deaths appeared unrelated to the experiment since immunohistological analyses did not detect influenza virus positive cells in tissue samples collected from these animals. Red numbers on the top right of each graph represent the HA inhibiting antibody titers against A/Indonesia/5/05 HA H103Y, T156A, Q222L, G224S of sera collected from the airborne recipient animals at the end of the experiment.



Supplemental Figure 2:
Receptor-Binding Properties of Wildtype and Mutant H5N1 Viruses in Direct and Indirect Binding Assays, Related to Figure 3 (A) Binding of wildtype and mutant A/Indonesia/5/05, A/duck/Bavaria/1/1977 and Hong Kong/1/1968 to sialylglycopolymers containing α-2,3-SA (red bars) or α-2,6-SA (blue bars). This analysis is similar to that shown in Figure 3B using fetuin. Error bars represent the standard deviation from the mean values of two replicates.
(B) Fetuin binding-inhibition assay indicating binding patterns of the mutant and wildtype A/Indonesia/5/05 viruses, A/duck/Bavaria/1/1977 (H1N1) and HongKong/1/1968 (H3N2) to the specified receptor analogs. Red color in different shades represents oligosaccharides containing α-2,3-SA, blue bars indicate an α-2,6-SA containing oligosaccharide. Data represent mean values and standard deviations of K_{ass} determined in the same experiment using two to four different concentrations of the inhibitor. 3'SLN: Neu5Acα2,3Galβ1-4GlcNAcβ; Su-3'SLN Neu5Acα2,3Galβ1-4(6-O-HSO_3)GlcNAcβ; SLe^x: Neu5Acα2,3Galβ1-4(Fucα1-3)GlcNAcβ; SLe^a: Neu5Acα2,3Galβ1-3(Fucα1-4)GlcNAcβ; SLe^c: Neu5Acα2,3Galβ1-3GlcNAcb; Su-SLe^x: Neu5Acα2,3Galb1-4(Fuca1-3)(6-O-HSO3) GlcNAcβ; and 6'SLN: Neu5Acα2,6Galβ1-4GlcNAcβ.

Supplemental Table 1:
Comparison of Nucleotide Variations between the Full Virus Genomes of A/H5N1$_{wildtype}$ and A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$ in the Original Virus Stock and the Nasal Turbinates of Ferrets at Passage 4, 8, and 10[1], Related to the Results

	Gene	nt pos	nt ref	nt mut	aa pos	aa ref	aa mut	H5N1$_{WT}$ Freq[2]	H5N1$_{WT}$ Reads[3]	H5N1$_{MUT}$ Freq	H5N1$_{MUT}$ Reads	H5N1$_{WT}$ Freq	H5N1$_{WT}$ Reads	H5N1$_{MUT}$ Freq	H5N1$_{MUT}$ Reads	H5N1$_{WT}$ Freq	H5N1$_{WT}$ Reads	H5N1$_{MUT}$ Freq	H5N1$_{MUT}$ Reads	H5N1$_{WT}$ Freq	H5N1$_{WT}$ Reads	H5N1$_{MUT}$ Freq	H5N1$_{MUT}$ Reads
1	PB2	94	A	C	23	Thr	Pro													9,9	141		
2		491	G	A	155	Ser	Asn											1,8	664				
3		1298	C	T	423	Ala	Val							0,4	1100								
4		1906	G	A	627[5]	Glu	Lys			100	83			100	8			100	12			99	105
5		2008	G	A	661	Ala	Thr									37,5	8			100,0	142		
6	PB1	319	C	T	99	His	Tyr							18,9	169			76,6	466			57,3	131
7		694	C	A	224	Leu	Met							45,9	641			5,2	1114				
8		787	G	A	255	Val	Ile													6,2	162		
9		885	G	T	287	Arg	Ser							20,8	317			11,1	719			27	196
10		982	G	A	320	Ala	Thr											24,8	149			38,7	168
11		1031	T	C	336	Val	Ala	0,7	578			6,8	190			1,0	308						
12		1036	A	G	338	Ser	Gly									8,6	290						
13		1126	A	G	368	Ile	Val							5,8	52			67,6	225			50,5	156
14		1132	G	T	370	Ala	Ser									18,5	384						
15	PA	636	A	T	204	Lys	Asn											0,6	495			2	253
16		1105	A	G	361	Lys	Glu	10,9	46			6,5	31			19,4	31			40,7	27		
17		1111	A	C	363	Thr	Pro	4,6	130			7,7	52	12,5	40	4,5	89			47,8	46	38,2	34
18		1583	A	T	520	Tyr	Phe			6,9	204									10,9	247		
19		1864	A	G	614	Asn	Asp			0,4	1600							50,1	1180			23,8	957
20	HA	215	G	A	47	Val	Met													7,3	150		
21		333	C	T	86	Thr	Ile							1,7	287			10	180			5,5	109
22		383	C	T	103	His	Tyr											97,4	190			88,9	99
23		446	G	A	124	Asp	Asn					5,5	419										
24		538	C	A	154	Asn	Lys															9,5	1241
25		542	A	G	156	Thr	Ala	0,2	3068			27,4	2098	95,1	2432	90,3	2532	94,1	2264	99,8	1054	93	1595
26		558	A	G	161	Lys	Arg	0,2	1643			10,5	1248			0,2	1470						
27		660	C	T	205	Thr	Ile							1,1	1463								
28		704	G	A	210	Val	Ile					18,4	2063			21,8	2620						
29		707	C	A	211	Pro	Thr	0,1	2831											13,2	676	0,4	1241
30		725	T	C	217	Ser	Pro					1,8	2132			6,3	2669						
31		741	A	T	222	Gln	Leu			99,8	1188			99,8	2496			99,9	2393			100	1518
32		746	G	A	224	Gly	Arg			99,9	2082			99,8	2798			99,9	2427			99,8	1683
33		748	A	C	224	Gly	Arg			99,9	1217			99,8	2557			99,7	2465			100	1713
34		977	G	A	301	Glu	Lys															12,9	178
35		1062	A	G	329	Lys	Arg											7,6	66				
36		1570	A	T	498	Leu	Phe													40	25		
37		1611	G	A	512	Gly	Glu							24,1	569			8,2	600			10,4	163
38	NP	341	G	A	99	Arg	Lys											2,9	1741			20,5	987
39		1430	G	A	462	Gly	Glu	0,7	3424	0,6	2112					0,1	2682	0,3	2834	16,9	1246	12,9	1295
40	NA	63	A	G	15	Met	Val													12,6	873		
41		324	A	G	102	Ile	Val							None[4]				0,2	1951				
42		447	G	A	143	Val	Met			0,3	1547			2,4	541			3	2181			1,8	1482
43		453	G	A	145	Glu	Lys									0,9	776						
44		736	A	G	239	Glu	Gly			1	305									5,3	188		
45		981	A	G	321	Asn	Asp															23,5	319
46	M	374	C	T	117	Leu	Phe													6,9	867		
47	NS	219	G	A	65	Val	Met							40,3	1136			16	1110			40,2	359
48		672	A	G	216	Lys	Glu									0,5	1137						

1. All viruses were described in Herfst et al., 2012.
2. Freq: percentage of sequence reads with the detected mutation.
3. Reads: total number of sequence reads covering the nucleotide position.
4. None: no coverage at that nucleotide position.
5. Engineered substitutions are depicted in grey font.

Supplemental Table 2:
Nucleotide Variations in the Full Virus Genome of A/H5N1$_{\text{HA Q222L, G224S PB2 E627K}}$ Passage 10 Nasal Wash Virus, as Compared to Those of Transmissible Viruses F5 and F6, Related to the Results

								P10 NW		F5 NS		F6 TS	
	Gene	nt pos	nt ref	nt mut	aa pos	aa ref	aa mut	Freq	Reads	Freq	Reads	Freq	Reads
1	PB2	94	A	C	23	Thr	Pro	13,3	166				
2		1298	C	T	423	Ala	Val			none		42,9	7
3		1906	G	A	627	Glu	Lys	99,6	223	100	25	96,2	26
4		319	C	T	99	His	Tyr	44,9	136	100	323	73,3	206
5		885	G	T	287	Arg	Ser	34,6	188				
6		982	G	A	320	Ala	Thr	39,2	102			56	182
7		1126	A	G	368	Ile	Val	50,8	177	100	163	57,5	228
8	PA	636	A	T	204	Lys	Asn	6	235				
9		1111	A	C	363	Thr	Pro	37,1	35				
10		1583	A	T	520	Tyr	Phe	15,5	226				
11		1864	A	G	614	Asn	Asp	26,4	700	0,5	994	0,2	1692
12	HA	333	C	T	86	Thr	Ile	6,4	110				
13		383	C	T	103	His	Tyr	87,6	113	100	133	100	148
14		538	C	A	154	Asn	Lys	12,2	736				
15		542	A	G	156	Thr	Ala	89,9	971	99	2231	98,8	3588
16		707	C	A	211	Pro	Thr	7	631				
17		741	A	T	222	Gln	Leu	99,9	931	99,8	2532	99,8	3979
18		746	G	A	224	Gly	Arg	99,7	997	99,8	2567	100	4032
19		748	A	C	224	Gly	Arg	99,9	1032	99,8	2608	99,8	4084
20		977	G	A	301	Glu	Lys	16	169				
21		1062	A	G	329	Lys	Arg	9,9	101				
22		1570	A	T	498	Leu	Phe	56	25				
23		1611	G	A	512	Gly	Glu	10,8	305				
24	NP	341	G	A	99	Arg	Lys	18,1	1190	99,7	928	39,3	1745
25		1079	G	A	345	Ser	Asn			99,1	814		
26		1430	G	A	462	Gly	Glu	22,2	1083	0,3	1451	0,3	2826
27	NA	324	A	G	102	Ile	Val	0,2	1208			64	2146
28		447	G	A	143	Val	Met	2,5	1526			67,1	2233
29		736	A	G	239	Glu	Gly	9	201				
30		981	A	G	321	Asn	Asp	26,9	268				
31	NS	219	G	A	65	Val	Met	41,9	329			56,8	44
32		337	A	C	104	Gln	Pro	7,9	126				

See footnotes to table S1 for explanation. NW; nasal wash, NS; nasal swab, TS; throat swab.

Supplemental Table 3:
Meta-Analysis of Studies on Airborne Transmission of Influenza A Virus in Ferrets, Related to the Experimental Procedures

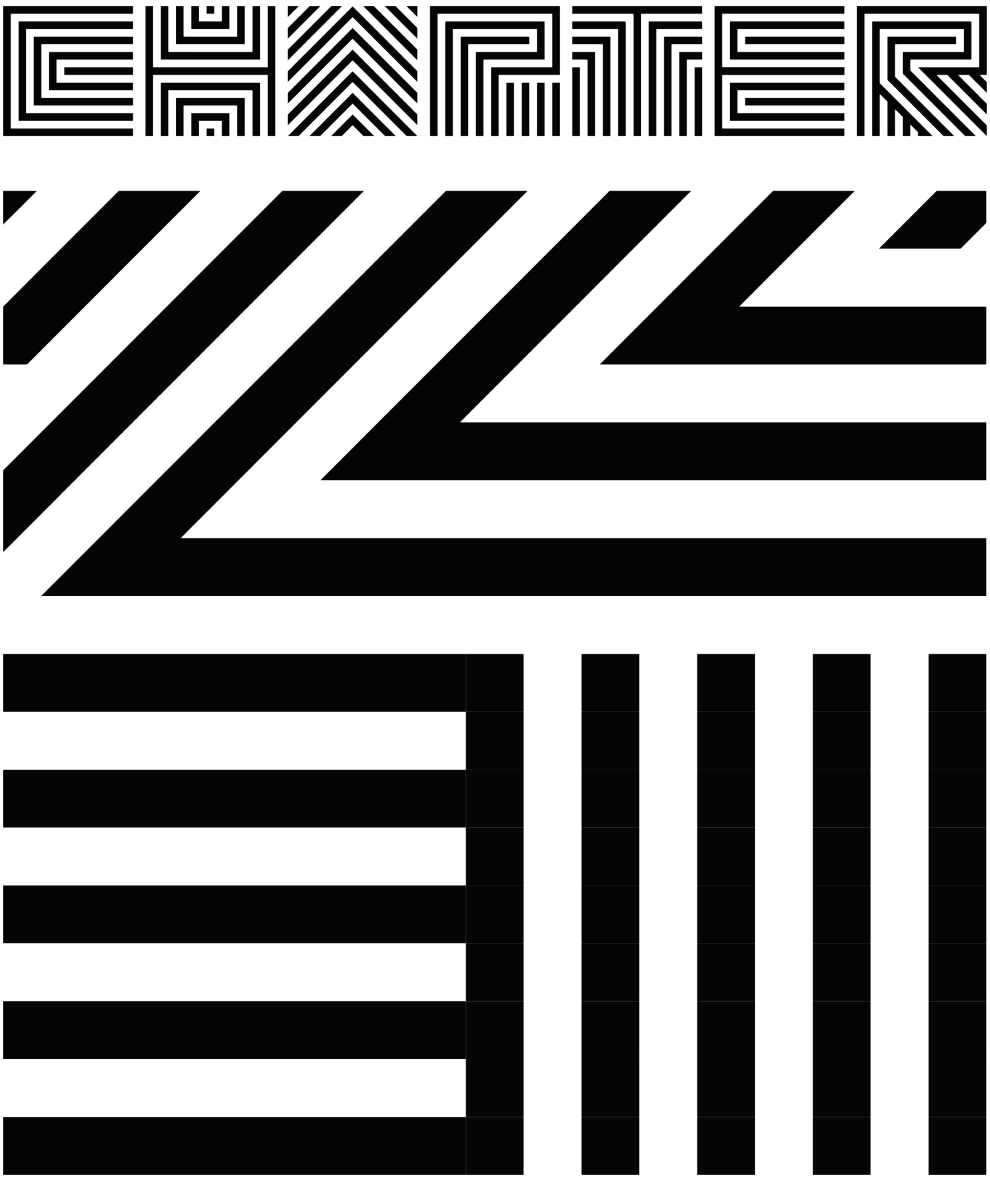
Subtype (origin)	Strain	Airborne transmission (N positive/N tested)	Reference
H5N1 (avian)	A/wild bird/HongKong/07035-1/2011	0/3	(Xu et al., 2013)
	A/Vietnam/1194/2004	0/3	(Xu et al., 2013)
	A/Vietnam/1203/04	0/3	(Xu et al., 2014)
	A/egret/Egypt/1162/2006	0/2	(Chen et al., 2012)
	A/Hong Kong/486/97	0/3	(Maines et al., 2011)
	A/Vietnam/1203/04	0/3	(Maines et al., 2011)
	A/Indonesia/5/05	0/2*	(Munster et al., 2009)
	A/Thailand/16/04	0/3	(Jackson et al., 2009)
	A/Hong Kong/486/97	0/3	(Maines et al., 2006)
	A/Hong Kong/213/03	0/3	(Maines et al., 2006)
H1N1 (avian)	A/shorebird/DE/300/2009	2/2	(Kocer et al., 2012)
	A/duck/Alberta/35/76	0/3	(Itoh et al., 2009)
	A/Duck/NewYork/96	0/3	(Van Hoeven et al., 2009)
	A/Turkey/South Dakota/7034/86	0/3	(Van Hoeven et al., 2009)
	A/Turkey/Kansas/4880/80	0/3	(Van Hoeven et al., 2009)
	A/Mallard/Alberta/119/98	0/3	(Van Hoeven et al., 2009)
	A/Duck/Alberta/35/76	0/3	(Van Hoeven et al., 2009)
	A/Duck/NY/15024–21/96	0/3	(Van Hoeven et al., 2009)
	A/duck/Alberta/35/76	0/3	(Tumpey et al., 2007)
H2N1 (avian)	A/Mallard/Potsdam/177-4/1983	0/2	(Jones et al., 2014)
H2N2 (avian)	A/Mallard/New York/6750/78	0/3	(Pappas et al., 2010)
	A/Mallard/MT/Y61	0/2	(Jones et al., 2014)
	A/Mallard/Potsdam/179/1983	0/2	(Jones et al., 2014)
	A/Duck/Hong Kong/319/1978	0/2	(Jones et al., 2014)
	A/Chicken/Jena/4705/1984	0/2	(Jones et al., 2014)
	A/Duck/NewJersey/7872-27/1995	0/2	(Jones et al., 2014)
	A/Duck/Hong Kong/273/1978	0/2	(Jones et al., 2014)
	A/GuineaFowl/NewYork/13824/1995	0/2	(Jones et al., 2014)
	A/Chicken/NewYork/13824/1995	0/2	(Jones et al., 2014)
H7N2 (avian)	A/Turkey/Virginia/4529/02	0/3	(Belser et al., 2008)
	A/NewYork/107/03	0/3	(Belser et al., 2008)
	A/chicken/Conneticut/260413-02/03	0/3	(Belser et al., 2008)
H7N3 (avian)	A/mallard/Alberta/24/01	0/3	(Song et al., 2009)
	A/Canada/504/04	0/3	(Belser et al., 2008)
H7N7 (avian)	A/Netherlands/219/03	0/3	(Belser et al., 2008)
	A/Netherlands/230/03	0/3	(Belser et al., 2008)
avian H7N9 (before 2013)	A/duck/Gunma/466/2011	0/3	(Watanabe et al., 2013)
2013 H7N9 (avian)	A/Anhui/1/2013	1/3	(Xu et al., 2014)
	A/Anhui/1/2013	3/4	(Richard et al., 2013)
	A/Anhui/1/2013 variant	1/4	(Richard et al., 2013)
	A/chicken/Shanghai/S1053/2013	0/3	(Zhang et al., 2013)
	A/pigeon/Shanghai/S1069/2013	1/3	(Zhang et al., 2013)
	A/Shanghai/1/2013	1/3	(Zhang et al., 2013)
	A/Shanghai/2/2013	1/3	(Zhang et al., 2013)
	A/Anhui/1/2013	6/6	(Zhang et al., 2013)
	A/Anhui/1/2013	2/6	(Belser et al., 2013a)
	A/Shanghai/1/2013	1/3	(Belser et al., 2013a)
	A/Anhui/1/2013	1/3	(Watanabe et al., 2013)
	A/Shanghai/2/2013	1/3	(Zhu et al., 2013)
H9N2 (avian)	A/Guinea fowl/Hong Kong/WFD10/99	0/2	(Wan et al., 2008)

* 0/8 transmission events when A/Indonesia/5/05 wt passaged and PB2-627K HA-222L, 224S is included

Subtype (origin)	Strain	Airborne transmission (N positive/N tested)	Reference
pH1N1 (human)	A/California/4/2009	3/3	(Watanabe et al., 2013)
	A/New York/1682/2009	2/3	(Zhou et al., 2013)
	A/California/4/2009	3/3	(Zhu et al., 2013)
	A/California/4/2009	2/2	(Martinez-Romero et al., 2013)
	A/England/195/09	3/3	(Roberts et al., 2012)
	A/Sichuan/1/2009	2/2	(Zhang et al., 2012)
	A/California/04/2009	3/3	(Imai et al., 2012)
	A/California/07/2009	7/9	(Lakdawala et al., 2011)
	A/England/195/2009	3/3	(van Doremalen et al., 2011)
	A/Netherlands/602/2009	4/4	(van der Vries et al., 2011)
	A/California/4/2009	2/3	(Belser et al., 2011)
	A/California/4/2009	3/3	(Yen et al., 2011b)
	A/HK/415742/09	3/3	(Yen et al., 2011b)
	A/Quebec/147023/2009	4/4	(Hamelin et al., 2011)
	A/California/4/09	3/3	(Yen et al., 2011a)
	A/California/4/09	2/3	(Jayaraman et al., 2011)
	A/Netherlands/602/09	4/4	(Schrauwen et al., 2011)
	A/Osaka/164/2009	3/3	(Kiso et al., 2010)
	A/Vietnam/HCM9727/2009	3/3	(Kiso et al., 2010)
	A/Netherlands/602/09	4/4	(Chutinimitkul et al., 2010a)
	A/California/04/2009	1/1	(Seibert et al., 2010)
	A/Hansa Hamburg/01/2009	1/1	(Seibert et al., 2010)
	A/Denmark/524/09	1/2	(Duan et al., 2010)
	A/Netherlands/602/09	4/4	(Herfst et al., 2010)
	A/California/04/09	3/3	(Itoh et al., 2009)
	A/Kawasaki/UTK-4/09	1/2	(Itoh et al., 2009)
	A/Netherlands/602/2009	4/4	(Munster et al., 2009)
	A/California/04/2009	3/3	(Maines et al., 2009)
	A/Texas/15/2009	3/3	(Maines et al., 2009)
	A/Mexico/4482/2009	3/3	(Maines et al., 2009)
sH1N1 (human)	A/Brisbane/59/07	1/3	(Belser et al., 2012)
	A/Netherlands/26/2007	3/4	(Munster et al., 2009)
	A/Brisbane/59/2007	3/3	(Maines et al., 2009)
	A/Solomon Islands/3/06	3/3	(Van Hoeven et al., 2009)
	A/Texas/36/91	3/3	(Tumpey et al., 2007)
sH3N2 (human)	A/Perth/16/2009	2/3	(Houser et al., 2013)
	A/Indiana/08/2011	3/3	(Houser et al., 2013)
	A/Texas/50/2012	3/3	(Belser et al., 2013a)
	A/Panama/2007/99	1/3	(Belser et al., 2012)
	A/Wuhan/359/95	2/3	(Yen et al., 2011b)
	A/Wuhan/359/95	2/3	(Yen et al., 2011a)
	A/Victoria/03/75	2/2	(Itoh et al., 2009)
	A/Wyoming/3/03	2/2	(Jackson et al., 2009)
	A/Memphis/14/98	2/2	(Wan et al., 2008)
	A/Panama/2007/99	3/3	(Maines et al., 2006)
	A/Victoria/3/75	3/3	(Maines et al., 2006)
'Asian' H2N2 (human)	A/Albany/6/58	3/3	(Pappas et al., 2010)
	A/El Salvador/2/57	1/3	(Pappas et al., 2010)
'Spanish' H1N1 (human)	A/South Carolina/1/18	3/3	(Tumpey et al., 2007)

Supplemental Table 4:
Primers Used to Amplify the Complete A/H5N1 Virus Genome for Deep Sequencing, Related to Figure 2 and Tables S1 and S2

Segment	Fragment no.	Sense	Sequence
PB2	1	Forward	CRAAAGCAGGTCAAWTATATTC
		Reverse	TCCATGATRACATCTTGTGCTTC
	2	Forward	AAGGTTGAAAGRTTAARACA
		Reverse	RTTTTGYCTAAGRATGTCYACC
	3	Forward	ATTGTNAGGAGRGCAACRG
		Reverse	CCATGACATTRTCRATDGGTTC
	4	Forward	AACAGAGCRAACCAAMG
		Reverse	GTTCTCACAAAYCCACTRTATTG
	5	Forward	CARTGGTCYCARGAYCC
		Reverse	AGTAGAAACAAGGTCGTTT
PB1	1	Forward	RAAAGCAGGCAAACCAYTTGAATG
		Reverse	TTCTTCCTTATCCATTGATTCC
	2	Forward	CYGCTTTGGCCAAYACYATAG
		Reverse	CKAGGRTTYTGATTCTCATTCC
	3	Forward	GCTAAAYTGGCAAAYGTHGTRAG
		Reverse	TTCTTYTTRCTCATRTTGATYCC
	4	Forward	CAATCYTCYGATGAYTTYGC
		Reverse	CTDCCYTGRTARTCTTCATCC
	5	Forward	AGGACTGTTGRKTTCDGATGG
		Reverse	TTTTTTCAYGAAGGACAAGC
PA	1	Forward	CRAAAGCAGGTACTGATYC
		Reverse	GTGWAYAGYCTGGTTTTRATTC
	2	Forward	CAYATYCACATHTTCTCATTCAC
		Reverse	TYAATTTHAGRGCATCCATCAG
	3	Forward	TTCYCAAATGTCVAAAGARG
		Reverse	RCTYGCDATGTGCTCAATTGG
	4	Forward	CARGAYATTGAAAATGAGG
		Reverse	TTYTCCCAYTTGTGTGGYTCC
	5	Forward	GATGTGGTAAACTTTGTGAGTATGG
		Reverse	ATCGAARGTHCCAGGTTCCAG
	6	Forward	CATGATYGARGCHGARTCTTC
		Reverse	AGTAGAAACAAGGTACYTTTT
HA	1	Forward	AAAGCAGGGGTHYDATCTGTC
		Reverse	TTGTARCTYCTCTTTATBGTBGG
	2	Forward	GRGTRAGCKCAGCATGTCC
		Reverse	GDGTTTGRCACTTGGTGTTGC
	3	Forward	AGTAATGGRAATTTCATTGCYCC
		Reverse	ATTYTCCATKAGAACYAGRAGTTC
	4	Forward	ACTCARTTTGARGCHGTTGG
		Reverse	AGTAGAAACAAGGGTGTTTT
NP	1	Forward	AGCRAAAGCAGGGTDKATA
		Reverse	GCATCATTYAGRTTKGAATGCC
	2	Forward	GAATGGTNCTCTCTGCVTTTG
		Reverse	TGAGTGCAGACCGHGCCAG
	3	Forward	RAAATTYCAAACAGCAGCAC
		Reverse	CTKATYTGYCCTGCVGATGC
	4	Forward	GTTCAAATTGCTTCAAATG
		Reverse	AGTAGAAACAAGGGTATTTT
NA	1	Forward	CRAAAGCAGGAGTTYAAAATG
		Reverse	GCAACAGACTCAAAYCTYGAG
	2	Forward	AGCCYTRYTGAATGACAARC
		Reverse	YTGATTGAAAGAYACCCATGG
	3	Forward	TTACTGTWATGACWGAYGGACC
		Reverse	ACCCARAARCAAGGTCTYATGC
	4	Reverse	AATAGGRTAYATATGCAGYGG
		Forward	AGTAGAAACAAGGAGTTTTT
MA	1	Reverse	AAAGCAGKTAGATRTTGAAARATG
		Forward	ACCATTCTGTTYTCATGYCTG
	2	Reverse	TAKTRTGTGCCACTTGTGAGC
		Reverse	AGTAGAAACAAGGTARKTTTT
NS	1	Forward	CRAAAGCAGGGTGACAAAVAC
		Reverse	CCAATTGCAWTYTTGACATCCTC
	2	Forward	DCTKAGRGCVTTYACAGA
		Reverse	AGTAGAAMCAAGGGTGTTTT



Adaptation of Pandemic H2N2 viruses in Humans

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ABSTRACT

The 1957 H2N2 influenza virus caused an estimated 2 million fatalities during the pandemic. Since viruses of the H2 subtype continue to infect avian species and pigs, the threat of reintroduction into humans remains. To determine factors involved in the zoonotic origin of the 1957 pandemic we performed analyses on genetic sequences of 167 newly sequenced human and avian H2N2 virus isolates and all publically available influenza virus genomes.

TEXT

Influenza A viruses are ecologically successful pathogens that infect a wide range of host species and that have periodically emerged in humans to cause pandemics (75). The “Asian pandemic” of 1957 was caused by a H2N2 influenza A virus generated by reassortment of the previously circulating human H1N1 virus and an avian H2N2 virus that contributed the polymerase basic 1 (PB1), haemagglutinin (HA), and neuraminidase (NA) genes to the pandemic strain (60). Here we investigate genetic markers that are linked to the generation of human H2N2 viruses, by studying the genetic variation of H2N2 influenza A viruses in human hosts from 1957-1968 based on newly generated sequences from this study and all available sequences in public databases. The combined knowledge of which virus subtypes can potentially become pandemic and the characterization of changes essential for adaptation of individual viruses to humans will improve pandemic preparedness by allowing surveillance activities to identify specific genetic and phenotypic changes and allowing vaccine preparation for the most urgent threats.

The complete genomes of the archived H2N2 influenza viruses were sequenced using a high-throughput next-generation sequencing pipeline on a 454/Roche GS-FLX and Illumina HiSeqTM 2000 platform, as described previously (178-180), and consensus sequences were deposited in GenBank (Table S1). Nucleotide sequences of each gene segment were initially aligned using MAFFT (181) then manually corrected and assembled to only include coding regions. To determine the closest related avian and human clade, Maximum likelihood trees were inferred based on nucleotide alignments using the program FastTree version 2.1.5 (182) under the generalized time reversible (GTR) model with gamma-distributed rates among sites (GTR+Γ) (Supp. Figure S1). The ML trees were used to select the avian viruses most closely related to the human H2N2 viruses for further analysis. Temporal phylogenies were then inferred using Bayesian Markov chain Monte Carlo (MCMC) methods in BEASTv1.8.0. Nucleotide substitution rates and time to most recent common ancestor (TMRCA) for H2 viruses were estimated (Figure 1 A-B, Supp. Figure S2) under a relaxed clock model as described previously (183, 184).

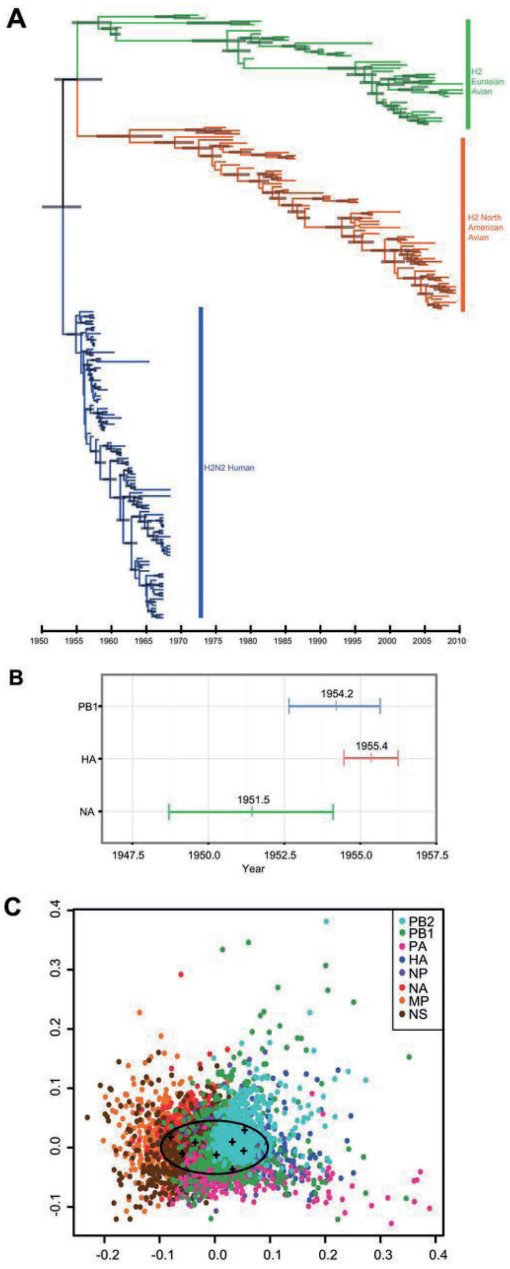

Figure 1:
Evolution and reassortment of H2 viruses. Maximum-clade credibility temporal trees of H2N2 influenza A virus segments (A) HA and (B) NA of avian (brown) and human (blue) virus isolates. Grey bars represent the 95% highest posterior density (HPD) of age for each node. (C) Multidimensional scaling (MDS) plot of uncertainty of TMRCAs between samples of 500 trees for each segment of pandemic H2N2 viruses (n=54) sampled between 1957 - 1968. In this analysis the tree-to-tree variation in posterior distribution of 500 trees for each segment is plotted as a cloud of points where the mean is represented by the centroid of the cloud, while the spread of points indicate the degree of statistical uncertainty in the phylogenetic history of each gene segment. The space occupied by human H3N2 viruses is indicated by the oval (190).

Table 1: Nucleotide substitution rates and times to most recent common ancestor (TMRCAs) of H2N2 segments analyzed

			Nucleotide substitution rate (subs/site/yr)			TMRCA (yr)		
				95% HPD			95% HPD	
Origin and gene		**Lineage**	**Mean**	**Lower**	**Upper**	**Mean**	**Lower**	**Upper**
Avian								
	HA	Human H2N2	3.59E−03	3.00E−03	4.22E−03	1955.39	1956.25	1954.47
		Avian H2N2	3.50E−03	3.00E−03	4.06E−03	1949.03	1956.77	1940.95
	NA	Human H2N2	0.91E−03	0.68E−03	1.15E−03	1951.47	1954.11	1948.73
		Avian H2N2	2.57E−03	2.24E−03	2.89E−03	1954.89	1961.33	1948.16
	PB1	Human H2N2	1.13E−03	0.89E−03	1.38E−03	1954.24	1955.66	1952.66
		Avian H2N2	1.76E−03	1.55E−03	1.98E−03	1973.03	1975.40	1970.58
Human								
	PB2	H1N1, H2N2	1.46E−03	1.25E−03	1.67E−03	1923.83	1928.70	1918.34
	PA	H1N1, H2N2	1.30E−03	1.09E−03	1.51E−03	1923.01	1928.30	1917.38
	NP	H1N1, H2N2	1.46E−03	1.20E−03	1.73E−03	1926.06	1929.83	1922.18
	MP	H1N1, H2N2	1.08E−03	8.69E−04	1.29E−03	1923.58	1928.46	1918.52
	NS	H1N1, H2N2	1.73E−03	1.27E−03	2.19E−03	1925.06	1929.20	1920.55

Table 2: Selection pressures of H2N2 segments compared to their respective avian or H1N1 precursors

			Global d_N/d_S						
				95% CI*d*		Branch model d_N/d_S			
Origin	**and gene**	**Lineage**	**Mean**	**Lower**	**Upper**	**External**	**Internal**	**Internal/external ratio**	**Sites under positive selection***a*
Avian									
	HA	Human H2N2	0.448	0.402	0.497	0.287	0.272	0.948	
		Avian H2N2	0.120	0.110	0.129	0.114	0.062	0.538	**5** (S72R/I; N181I; N192D/E/K; E211K/D; K217E*b*)
	NA	Human H2N2	0.347	0.295	0.405	0.324	0.330	1.020	
		Avian H2N2	0.170	0.158	0.182	0.473	0.210	0.444	
	PB1	Human H2N2	0.130	0.106	0.158	0.068	0.054	0.794	
		Avian H2N2	0.031	0.028	0.035	0.111	0.080	0.723	
Human									
	PB2	H2N2	0.116	0.094	0.142	0.067	0.045	0.671	
		H1N1	0.072	0.061	0.083	0.079	0.052	0.655	
	PA	H2N2	0.147	0.119	0.179	0.112	0.098	0.871	
		H1N1	0.085	0.074	0.097	0.076	0.066	0.880	
	NP	H2N2	0.221	0.181	0.267	0.133	0.123	0.924	
		H1N1	0.165	0.132	0.204	0.166	0.104	0.627	
	M1	H2N2	0.224	0.167	0.291	0.161	0.083	0.513	
		H1N1	0.128	0.105	0.154	0.157	0.073	0.467	
	M2*c*	H2N2	0.309	0.187	0.475	0.163	0.115	0.708	
		H1N1	0.405	0.322	0.502	0.534	0.435	0.814	
	NS1	H2N2	0.500	0.398	0.618	0.406	0.321	0.790	
		H1N1	0.287	0.251	0.326	0.281	0.239	0.852	
	NS2*c*	H2N2	0.300	0.200	0.428	0.371	0.569	1.534	
		H1N1	0.215	0.170	0.268	0.182	0.192	1.053	

a Only sites that were significant in both the MEME method, with a P cutoff of 0.05, and the Tdg09 method, with a false discovery rate cutoff of 0.05, are reported.
b Letters refer to single-letter amino acid abbreviations.
c The MP and NS comprise two open reading frames with partial overlap, giving rise to two gene products of significant length. Selection pressures reflected in the overlapping par in either gene segment cannot be attributed to one of the two open reading frames, and the reported dN/dS values should be interpreted with caution.
d CI, confidence interval.

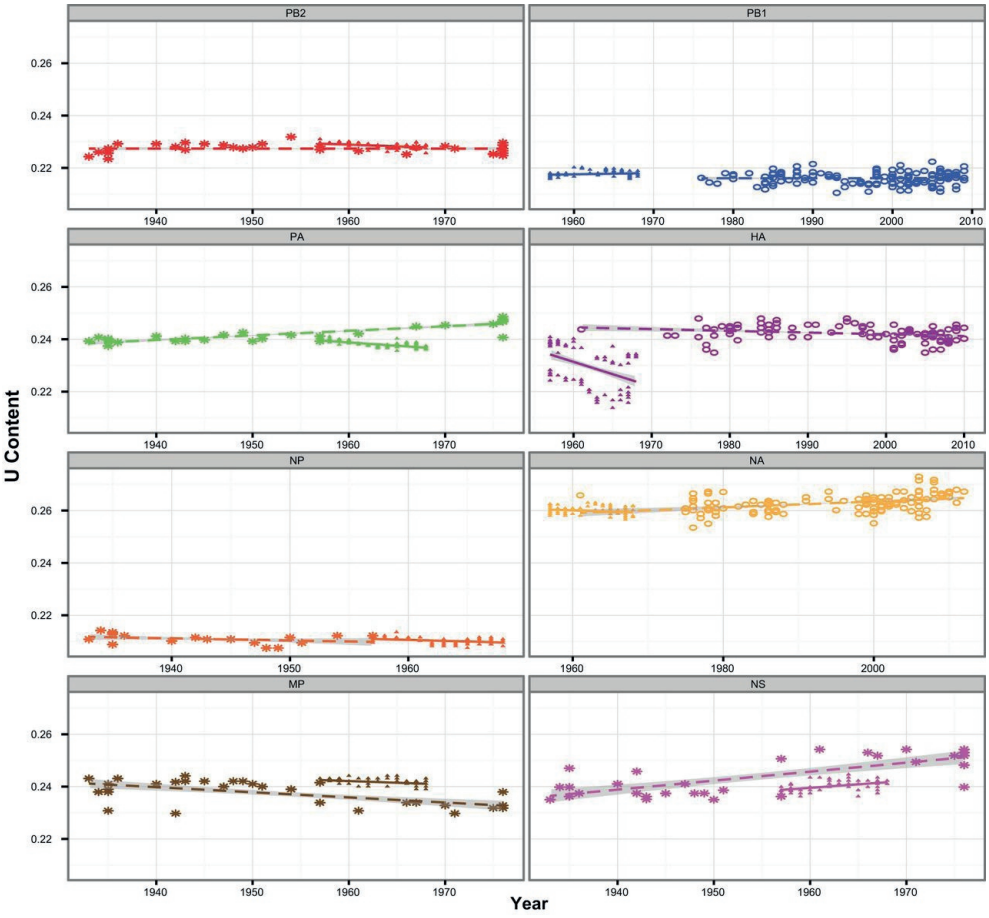

Figure 2:
Uracil content patterns. Uracil content in H2N2 human (solid triangles) gene segments in comparison to the respective H2 avian (open circles) or H1 mammalian (asterisks) isolates from which each segment is derived, depicted per year. Best-fit regression lines are shown with the 95% confidence interval shaded gray.

The mean nucleotide substitution rate of human H2N2 HA genes from 1957-1968 was estimated at 3.63×10^{-3} substitutions/site/year (subs/site/yr) (Table 1), comparable to the rate within the avian cluster (3.76×10^{-3} subs/site/yr) and which is consistent with previous reports (185, 186). However, the substitution rates of human H2N2 PB1 and NA genes (0.90×10^{-3} and 0.85×10^{-3} subs/site/yr, respectively) were lower than previous estimates and those of avian PB1 and NA (1.76×10^{-3} and 2.83×10^{-3} subs/site/yr, respectively). The mean TMRCAs of the novel origin genes provide evidence suggesting that the PB1 and NA genes (late 1952 and early 1951, respectively) circulated in mammals before the HA was acquired (mid-1955) indicating a series of reassortment events in the years before pandemic emergence, consistent with previous analyses (184). This hypothesis is further supported by the significantly lower substitution rates observed in the human H2N2 PB1 and NA segments compared to the HA (Bayes factors >1000, calculated as previously described (187)) suggesting prior mammalian adaptation. The lack of avian viruses isolated before 1957 precludes further validation of this hypothesis (184, 188).

To determine the degree of reassortment present in human H2N2 viruses, we used a multidimensional scaling (MDS) plot of uncertainty of TMRCAs between samples of 500 trees for each gene segment, as previously described (189, 190). In this analysis we assume that gene segments with similar evolutionary histories will occupy similar positions in the MDS plot. Our analysis showed considerable overlap in the phylogenetic histories of all H2N2 gene segments during the 11-year period they circulated in humans, indicating a relatively homogenous virus population with little evidence for ongoing reassortment in the human population (Figure 1C).

We investigated natural selection on the virus populations by estimating the global ratio of nonsynonymous (dN) and synonymous (dS) substitutions per codon (dN/dS ratio) for each gene using single-likelihood ancestor counting (SLAC) as implemented in Datamonkey (191, 192). Results indicate that H2N2 pandemic virus genes were generally under higher selection pressures than those observed in avian hosts (Table 2). In particular, the H2N2 PB1, HA and NA genes had a higher dN/dS ratio in the human clade compared to the avian group, while the human H1N1-derived genes show increased dN/dS compared to the ancestral H1N1 viruses circulating in mammals. These results likely reflect increased adaptive pressure on the newly generated H2N2 strain to both the new genome constellation and the new host.

A comparison of branch-wise dN/dS ratios of internal branches (ancestral nodes) and external branches (tips of the tree) was made using the 2-ratio model in CODEML (193). Pandemic H2N2 PB1, HA and NA genes have a higher internal/external ratio compared to the avian background, indicating accumulation of amino acid mutations in these genes, probably as a consequence of adaptation to the new host (Table 2). The lower external

dN/dS ratios suggest higher selective constraints resulting in deleterious nonsynonymous changes that were ultimately not fixed in the population (194), and may explain the lower evolutionary rates observed for this lineage. The internal versus external dN/dS ratios of the remaining gene segments that previously circulated in humans are comparable between the H2N2 and H1N1 backgrounds, indicating the absence of particular adaptive pressures after reassortment.

Previous studies on antibody profiling and comparative alignments of avian and human H2N2 virus isolates have reported amino acid differences between the two virus populations and suggested that they may be adaptive (60, 119, 186, 195, 196). Here, we analyze the significance of these changes by comparing selection pressures between human and avian viruses. Site-specific selection on amino acids was analyzed using two different methods and was considered relevant if both methods detected the same codons. The mixed effects model of evolution (MEME) method detects both fixed and episodic diversifying selection between branches (197), while the Tdg09 program (198) relies on the designation of host-specific lineages to determine amino acid mutations that might have been important for the host-shift event. Our analyses detected five positively selected sites in the HA gene only (Table 2). Of these, two residues – Asn-192-Asp/Glu/Lys and Gly-223-Ser – were in the receptor-binding site (RBS) of the HA protein (Supp Figure S3A). The remaining three sites – Ser-72-Arg/Ile, Asn-181-Ilu and Glu-211-Lys/Asp – are in antigenic regions of the HA protein (185, 186, 196). The Gly-223-Ser mutation (position 228 in H3 numbering) is known to be significant in the adaptation of the H2N2 viruses in humans (70, 199), and results in increased binding affinity to α-2,6 linked sialic acid host receptors in H3N2 viruses (70, 199, 200). It is also worth noting that, although not identified as being under significant positive selection in our analysis, most of the avian precursor and human H2N2 virus HA genes had a Glu-221-Lys mutation (position 226 in H3 numbering) that is also associated with a mammalian-like receptor-binding switch (70, 199).

Studies on the adaptation of influenza A H1 and H3 viruses describe a decrease in the observed to expected CpG content as an evolutionary mechanism to evade immune responses through viral mimicry of the host genome (201). Similarly, an increase in the uracil content of avian derived influenza A viruses following establishment and circulation in mammalian hosts is thought to reflect mammalian adaptation (202). However, our results show either no change, or a slight decrease, in uracil content of the avian derived PB1, HA and NA genes (Fig 2). Furthermore, analysis of CpG content showed no consistent pattern with the PB1, HA and NA gene respectively showing no change, a decrease and an increase in CpG content (Supp Figure S3A).

This analysis provides the first large-scale comparative genomic analysis of the adaptation of pandemic H2N2 viruses to humans. We found that pandemic H2N2 viruses have higher selection pressures across the genome in comparison to their source populations, reflecting relaxed selective constraints on the viruses during adaptation to humans. We identified five amino acid residues under significant positive selection in the RBS and antigenic regions of the HA protein involved in efficient binding to human-like receptors and immune escape (60, 119, 196). Further means of viral adaptation to humans such as decreased CpG and uracil content were inconclusive, possibly due to the short circulation of H2N2 viruses in humans (11 years), whereas previous studies looked at H1 and H3 viruses over a period of 20 - 70 years (201, 202). Finally, our analysis on the temporal phylogenies of the avian-origin genes supports, as previously reported, the staggered introduction of each novel segment over 2 - 6 years prior to the emergence of the 1957 pandemic (184).

Jones et al. (170) provide a comprehensive risk assessment for the potential reintroduction in humans of H2N2 viruses currently circulating in wild birds and found that while the risk was low, these viruses exhibited pathogenicity, replicative competency and direct-contact transmission in experimental mammalian systems. Moreover, a lack of sustained systematic global influenza surveillance in birds and other animals severely limits the power of any risk assessment. Consequently, as periodic bottlenecks can greatly influence the genetic characteristics of circulating avian influenza viruses (202), the deduced characteristics of the current avian population (i.e. after 1980) may not be the same as the virus population that provided genes to the H2N2 pandemic strain. Although the exact mechanism of adaptation from the zoonotic ancestor remains unknown, historical evidence of successful H2N2 infection and transmission in humans, combined with an immunologically naïve population for H2N2 below approximately 50 years of age, highlights the need for continued monitoring of this subtype for pandemic preparedness planning.

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FULL METHODS SUMMARY

Virus Sequencing

The complete genomes of the influenza viruses were sequenced using a high-throughput Next-generation sequencing pipeline at the JCVI that includes the 454/Roche GS-FLX and the Illumina HiSeqTM 2000. Viral RNA was isolated using the ZR 96 Viral RNA kit (Zymo Research Corporation, Irvine, CA, USA). The influenza A genomic RNA segments were simultaneously amplified from 3 µL of purified RNA using a multi-segment RT-PCR strategy (180, 203). The influenza M-RTPCR amplicons were barcoded and amplified using an optimized SISPA protocol (178, 179, 204). Subsequently, the SISPA amplicons were purified, pooled and size selected (~800 bp or ~200 bp) and the pools were used for both Roche 454 (Roche Diagnostics Corporation, Indianapolis, IN) and Illumina (Illumina, Inc., San Diego, CA, USA) library construction (~50) viruses per library. Samples were sequenced on the 454/Roche-FLX and Illumina HISeq2000 platforms. Libraries were prepared for sequencing on the 454/Roche GS-FLX platform using Titanium chemistry or for sequencing on the Illumina HiSeqTM 2000. The sequence reads were sorted by barcode, trimmed, searched by TBLASTX against custom nucleotide databases of full-length influenza A segments downloaded from GenBank to filter out both chimeric influenza sequences and non-influenza sequences amplified during the random hexamer-primed amplification. The reads binned by segment, and Illumina reads were de novo assembled using CLC Bio's clc_novo_assemble program. The resulting contigs were searched against the corresponding custom full-length influenza segment nucleotide database to find the closest reference sequence for each segment. Both 454/Roche GS-FLX and Illumina HiSeqTM 2000 reads were then mapped to the selected reference influenza A virus segments using the clc_ref_assemble_long program. At loci where both GS-FLX and Illumina sequence data agreed on a variation (as compared to the reference sequence), the reference sequence was updated to reflect the difference. A final mapping of all next-generation sequences to the updated reference sequences was then performed. Any regions of the viral genomes that were poorly covered or ambiguous after Next Generation sequencing were amplified and sequenced using standard Sanger sequencing approach. The consensus sequences were deposited in GenBank (Table S1).

Sequence Selection for Phylogenetic Analysis. We downloaded complete influenza A gene segments of different subtypes and of different host origin from NCBI Influenza database on 28 November 2013. 11285 sequences were downloaded, supplemented by full genomes of 175 isolates newly sequenced for this project, adding to a total of 12685 sequences used. Nucleotide sequences of the coding regions of each of the segments were aligned using MAFFT (181) and were further manually adjusted and assembled to only include coding regions of each gene segment.

Phylogenetic Analysis

Maximum likelihood trees for the large dataset were inferred based on nucleotide alignments using the program FastTree 2.1.5 under the generalized time reversible (GTR) model with gamma-distributed rates among sites (GTR+Γ) (205). To conduct a temporal phylogenetic analysis, sequences were also downloaded with associated metadata of date of isolation. If the exact day and month of the year was not available, sequences were given a mid-year root. However, since we were interested in the adaptation involved in the host species jump, greater temporal resolution was required for the first year of pandemic transmission. Hence, dates of isolation were extrapolated (to month of transmission) from available epidemiological and scientific reports of the period (205-207). Temporal phylogenies and rates of evolution were inferred with the program BEAST v1.8.0 using a relaxed clock model that allows rates to vary among lineages within a Bayesian Markov chain Monte Carlo (MCMC) framework (183). This method was used to sample phylogenies and dates of divergences between viruses from their joint posterior distribution, in which the sequences are constrained by their known date of sampling. For the analyses using Bayesian MCMC sampling, in all cases chain lengths of at least 100 million steps were used and a 10-20% 'burn-in' was removed. At least two independent runs of each were performed and compared or combined to ensure adequate sampling.

Reassortment Analysis. Reassortment within the H2N2 pandemic clade (n=49) was evaluated by comparing the patristic distances of trees estimated independently for each gene segment. A distance matrix was calculated from the posterior distributions of a sample of 500 trees obtained from a MCMC analysis with a GTR+G substitution model as described above. The resulting distance matrix was visualized with a multi-dimensional scaling plot to characterize the phylogenetic relationships between the gene segments using the R statistical software package (208).

Selection Analysis

To determine the degree of natural selection, the ratio of nonsynonymous (dN) and synonymous (dS) substitutions per codon (dN/dS ratio) were estimated for the entire tree (global model) using single-likelihood ancestor counting (SLAC) method (192), implemented through the Datamonkey webserver (191). The selection pressures acting on the internal and external branches of the tree were measured using the 2-ratio model of the CODEML program in the PAML suite (193). Selection on specific sites was analyzed using two methodologies and sites were considered relevant if both methods detected the same positively selected codons. The first method used was the mixed effects model of evolution (MEME), which is a useful tool in the detection of both fixed and episodic diversifying selection between branches (197). A

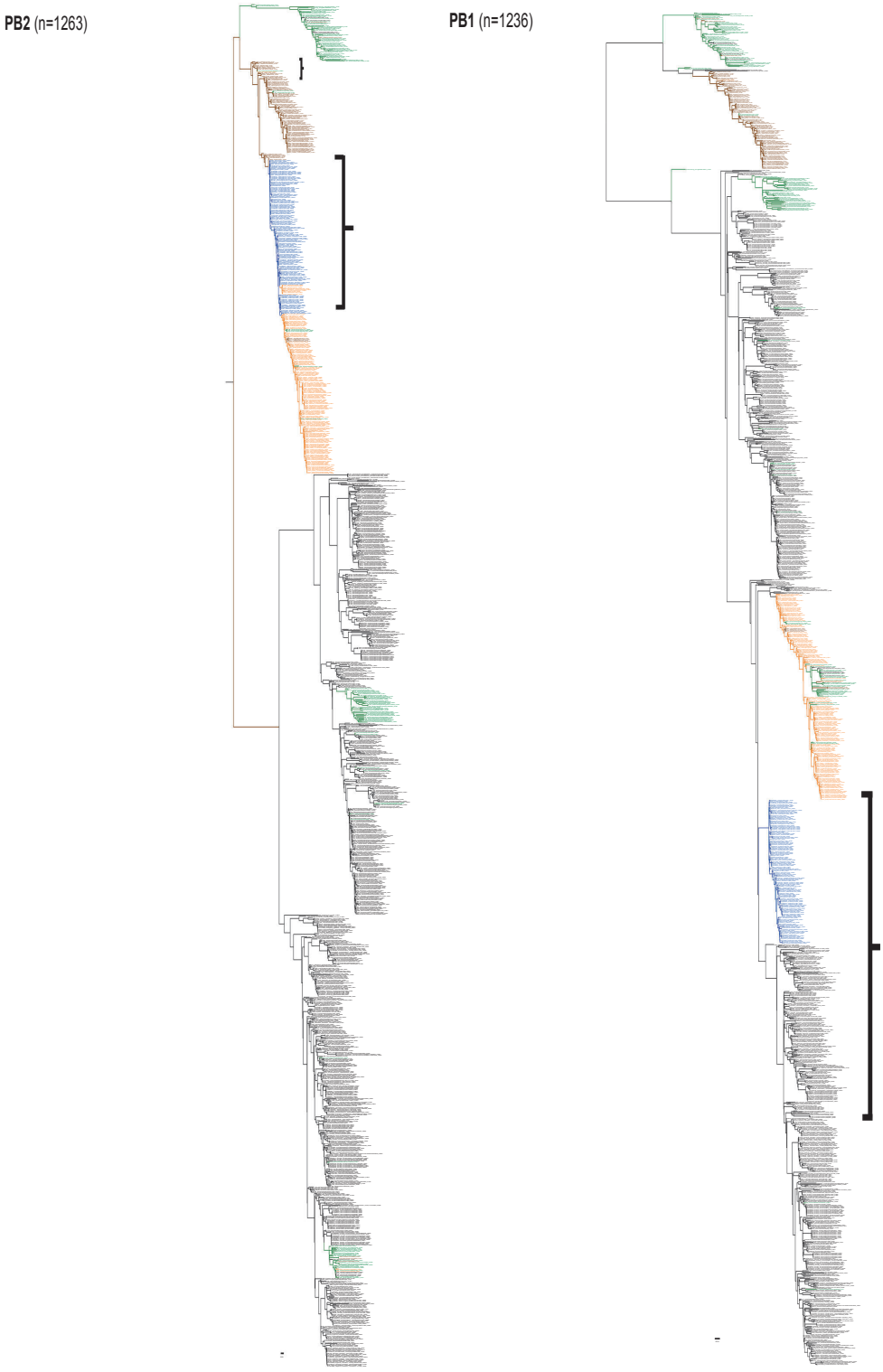
p-value of 0.05 was used as a statistical cutoff for a positively selected site in this method. The second method used to detect specific sites undergoing selective pressure was employed through the Tdg09 program (198), which allows for the definition of host-specific linages to determine sites that contribute to the host-shift event. Sites with a false discovery rate (fdr) less than 0.05 was used reported as significant in this method.

Uracil and CpG Content

The nucleotide content of pandemic H2N2 gene segments were assessed to determine adaptation levels of adaptation in a human/mammalian hosts. To estimate the observed to expected CpG ratios, a custom perl script was executed using the following formula: 100*('CG'/(G*C)*(A+U+G+C)), where A, U, G, C represents the counts of the respective nucleotides and 'CG' the number of adjacent C and G representing the CpG pattern (209). Uracil content values were measured using PAUP* 4.0b10 (194), as it has been postulated that the uracil content of the Influenza A virus genomes tends to increase steadily in mammalian hosts (202, 210). Uracil content values and CpG ratios were visualized and plotted using the R statistical software package (208).

Positions of Positively Selected Sites in HA Structure

The positions identified as significant positively selected sites were plotted on the crystal structure of the A/Singapore/1/1957(H2N2) virus HA [PDB accession code 2WR7] using MacPyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) for visualization of the trimer.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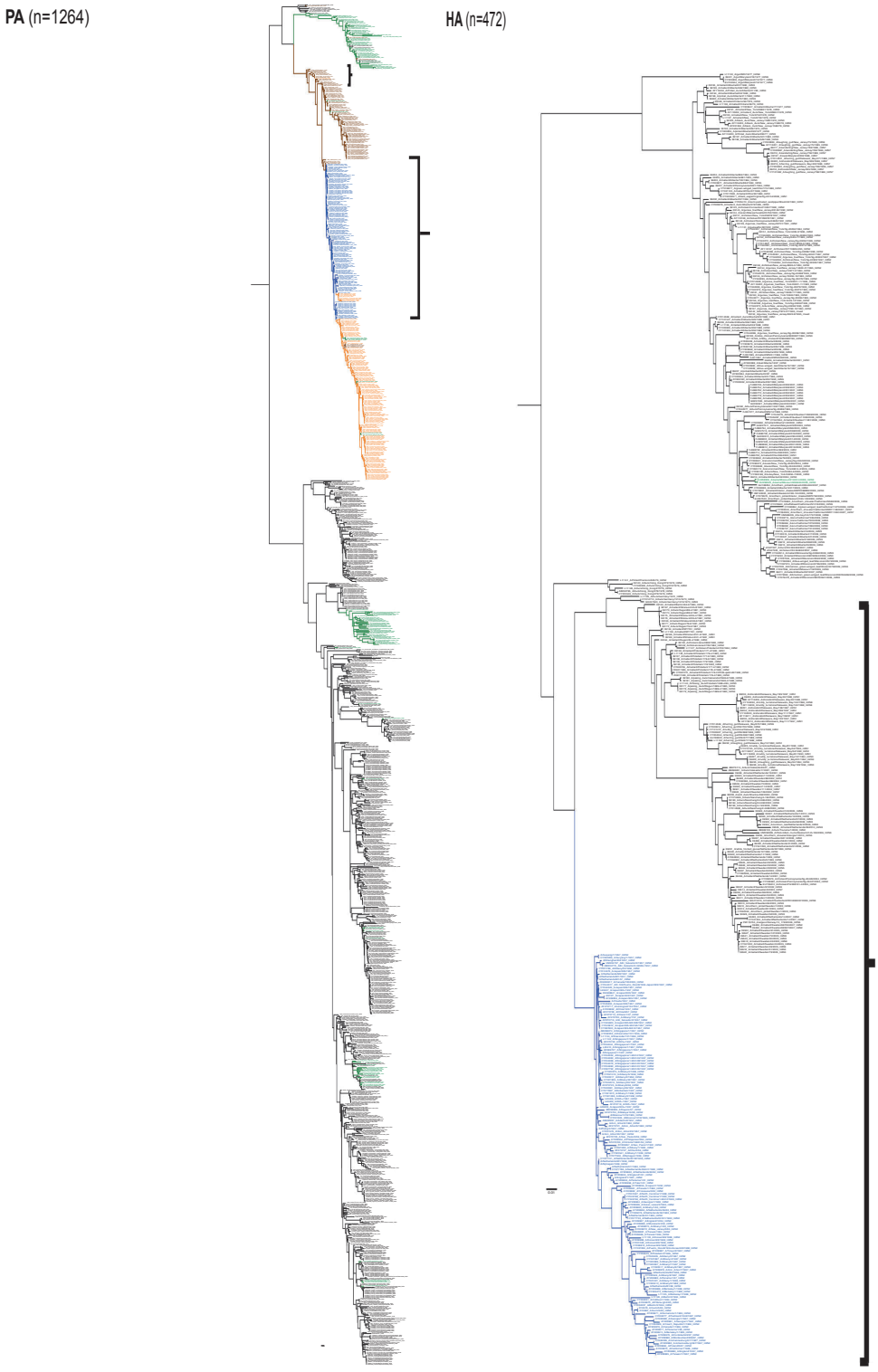
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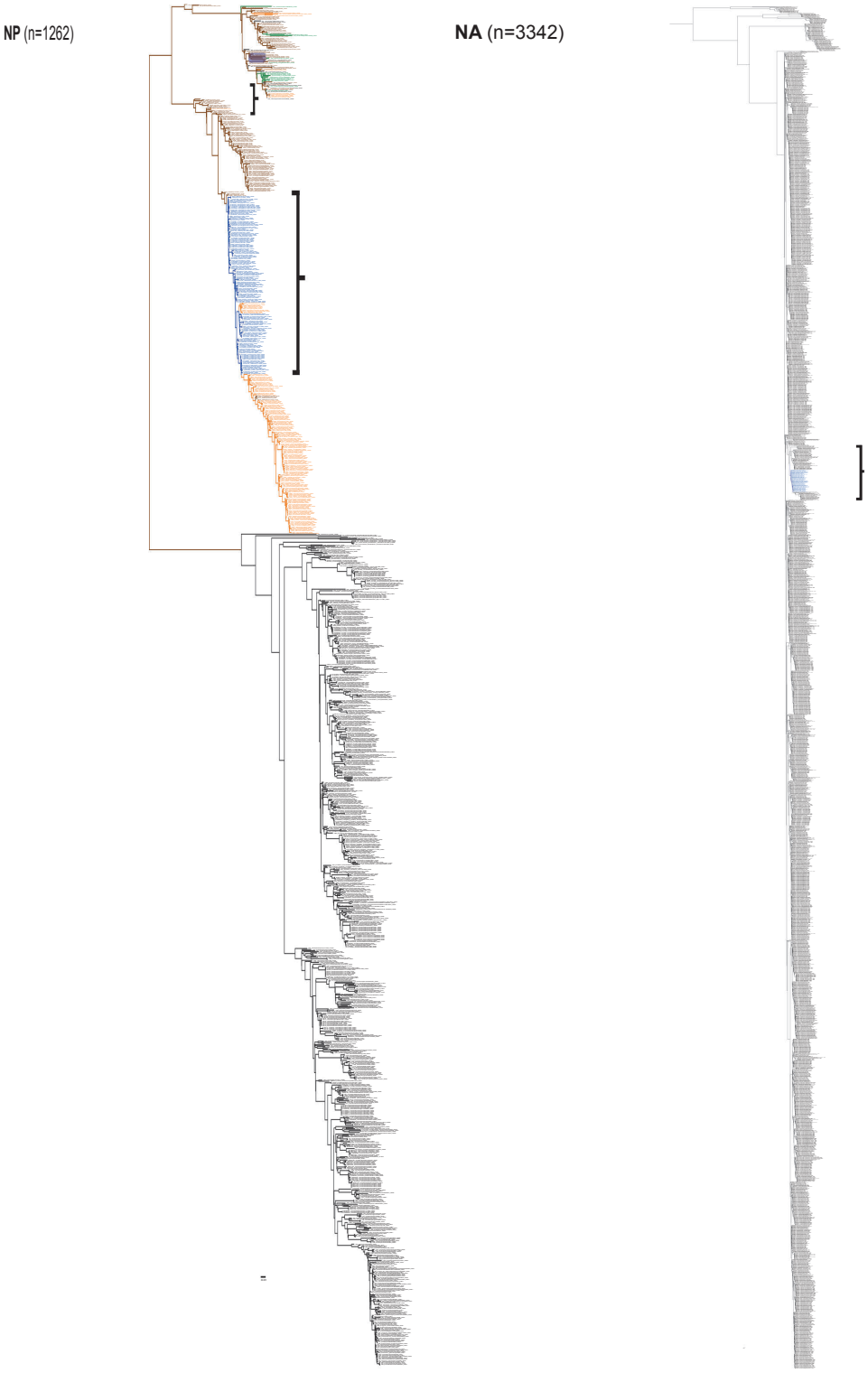
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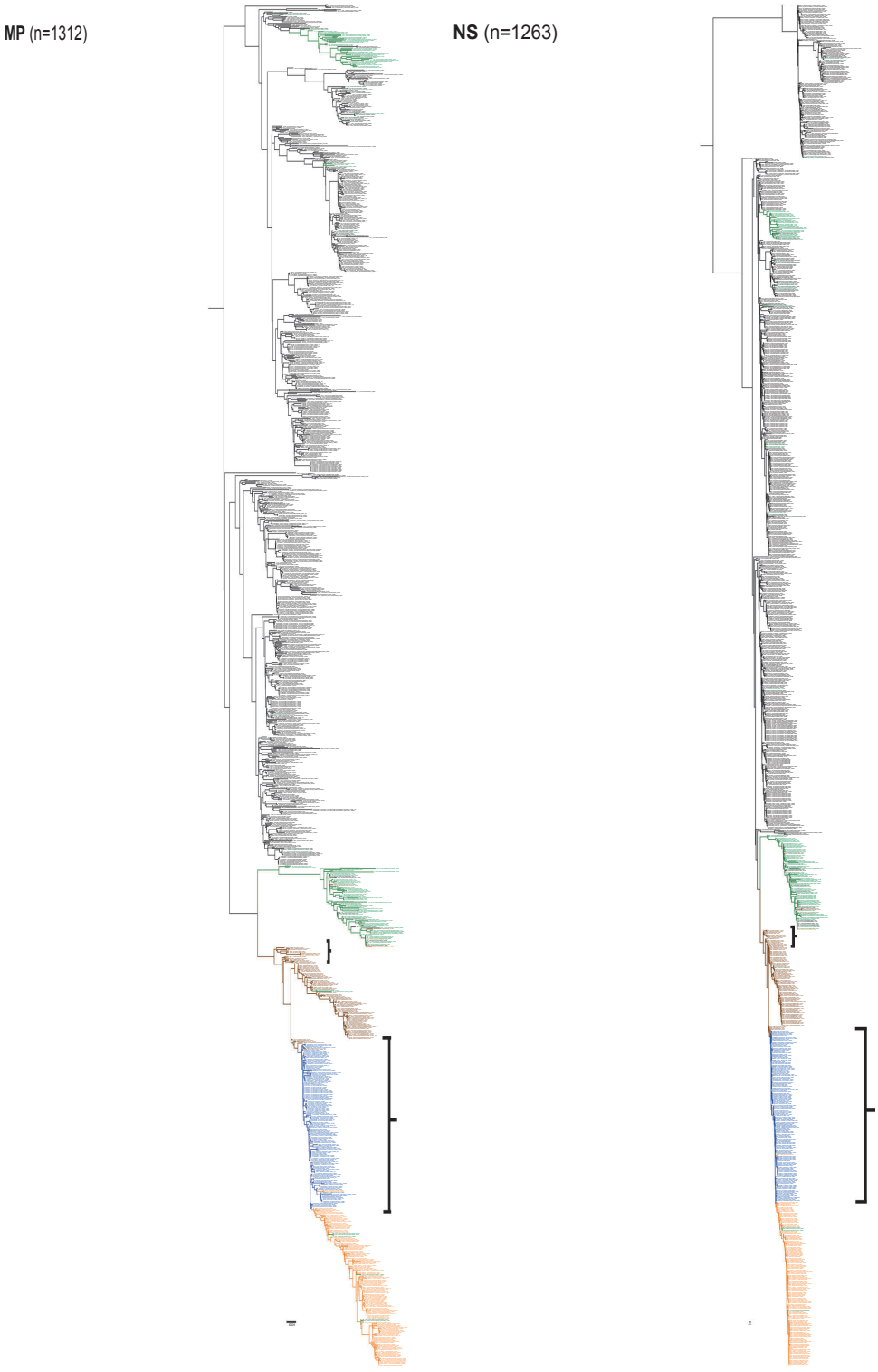
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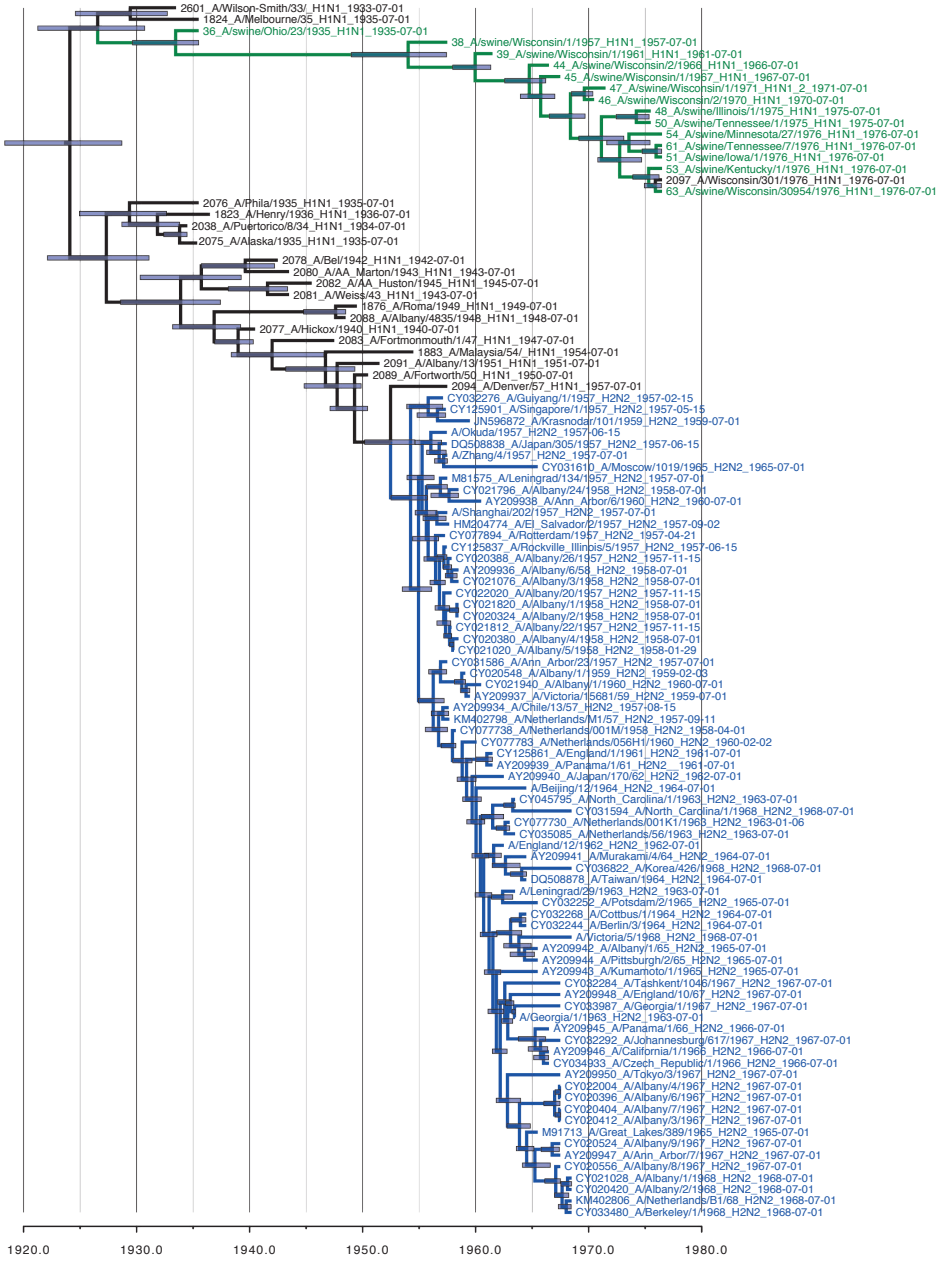
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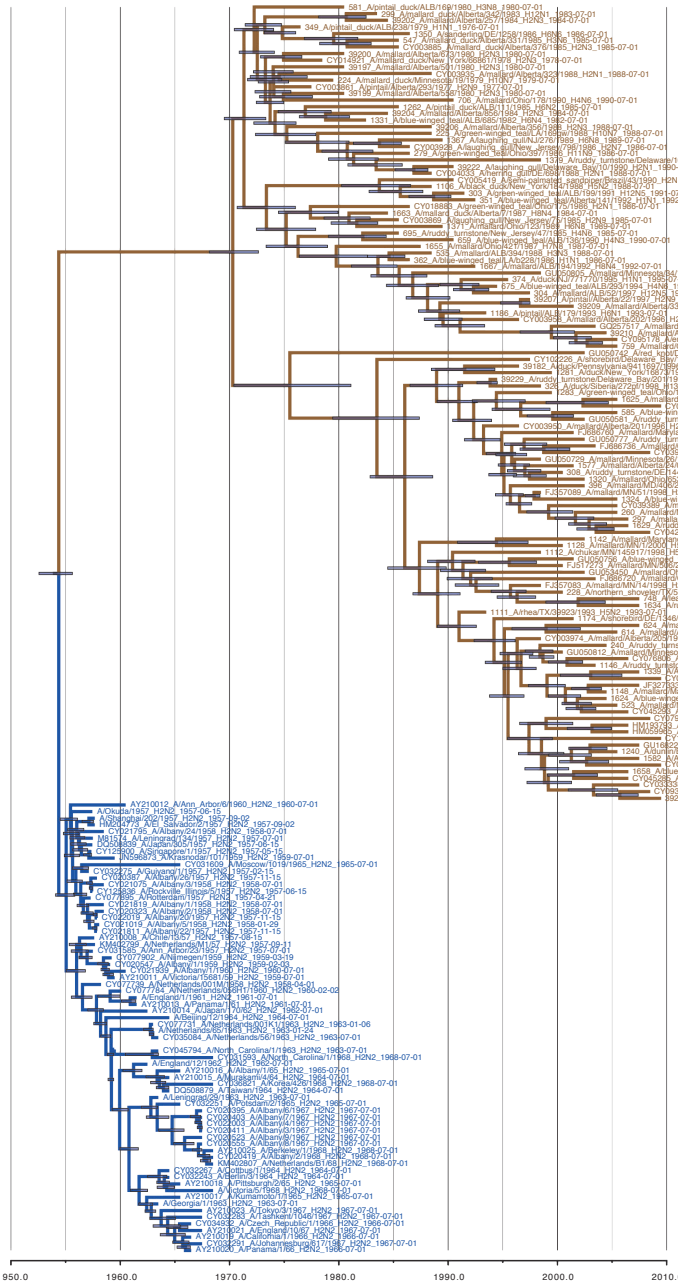

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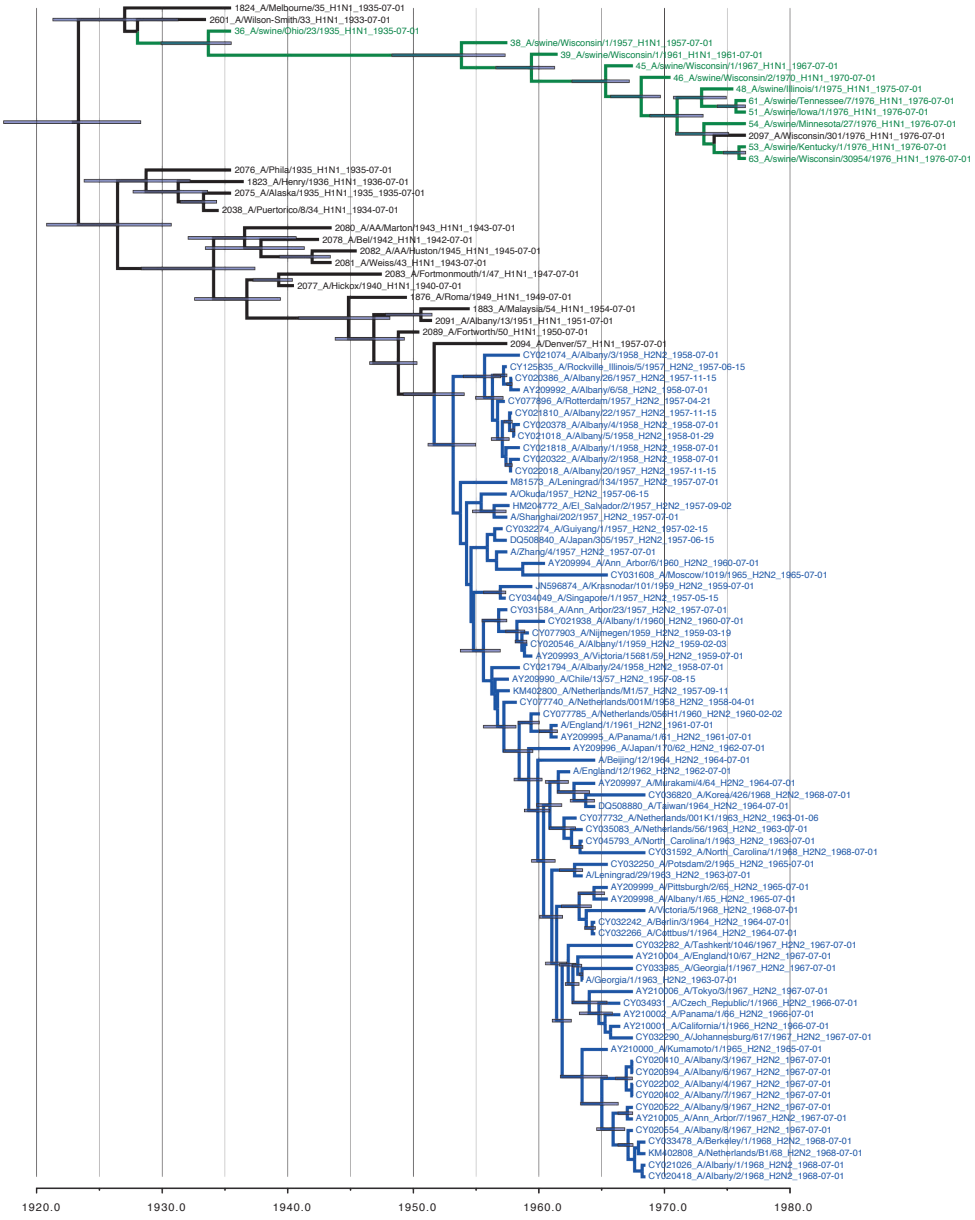
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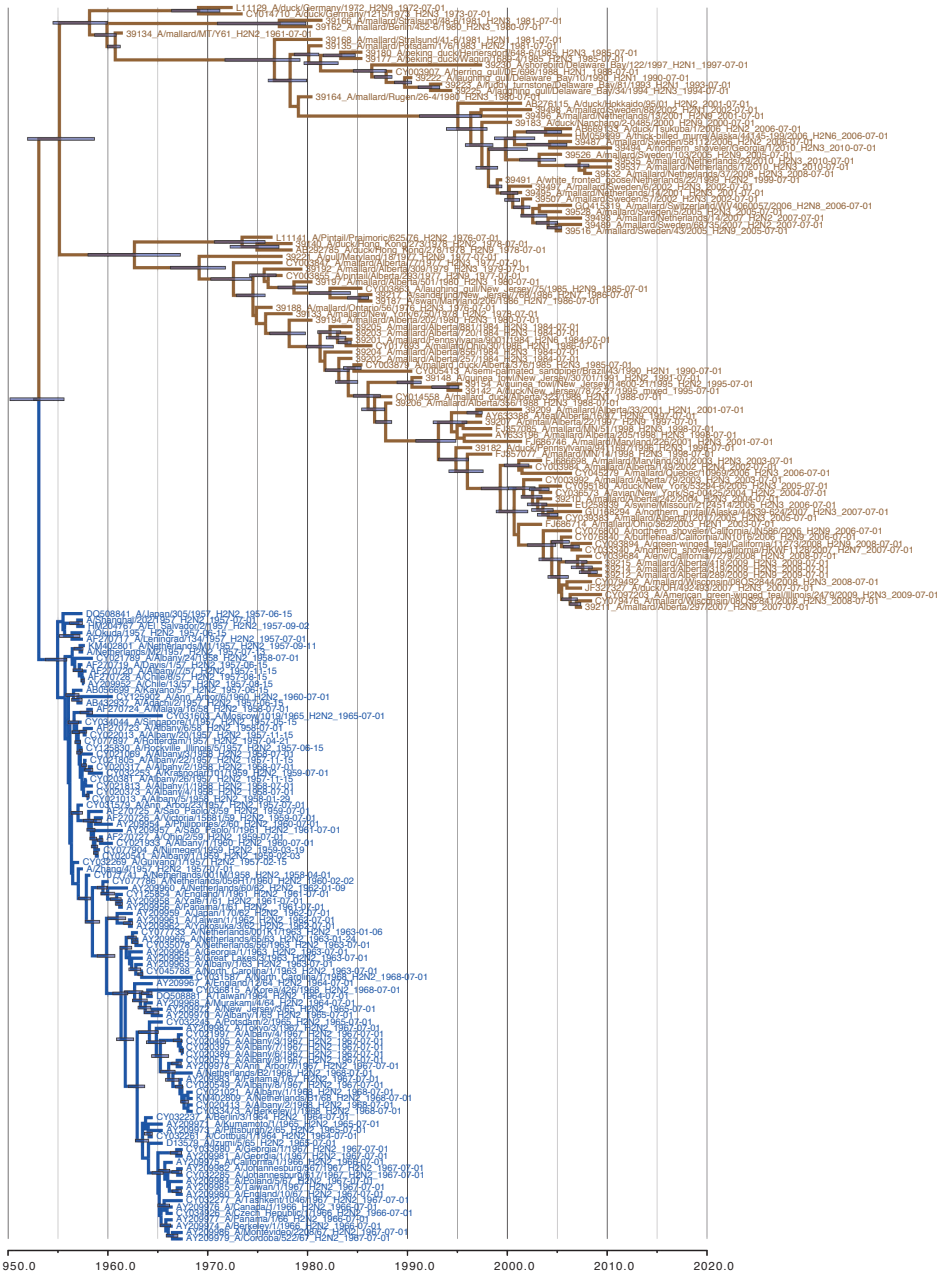

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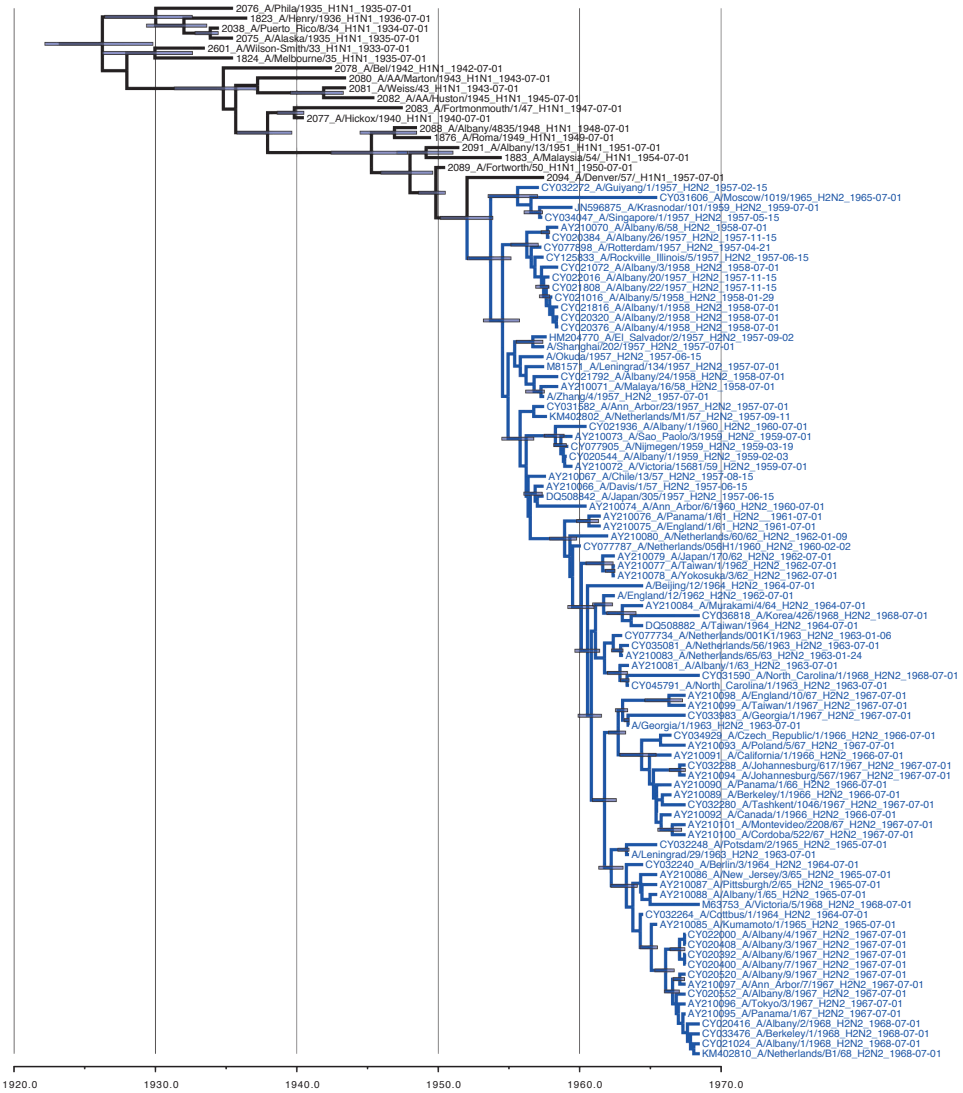
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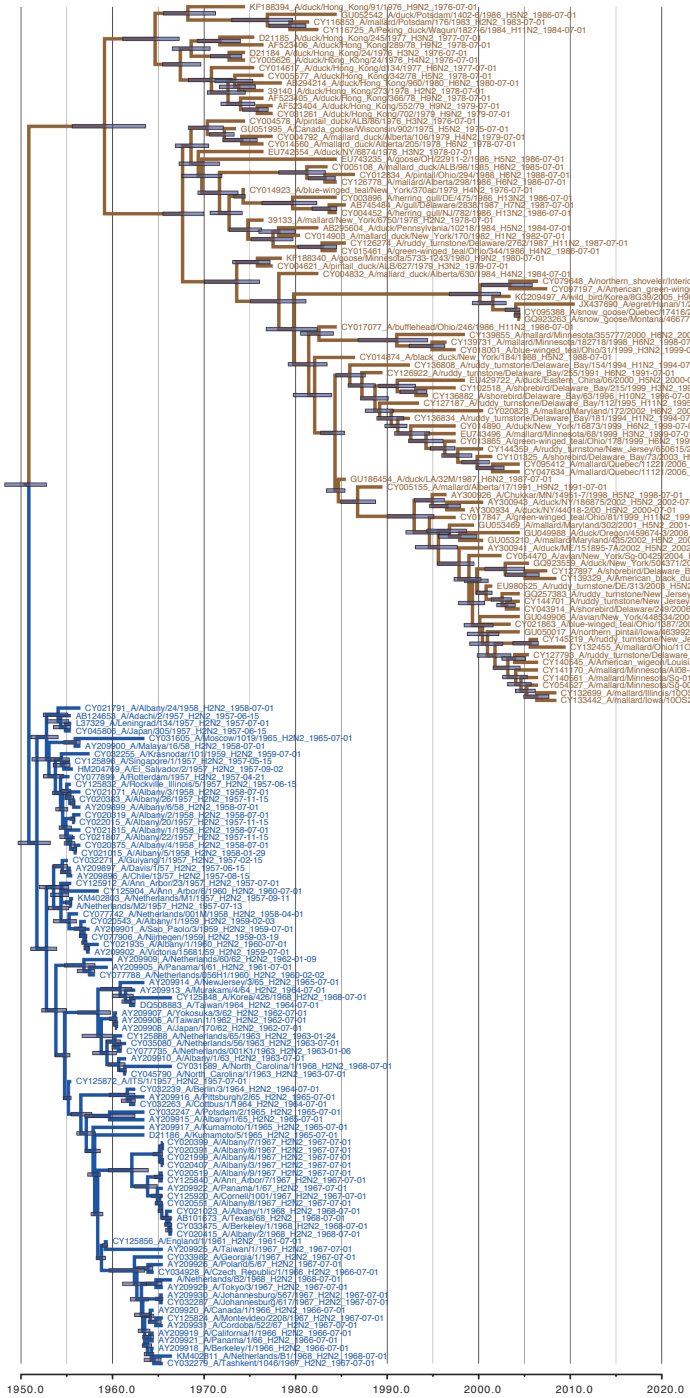

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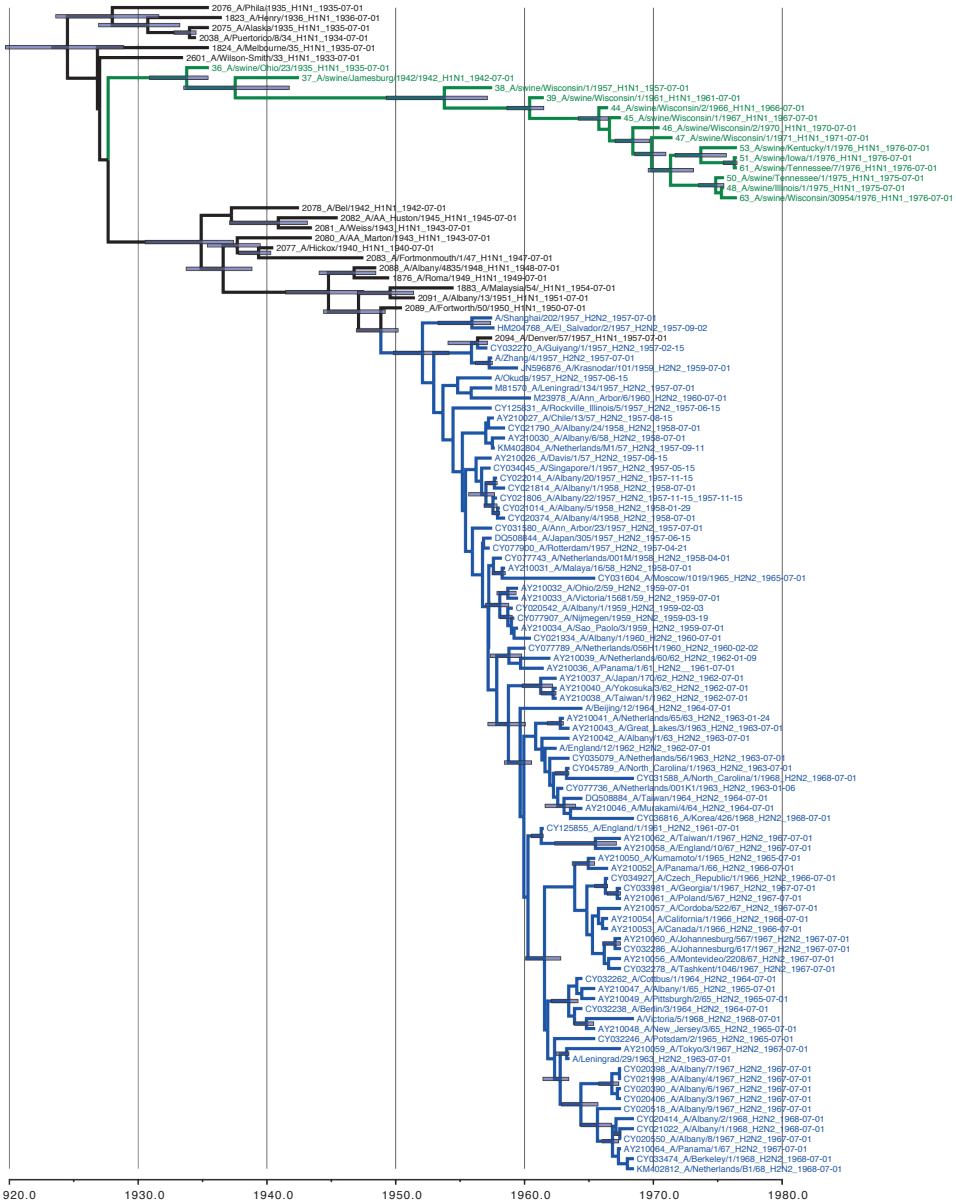
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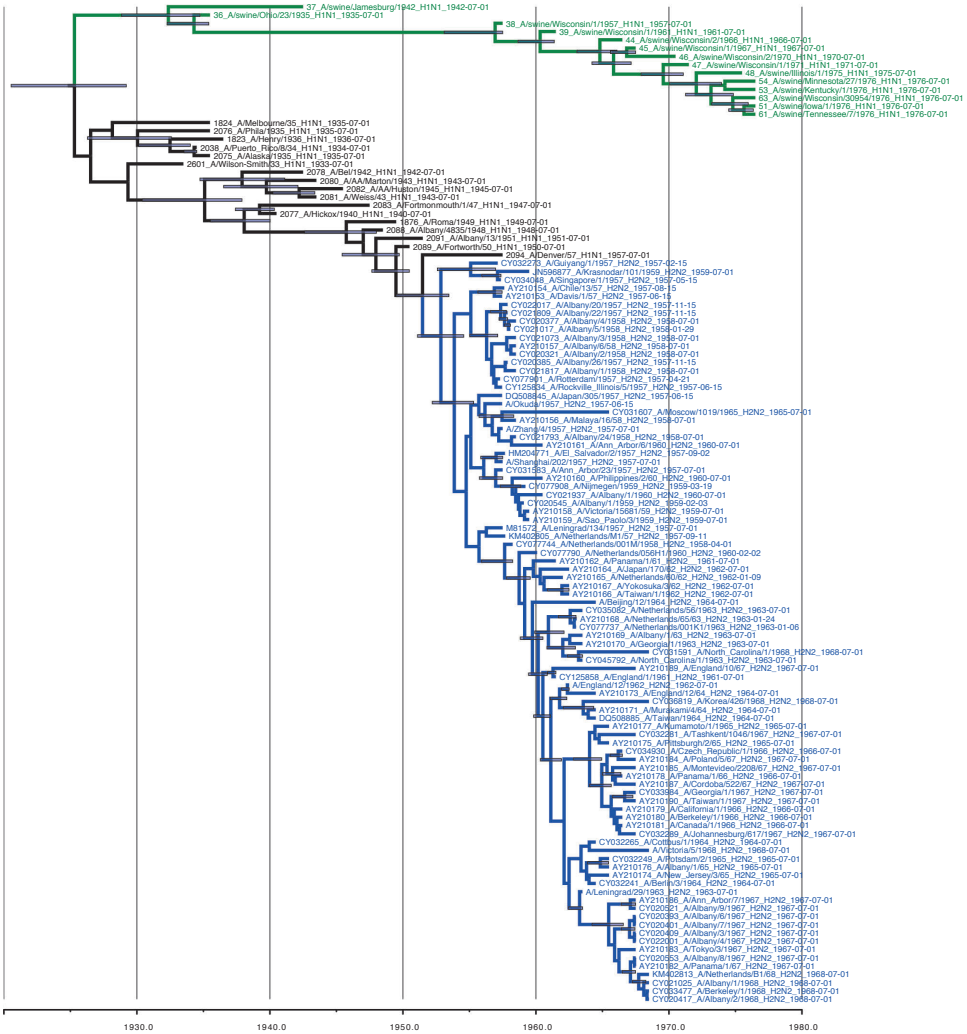
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1878_A/Roma/1949_H1N1_1949-07-01
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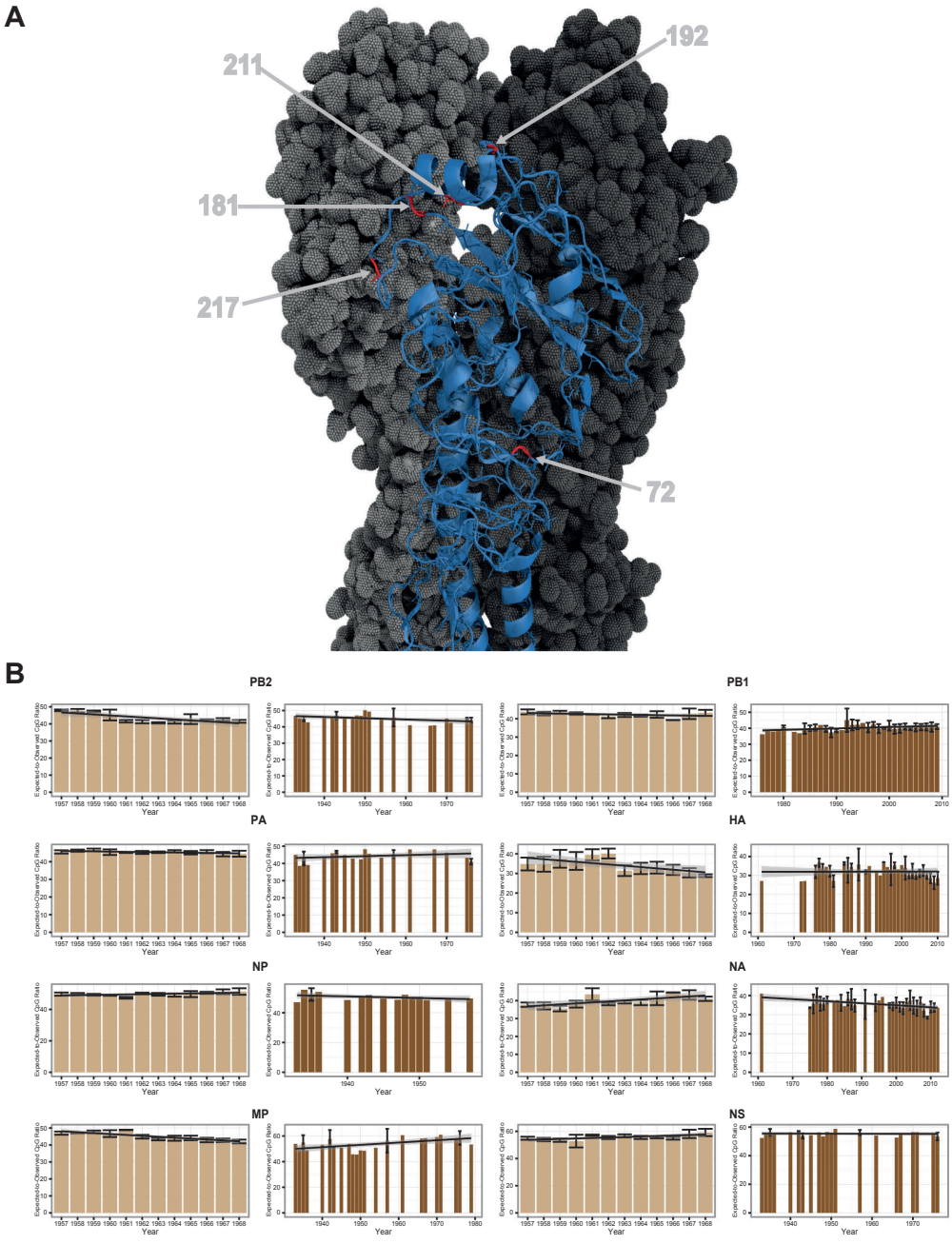
CY020417_A/Albany/2/1968_H2N2_1968-07-01
1930.0
1940.0
1950.0
1960.0
1970.0
1980.0

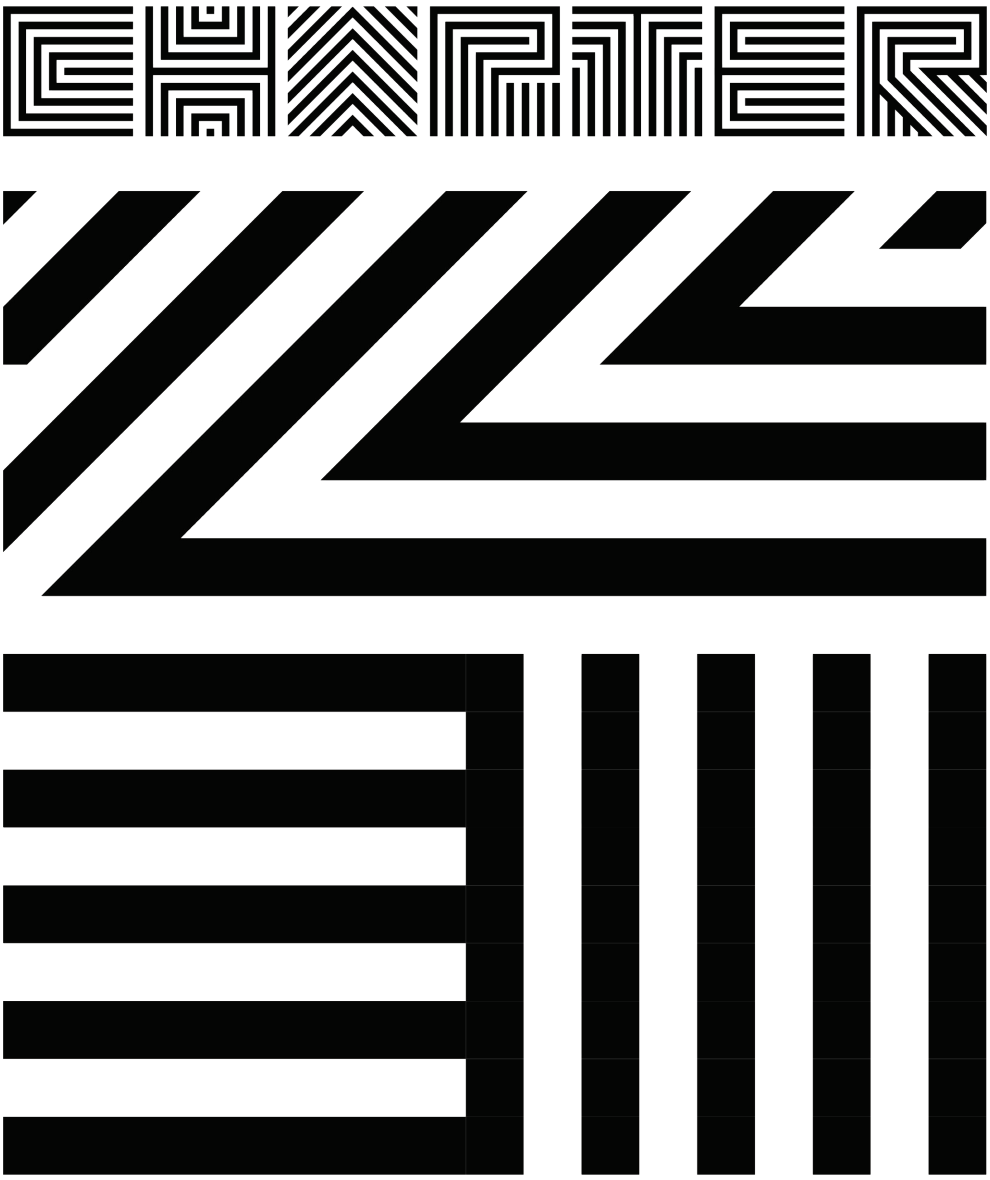
Supplemental Figure 1:
Maximum likelihood (ML) trees of the PB2 (A), PB1 (B), PA (C), HA (D), NP (E), NA (F), M (G), and NS (H) segments used in this study. ML trees inferred using FastTree v2.1.5 under the generalized time reversible (GTR) nucleotide substitution model for all 8 segments of Influenza A isolates. Human H2N2 isolates represented in blue; human H3N2 isolates represented in orange; human H1N1 isolates represented in brown; all swine isolates represented in green; and all avian isolates represented in black.
Isolates used for further temporal phylogenetic and selection analyses indicated by large bracket lines. Images can be zoomed in in the online version to visualize details.

Supplemental Figure 2:
Temporal Phylogenies of human H2N2 (blue) virus gene segments and their associated avian H2N2 (brown) or human H1N1 (black) sister clades. Swine sequences denoted in green. Temporal phylogenies inferred using an uncorrelated log- normal distribution (ucld) clock model and a Gaussian Markov random field (GMRF) Bayesian skyride coalescent tree prior. Blue horizontal bars at nodes represent the 95% HPDs of TMRCAs.

Supplemental Table 1:
For the list of GenBank accession numbers used in this study, please refer to the online version of the manuscript.

Supplemental Figure 3:
A. The positions identified as significant positively selected sites plotted on the crystal structure of the A/Singapore/1/1957(H2N2) virus HA trimer [PDB accession code 2WR7] using MacPyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). The positions that are predicted to be significant in adaptation to human hosts in red. B. Observed to Expected CpG ratios in each segment of the human H2N2 isolates (light brown bars) and the respective background/sister clade (dark brown) plotted by year. Error bars represent the standard deviation (SD) by year. Best-fit regression lines (black) shown with 95% confidence interval (grey).


A
192
211
181
217
72
B
PB2
PB1
PA
HA
NP
NA
MP
NS
Expected-to-Observed CpG Ratio
Year



The Molecular Basis for Antigenic Drift of Human A/H2N2 Influenza Viruses

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ABSTRACT

Influenza A/H2N2 viruses caused a pandemic in 1957 and continued to circulate in humans until 1968. The antigenic evolution of A/H2N2 viruses over time and the amino acid substitutions responsible for this antigenic evolution are not known. Here, the antigenic diversity of a representative set of human A/H2N2 vi ruses isolated between 1957 and 1968 was characterized. The antigenic change of influenza A/H2N2 viruses during the 12 years that this virus circulated was modest. Two amino acid substitutions, T128D and N139K, located in the head domain of the H2 hemagglutinin (HA) molecule, were identified as important determinants of antigenic change during A/H2N2 virus evolution. The rate of A/H2N2 virus antigenic evolution during the 12-year period after introduction in humans was half that of A/H3N2 viruses, despite similar rates of genetic change.

IMPORTANCE

While influenza A viruses of subtype H2N2 were at the origin of the Asian influenza pandemic, little is known about the antigenic changes that occurred during the twelve years of circulation in humans, the role of preexisting immunity, and the evolutionary rates of the virus. In this study, the antigenic map derived from hemagglutination inhibition (HI) titers of cell-cultured virus isolates and ferret postinfection sera displayed a directional evolution of viruses away from earlier isolates. Furthermore, individual mutations in close proximity to the receptor-binding site of the HA molecule determined the antigenic reactivity, confirming that individual amino acid substitutions in A/H2N2 viruses can confer major antigenic changes. This study adds to our understanding of virus evolution with respect to antigenic variability, rates of virus evolution, and potential escape mutants of A/H2N2.

Influenza A viruses of the H2N2 subtype initiated a pandemic in 1957, causing morbidity and mortality in humans, an event also known as the “Asian flu pandemic” (188, 211, 212). No surveillance systems were in place in 1957 to accurately detect and record the A/H2N2 pandemic outbreak scenario. Based on death certificates and newspaper articles, excess mortality was found to occur in waves, with the highest number of events between October 1957 and March 1958 in 5to 14-year-olds (213). The A/H2N2 virus originated upon reassortment between a previously circulating seasonal human A/H1N1 virus and an avian A/H2N2 virus. The latter virus contributed the HA, neuraminidase (NA), and polymerase basic protein 1 (PB1) gene segments to the pandemic A/H2N2 virus (60, 61, 186). This virus circulated in the human population until it was replaced by an A/H3N2 influenza virus in 1968. Today, more than 50 years after the last detected A/H2N2 virus infection in humans, immunity against A/H2N2 viruses is waning. The threat

of reintroduction and spread of H2 viruses in humans remains, because A/H2N2 viruses and other influenza A viruses with combinations of H2 and various NA genes continuously circulate in avian species and incidentally in swine (62, 214, 215). Several vaccine candidates have been developed for pandemic preparedness (216-218), and prophylactic vaccination of individuals at increased risk has been proposed (219).

The HA glycoprotein of influenza A viruses is the major target for neutralizing antibodies and continuously undergoes antigenic evolution by acquiring substitutions to escape antibody-mediated immunity (137). Five antigenic sites in the HA molecule have been identified to determine antigenic properties of seasonal human influenza viruses (220-222). In the case of A/H2N2 influenza viruses, six antigenic sites (I-A to I-D, II-A, and II-B) in the HA have been recognized to play a major role in antigenic change (196). These sites structurally correspond to the five sites described for A/H3N2 influenza viruses (designated A to E). Site II-A is unique for A/H2N2 influenza viruses, highly conserved, and located in the HA stem domain.

After seminal studies that described the structural importance of the HA receptor binding site (RBS) for antigenic variation (19, 155, 221), it was shown recently that a mere seven amino acid positions on HA located immediately adjacent to the receptor binding site (RBS) largely determined antigenic changes that occurred during A/H3N2 influenza virus circulation in humans from 1968 to 2003 (223). Similarly, a study on clade 2.1 A/H5N1 viruses showed that substitutions in close proximity to the RBS dictated antigenic change of avian A/H5N1 influenza viruses emerging in poultry (224), and amino acid changes close to the RBS were found to induce antigenic change in A/H1N1pdm09 viruses (225-227). Substitutions in the head domain of the HA molecule have also been demonstrated to determine the antigenic phenotype of equine and swine influenza A viruses (228, 229). Combined, these studies demonstrate the importance of RBS-proximal substitutions for antigenic drift of influenza A viruses.

In this study, the antigenic properties of a representative set of human A/H2N2 virus isolates spanning the period from 1957 to 1968 were assessed with respect to their reactivity to ferret postinfection sera in hemagglutination inhibition (HI) assays. The substitutions responsible for major antigenic differences between A/H2N2 influenza viruses were mapped by site-directed mutagenesis and generation of recombinant viruses.

Figure 1:
Maximum likelihood phylogenetic tree based on HA1 amino acid sequences of human A/H2N2 viruses. Virus isolates used for antigenic characterization are highlighted in red.

RESULTS

Genetic and antigenic diversity of A/H2N2 viruses

The genetic variation of human A/H2N2 influenza viruses isolated between 1957 and 1968 was assessed by maximum likelihood algorithms in an HA1 amino acid phylogenetic tree (Fig. 1). The tree displays a ladder-like structure that indicates gradual accumulation of mutations over time. A set of 18 human A/H2N2 influenza virus isolates representative of genetic variation over the 12-year period and that was available in our laboratory was compiled (highlighted in red in Fig. 1).

HI titers of the set of 18 A/H2N2 viruses and 6 A/H2N2 ferret postinfection sera revealed a typical pattern of influenza virus antigenic drift, with high antibody titers of antisera against homologous and contemporary viruses and lower titers against noncontemporary strains (Table 1). HI titers were processed using antigenic cartography methods to yield an antigenic map (Fig. 2), revealing directional antigenic progression of later isolates away from early strains over time. Viruses isolated in the same or subsequent years generally grouped together in the map and thus were antigenically similar. Exceptions were A/Sydney/2/64 and the latest A/H2N2 viruses. In this study, the maximum antigenic distance between any pair of wild-type viruses was 6.4 antigenic units (AU) between A/Netherlands/M1/1958 (NL/M1/58) and A/Netherlands/B2/1968 (NL/B2/68). Viruses isolated in 1964 were antigenically highly diverse; whereas A/England/12/1964 (EN/12/64) and A/Taiwan1/64 (TW/1/64) drifted 3.9 antigenic units away from NL/M1/57, A/Sydney/2/1964 (SY/2/64) was only 1.6 antigenic units away from NL/M1/57. The three viruses isolated shortly before the introduction of the first A/H3N2 virus in 1968 (NL/61/68, NL/B1/68, and NL/B2/68) were particularly divergent in the antigenic map, with 3.2 antigenic units of difference between NL/61/68 and NL/B2/68.

Molecular basis of antigenic change in A/H2N2 viruses

The head domain of the HA molecule is the main target of neutralizing antibodies (221, 230). Previous studies indicated that amino acid substitutions near the RBS and exposed on the surface of the HA molecule were responsible for major antigenic drift of influenza A/H3N2, A/H5N1 viruses, and influenza B virus (224, 226, 231). Amino acid changes on positions 100 to 250 were compared as a coarse outline of the globular head domain, including the RBS area of the H2 HA. A set of 7 amino acid substitutions (T126E, T128D, R132K, N139K, S154P, A184T, and A188T) was consistently found in later A/H2N2 virus isolates compared to the earlier strains and hence could explain the antigenic differences between early and late strains. Throughout this study, amino acid positions are numbered as suggested by Burke and Smith (232).

Table 1 :
Hemagglutination inhibition titers for wild-type viruses toward A/H2N2 postinfection ferret antisera [a]

	Titer toward ferret postinfection antiserum					
Virus isolate	**JP/305/57**	**SP/1/57**	**NL/K1/63**	**EN/1/66**	**TY/3/67**	**NL/B1/68**
A/NETHERLANDS/M1/57 [NL/M1/57]	**2,560**	**1,600**	**1,280**	**320**	**40**	**160**
A/NETHERLANDS/M2/57 [NL/M2/57]	960	1,600	960	240	40	80
A/SINGAPORE/1/57 [SP/1/57]	1,600	1,920	1,280	160	35	60
A/NETHERLANDS/M1/58 [NL/M1/58]	960	960	960	160	30	60
A/NETHERLANDS/N1/59 [NL/N1/59]	1,920	1,920	1,920	800	240	240
A/NETHERLANDS/H1/60 [NL/H1/60]	1,920	1,600	2,560	480	120	200
A/NETHERLANDS/67/63 [NL/67/63]	560	1,280	4,480	560	240	800
A/NETHERLANDS/K1/63 [NL/K1/63]	1,600	1,120	5,760	960	200	800
A/ENGLAND/12/64 [EN/12/64]	60	240	2,240	2,880	320	1,120
A/SYDNEY/2/64 [SY/2/64]	480	800	480	200	60	160
A/TAIWAN/1/64 [TA/1/64]	80	320	1,120	1,920	320	1,120
A/MOSCOW/56/65 [MW/56/65]	560	240	2,560	480	240	1,280
A/ENGLAND/1/66 [EN/1/66]	640	320	3,840	6,400	320	2,240
A/ENGLAND/10/67 [EN/10/67]	1,120	320	2,560	800	240	1,120
A/TOKYO/3/67 [TY/3/63]	80	80	160	320	960	320
A/NETHERLANDS/61/68 [NL/61/68]	320	160	1,120	2,240	160	800
A/NETHERLANDS/B1/68 [NL/B1/68]	**20**	**80**	**960**	**960**	**320**	**2 ,880**
A/NETHERLANDS/B2/68 [NL/B2/68]	50	60	320	640	640	640

a One serum per isolate was selected to represent the two individual ferret sera since variation in HI titers between repeat sera was negligible. Viruses emphasized in Fig. 3 are in bold and homologous HI titers are underlined.

Single amino acid substitutions and combinations thereof were introduced and tested in recombinant viruses harboring the HA gene of NL/M1/57 or NL/B1/68 in a backbone of A/Puerto Rico/8/34 (A/H1N1). All reverse genetics viruses were rescued, with the exception of NL/B1/68 HA K132R mutant virus, which was not rescued despite three independent rescue attempts.
A substitution at position 139 was responsible for a substantial antigenic change of 2.9 antigenic units (AU) when tested in viruses containing HA genes of NL/M1/57 and NL/B1/68 (Fig. 3A and B, Table 2). This position is surface exposed and located on a protruding loop adjacent to the RBS (Fig. 3E). All other individual mutations in NL/ M1/57 and NL/B1/68 had an antigenic effect of less than 1.7 AU compared to the wild-type virus. The effect of N139K in NL/M1/57 increased with the addition of T128D to a 3.6-AU distance from the NL/M1/57 virus carrying the NL/M1/57 wild-type HA (Fig. 3C). When the combination of K139N and D128T was tested in NL/B1/68 HA, only a rather small difference in antigenic effect (1.1 AU) was measured compared to K139N alone (Fig. 3D). Here, a combination of six amino acid substitutions (E126T, D128T, K139N, S154P, A184T, and A188T) was necessary in order for the virus to be antigenically similar to NL/M1/57 and located 5.4 antigenic units from NL/B1/68. Each substitution in addition to K139N had a rather small but incremental effect on the antigenic reactivity of the H2N2 HA.

Evolutionary rates of A/H2N2 and A/H3N2

Next, the genetic and antigenic change over time after introduction of the new influenza virus subtype in the human population was investigated (Fig. 4). The rate of evolution of A/H2N2 HA1 was estimated and compared to the rate of HA1 evolution during the first 12 years of A/H3N2 circulation after its introduction in the human population in 1968, based on phylogenetic trees generated here and by Smith et al. (137). The average rate of genetic evolution (nucleotide substitutions per site per year) estimated in this analysis was 8.47×10^{-3} for H2N2 and 7.53×10^{-3} for H3N2 (Fig. 4A). The average rate of antigenic evolution for A/H2N2 from 1957 to 1968 was 0.4 AUs per year, calculated from the slope of the best-fit regression line of the distances in the antigenic map (Fig. 4B). Using the A/H3N2 data set reported by Smith et al. (137), the maximum distance in the antigenic map during the first 12 years of circulation (1968 to 1979) was 13.3 AUs between isolates A/Bilthoven/16190/1968 and A/Bangkok/1/1979, resulting in an average evolutionary rate of 0.9 AUs per year, somewhat lower than the rate reported over the 35-year period from 1968 to 2003 of 1.2 AUs per year (137). Thus, the antigenic evolution of the A/H2N2 virus was approximately two times slower than antigenic evolution of A/H3N2 during the first 12 years of circulation and three times slower than that over the 35-year period.
To investigate if these differences in antigenic evolution were potentially due

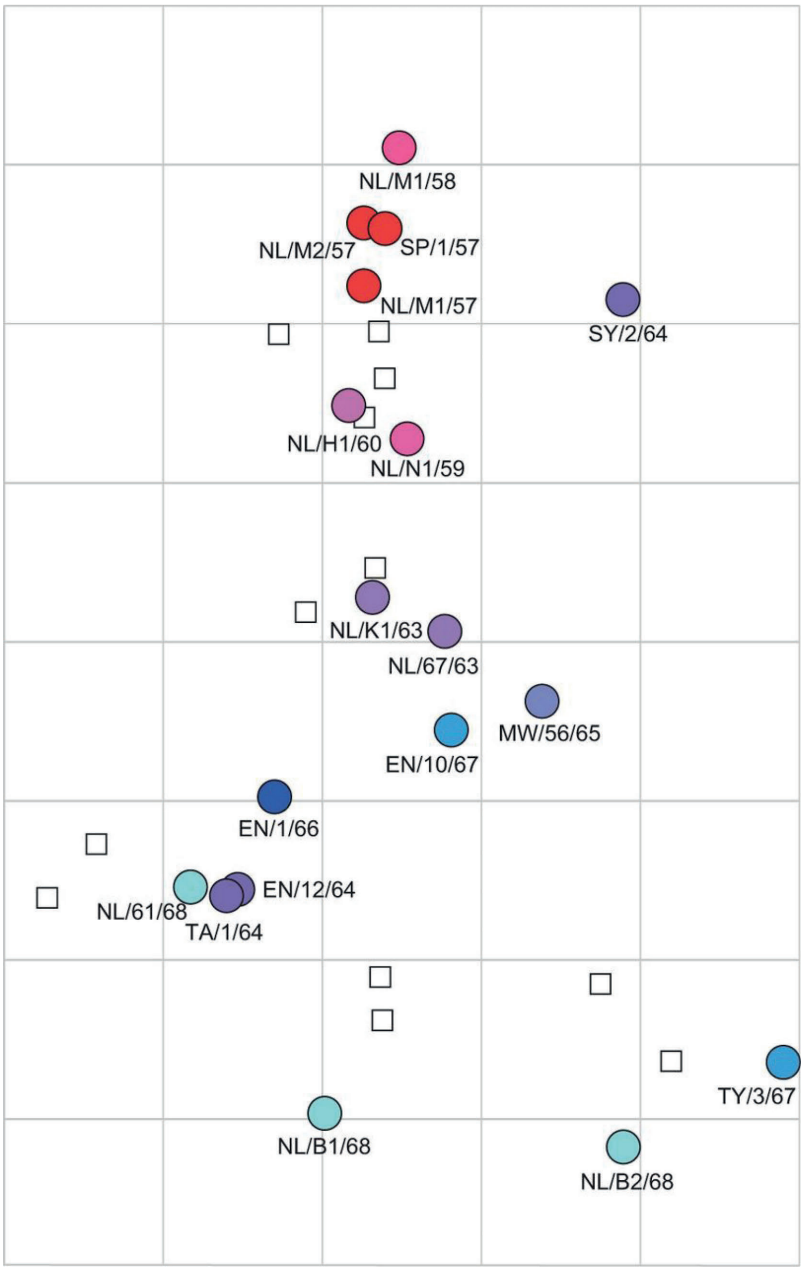

Figure 2:
Antigenic map of human A/H2N2 influenza viruses as measured in HI assays with ferret postinfection antisera. Circles indicate the position of viruses, squares represent two ferret antisera raised against each of the viruses A/Japan/305/57, A/Singapore/1/57, A/Netherlands/K1/63, A/England/1/66, A/Tokyo/3/67, and A/Netherlands/B1/68. The underlying grid depicts the scale of antigenic difference between the viruses, with each square representing one antigenic unit or a 2-fold difference in HI titer. Years of isolation of the A/H2N2 virus isolates are indicated, ranging from 1957 (red) to 1968 (blue).

to increased evolutionary pressures to select antigenic escape mutants or were the consequence of an overall increased rate of nucleotide substitution in HA1, the nucleotide substitution rates were estimated using Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.8.1 with a relaxed log-normal clock and the Bayesian skyride time-aware model. All available sequences in public databases were downloaded, which resulted after curation in alignments of 98 sequences for A/H2N2 virus HA1 and 103 sequences for H3N2 virus HA1. The mean rate of nucleotide substitution for A/H2N2 HA1 was determined to be 4.88 X 10^{-3} nucleotide substitutions per site per year (highest posterior density [HPD], 3.68 X 10^{-3} to 6.21 X 10^{-3}). The nucleotide substitution rate of A/H3N2 virus HA1 was determined to be 4.48 X 10^{-3} (HPD, 3.57 X 10^{-3} to 5.49 X 10^{-3}), comparable to previous results obtained for A/H3N2 HA1 at 5.15 X 10^{-3} (HPD, 4.62 X 10^{-3} to 5.70 X 10^{-3}) (230). This rate of A/H3N2 virus evolution was not statistically significantly different from the A/H2N2 virus rate (Bayes factors: H2 > H3, 1.533; H3 > H2, 0.651).

DISCUSSION

Using a unique and comprehensive collection of human A/H2N2 viruses with low passage history and matching ferret postinfection sera spanning the time of circulation of A/H2N2 viruses in humans, the antigenic evolution of A/H2N2 viruses over time was analyzed. Phylogenetic analysis of HA sequences of human A/H2N2 viruses resulted in the ladder-like structure of the phylogenetic tree (Fig. 1) due to the gradual accumulation of mutations characteristic for human influenza A viruses (184, 233). All H2N2 virus isolates available at our institute were amplified by PCR and sequenced. They were confirmed to be representative of the major genetic diversity and were tested in HI assays for their reactivity to corresponding ferret antisera and to construct antigenic maps. The antigenic evolution of A/H2N2 viruses did not demonstrate obvious clustering of virus isolates in contrast to A/H3N2 viruses (223) but a rather gradual pattern of antigenic change over time. However, the number of strains included in the current analysis and the short time span of A/H2N2 virus circulation may simply be insufficient for clustering to be obvious.

A single amino acid change from asparagine (N) to lysine (K) at position 139 in the HA molecule played a prominent role in determining the antigenic properties of A/H2N2 viruses. When introduced in either NL/M1/57 or NL/B1/68, this substitution had an antigenic effect of 2.9 antigenic units, describing roughly half of the observed antigenic diversity of A/H2N2 HA. No other single amino acid substitution was responsible for a greater antigenic effect than D128T in the context of NL/B1/68 (Fig. 3C and D). Both positions 128 and 139 are located close to the RBS in the HA protein, similarly to the substitutions that were previously shown to be important for major antigenic

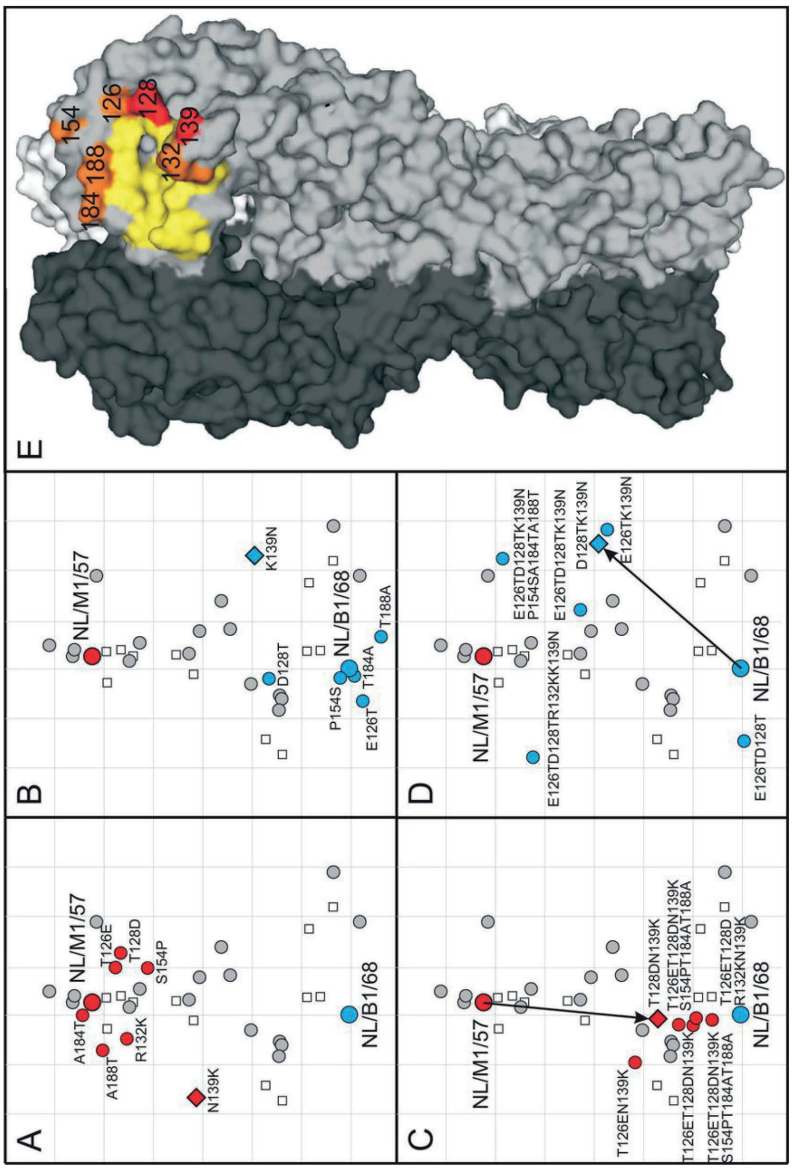

Figure 3:
Summary of substitutions responsible for antigenic differences between NL/M1/57 and NL/B1/68. Antigenic maps showing the antigenic change caused by individual amino acid substitutions introduced into NL/M1/57 (A) or NL/B1/68 (B) and combinations of mutations introduced into NL/M1/57 (C) or NL/B1/68 (D). Viruses are shown as circles of different colors, with a diamond indicating the mutant virus with the largest antigenic distance to the corresponding wild-type strain. Sera are indicated as open squares. The underlying map of wild-type viruses from Fig. 2 is shown in gray, and its positioning is kept constant. The arrows indicate the antigenic distance of a double mutant that spans a long distance between the earliest and latest isolates of A/H2N2. Structure of an HA trimer (E) with individual monomers in shades of gray, the RBS in yellow, and mutations near the RBS with a measurable effect on antigenicity in orange. The two mutations with the biggest combined effect in panel C are colored in red (T128D and N139K).

Table 2:
Hemagglutination inhibition titers for mutant viruses toward A/H2N2 postinfection ferret antisera [a]

	Titer toward ferret postinfection antiserum					
Virus isolate	**JP/305/57**	**SP/1/57**	**NL/K1/63**	**EN/1/66**	**TY/3/67**	**NL/B1/68**
NL/M1/57_T126E	1,600	1,440	640	320	80	560
NL/M1/57_T128D	1,600	960	640	280	80	640
NL/M1/57_R132K	2,240	1,280	640	800	80	320
NL/M1/57_N139K	**160**	**1,120**	**640**	**1,920**	**80**	**640**
NL/M1/57_S154P	1,920	1,280	1,280	560	360	280
NL/M1/57_T184A	1,600	1,920	1,120	320	20	160
NL/M1/57_T188A	1,280	1,600	1,920	480	20	160
NL/B1/68_E126T	40	80	640	960	160	1,280
NL/B1/68_D128T	1,120	240	1,440	2,240	160	2,240
NL/B1/68_K139N	**400**	**160**	**640**	**320**	**320**	**2,240**
NL/B1/68_P154S	20	160	640	800	320	3,200
NL/B1/68_A184T	40	120	480	640	160	2,560
NL/B1/68_A188T	20	100	160	320	240	1,920
NL/M1/57_T126EN139K	560	640	640	1,920	160	1,920
NL/M1/57_T128DN139K	**80**	**640**	**640**	**1,120**	**160**	**2,240**
NL/M1/57_T126ET128DN139K	100	640	320	800	140	2,880
NL/M1/57_T126ET128DR132KN139K	160	560	280	800	160	1,920
NL/M1/57_T126ET128DN139KS154PT184AT188A	80	320	960	800	160	3,200
NL/M1/57_T126ET128DR132KN139KS154PT184AT188A	80	320	640	800	160	2,560
NL/B1/68_E126TK139N	160	320	480	320	160	640
NL/B1/68_D128TK139N	**320**	**480**	**1,280**	**320**	**160**	**640**
NL/B1/68_E126TD128T	80	80	960	640	80	640
NL/B1/68_E126TD128TK139N	960	480	2,240	640	160	640
NL/B1/68_E126TD128TK139NK132R	640	400	2,240	320	60	320
NL/B1/68_E126TD128TK139NP154SA184TA188T	960	560	480	280	160	100
NL/B1/68_E126TD128TR132KK139NP154SA184TA188T	640	480	640	60	50	40

[a] Viruses emphasized in Fig. 3 are in bold.

change of other influenza A viruses and influenza B virus (223, 224, 226). Additional individual substitutions at positions 126, 132, 154, 184, and 188 had only minor antigenic effects, but collectively with positions 139 and 128 explained the major antigenic changes observed in A/H2N2 viruses. Also, these changes were located in close proximity to the RBS (Fig. 3). For the antigenic evolution of A/H3N2 virus, major antigenic change was caused by substitutions at only seven positions around the RBS, with relatively small effects of additional substitutions. For A/H2N2 virus, a single amino acid substitution also determined the antigenic phenotype of subsequent major drift variants, but the effect of additional substitutions was more substantial, potentially due to the different time scales at which the antigenic evolution was measured and the lack of clustering of strains in the A/H2N2 map.

Whereas A/H1N1pdm09 viruses remained remarkably antigenically stable since their introduction in humans (234, 235), A/H3N2 viruses displayed a more rapid accumulation of substitutions with major impact on antigenic evolution over time, possibly implying differential abilities of various HA subtypes to accommodate substitutions that affect antigenic properties (226, 236). The antigenic evolution of influenza B virus was also found to be relatively slow compared to that of A/H3N2 virus (223, 231). Here, the antigenic evolution of A/H2N2 was found to be two times slower than the antigenic evolution of A/H3N2 virus during its first twelve years of circulation, and three times slower than that during the period of A/H3N2 virus circulation from 1968 to 2003, while their respective nucleotide substitution rates differed only slightly in the first 12 years of virus circulation in humans. Although the exact factors contributing to this difference in rates of antigenic evolution are not known, antibody-mediated selection of escape mutants likely played an important role. Human sera obtained before 1957 from the elderly contained antibodies that react to A/H2N2 virus, suggesting that the pandemic of 1889 to 1890 was also caused by an influenza A virus of the H2 subtype (55). However, this preexisting immunity in the population apparently did not result in increased antibody-mediated selection for A/H2N2 virus variants, similar to the lack of rapid natural selection of escape mutants for A/H1N1pdm09 virus.

Combined, the genetic variability of A/H2N2 was comparable to that of other influenza A subtypes, whereas the antigenic evolution was relatively slow, indicating that population immunity to A/H2N2 did not facilitate rapid antigenic evolution at the time of virus introduction. The genetic data indicate that the size of the susceptible population, as well as virus turnover, was likely similar to that of other influenza virus subtypes. We hypothesize that a combination of factors, including the intrinsic capacity of the influenza virus HA to accumulate mutations responsible for antigenic evolution, preexisting immunity at the time of introduction, susceptible population size, and prior circulation of a certain subtype leading to human adaptation, have a combined effect on antigenic evolution of the HA.

This study describes directional antigenic evolution of A/H2N2 viruses during circulation in humans and highlights the importance of amino acid sites in close proximity to the RBS for antigenic reactivity of A/H2N2 HA. Rates of antigenic evolution in A/H2N2 viruses were lower than those in A/H3N2 viruses, possibly implying differences in the structural freedom of the HA molecules to evolve.

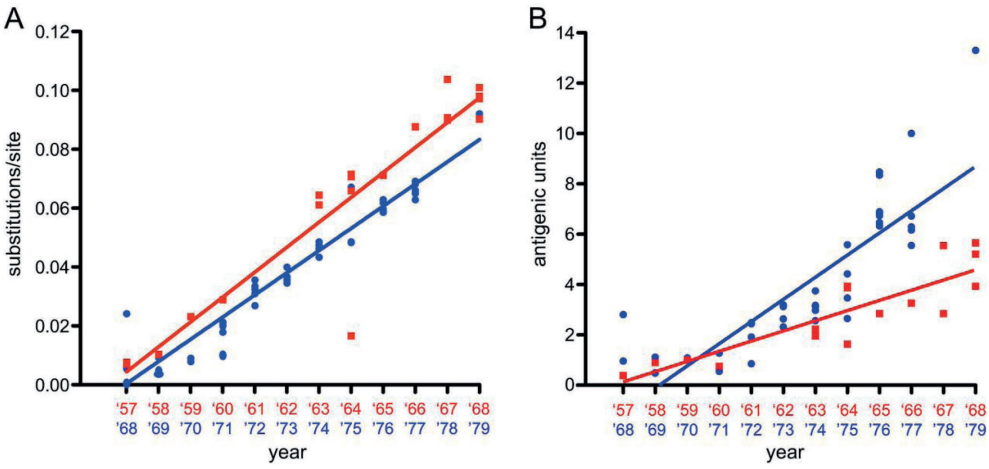

Figure 4:
Rates of genetic and antigenic evolution of A/H2N2 and A/H3N2 virus during 12 years of circulation in humans. Genetic (A) and antigenic (B) distances of the A/H2N2 (red squares) and A/H3N2 (blue circles) viruses from the first human virus isolates in 1957 (A/Netherlands/M1/1957) and 1968 (A/Bilthoven/16190/1968). Rates are derived from the slope of the best-fit regression line.

MATERIALS AND METHODS

Biosafety considerations

All experiments involving A/H2N2 viruses were conducted under biosafety level 3 (BSL3) conditions. Reassortant viruses in the backbone of A/Puerto Rico/8/34 (H1N1) harboring the HA gene of A/H2N2 viruses were used under BSL2 conditions.

Ferret antisera

Ferret postinfection antisera were prepared against virus isolates A/Japan/305/1957 (JP/305/57), A/Singapore/1/1957 (SP/1/57), A/Netherlands/K1/1963 (NL/K1/63), A/England/1/66 (EN/1/ 66), A/Tokyo/3/67 (TY/3/67), and A/Netherlands/B1/1968 (NL/B1/68). To this end, male ferrets (Mustela putorios furo) were obtained from an accredited ferret breeder. All animals tested negative prior to the start of the experiments for antibodies against H1, H2, and H3 influenza A viruses, influenza B virus, and Aleutian disease virus. Ferret antisera were prepared by intranasal inoculation of the animals with the respective viruses, and antisera were collected 14 days after inoculation. Ferret housing and animal experiments were conducted in strict compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animal Act, 1997). The experimental protocol was approved by an independent animal experimentation ethical review committee ("Stichting Dier Experimenten Commissie Consult"). Animal welfare was monitored daily, and all animal handling was performed under sedation to minimize discomfort.

Viruses and cells

The following 18 A/H2N2 viruses were used in this study (GenBank accession numbers for HA genes in parentheses): A/Netherlands/M1/57 (KM402801), A/Netherlands/M2/57 (KM885170), A/Singapore/1/57 (CY125894), A/Netherlands/M1/58 (CY077741), A/Netherlands/N1/59 (CY077904), A/Netherlands/H1/60 (CY077786), A/Netherlands/67/63 (CY125886), A/Netherlands/K1/1963 (CY077733), A/England/12/64 (AY209967), A/Sydney/2/64 (KP412320), A/Taiwan/1/1964 (DQ508881), A/Moscow/56/65 (CY031603), A/England/1/66 (KP412318), A/England/10/67 (AY209980), A/Tokyo/3/67 (AY209987), A/Netherlands/61/68 (KP412319), A/Netherlands/B1/68 (KM402809), and A/Netherlands/ B2/68 (KM885174). Human A/H2N2 virus samples from the Netherlands were collected from individuals with influenza-like symptoms during the years 1957 to 1968. From these samples, virus isolates were obtained by culture in tertiary monkey kidney cells (tMK) and Madin-Darby canine kidney cells (MDCK) for a maximum of five passages, without prior inoculation in embryonated

chicken eggs. Complete HA genes of viruses A/Netherlands/M1/1957 and A/Netherlands/B2/1968 were amplified from low-passage viruses and cloned in a modified pHW2000 expression plasmid as described previously (100). Recombinant viruses consisting of the HA gene of A/H2N2 and the 7 remaining gene segments of A/Puerto Rico/8/34 (A/H1N1) were generated by reverse genetics (130). Introduction of mutations in the HA gene was performed using the QuikChange multisite directed mutagenesis kit (Agilent Technologies, Amstelveen, The Netherlands) according to the manufacturer's instructions. The presence or absence of mutations was confirmed by sequence analysis of the HA gene. Virus stocks were generated by inoculation of MDCK cells with 293T transfection supernatant. The inoculum was removed after 2 h and replaced by MDCK infection medium, consisting of Eagle's minimal essential medium (EMEM), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM HEPES, nonessential amino acids, and 25 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin. Subsequently, cells were incubated at 37°C and 5% CO2, and virus-containing supernatant was harvested 3 days after inoculation.

293T cells were cultured in Dulbecco modified Eagle's medium (DMEM; Lonza Benelux, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and nonessential amino acids (MP Biomedicals). MDCK cells were cultured in Eagle's minimal essential medium (EMEM; Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM HEPES (Lonza), and nonessential amino acids (MP Biomedicals).

Hemagglutination inhibition assays

HI assays using a panel of postinfection ferret antisera were performed as described previously (137). Briefly, ferret antisera were treated with receptor-destroying enzyme (Vibrio cholerae neuraminidase) and incubated at 37°C overnight, followed by inactivation of the enzyme at 56°C for one hour. Twofold serial dilutions of the antisera, starting at a 1:20 dilution, were mixed with 25 µl phosphate-buffered saline (PBS) containing four hemagglutinating units of virus and were incubated at 37°C for 30 min. Subsequently, 25 µl 1% turkey erythrocytes were added, and the mixture was incubated at 4°C for one hour. HI titers were read and expressed as the reciprocal value of the highest dilution of the serum that completely inhibited agglutination of virus and erythrocytes.

Computational analyses

Amino acid sequences of human A/H2N2 HA1 were aligned and analyzed by maximum likelihood phylogeny using PhyML 3.0 software (237). The sequence of avian A/H2N2 isolate A/mallard/Netherlands/31/2006 (GenBank accession number ACR58563) was used as an outgroup.

Antigenic maps were constructed as described previously (137). Antigenic cartography is a method for the quantitative analysis and visualization of HI data. In an antigenic map, the distance between antiserum point S and antigen point A corresponds to the difference between the log2 of the maximum titer observed for antiserum S against any antigen and the log2 of the titer for antiserum S against antigen A. Each titer in an HI table can be thought of as specifying a target distance for the points in an antigenic map. Modified multidimensional scaling methods are used to arrange the antigen and antiserum points in the antigenic map to best satisfy the target distances as specified by the HI data. The result is a map in which the distance between the points represents antigenic distance as measured by the HI assay, in which the distances between antigens and antisera are inversely related to the log2 HI titer. Since antisera are tested against multiple antigens, and antigens are tested against multiple antisera, many measurements can be used to determine the position of the antigen and antiserum in an antigenic map, thus improving the resolution of interpretation for HI data.

The amino acid positions responsible for major changes in HI patterns were plotted on the surface of the crystal structure of A/Singapore/1/1957 HA (PDB accession number 2WR7) (154) using MacPyMOL (The PyMOL Molecular Graphics System version 1.3; Schrödinger, LLC).

Overall rates of evolutionary change (nucleotide substitutions per site per year) were estimated using the BEAST program version 1.8.1 (238), the uncorrelated log-normal relaxed molecular clock, and the HKY85 substitution model (239). This analysis was conducted with a time-aware linear Bayesian skyride coalescent tree prior (240) over the unknown tree space, with relatively uninformative priors on all model parameters using the GTR+G+I model with no codon positions enforced. Two independent Bayesian Markov chain Monte Carlo (MCMC) analyses for HA1 for 50 million states, sampling every 5,000 states, were performed. Convergences and effective sample sizes of the estimates were checked using Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/), and the first 10% of each chain was discarded as burn-in. Uncertainty in parameter estimates is reported as values of the 95% highest posterior density (HPD).

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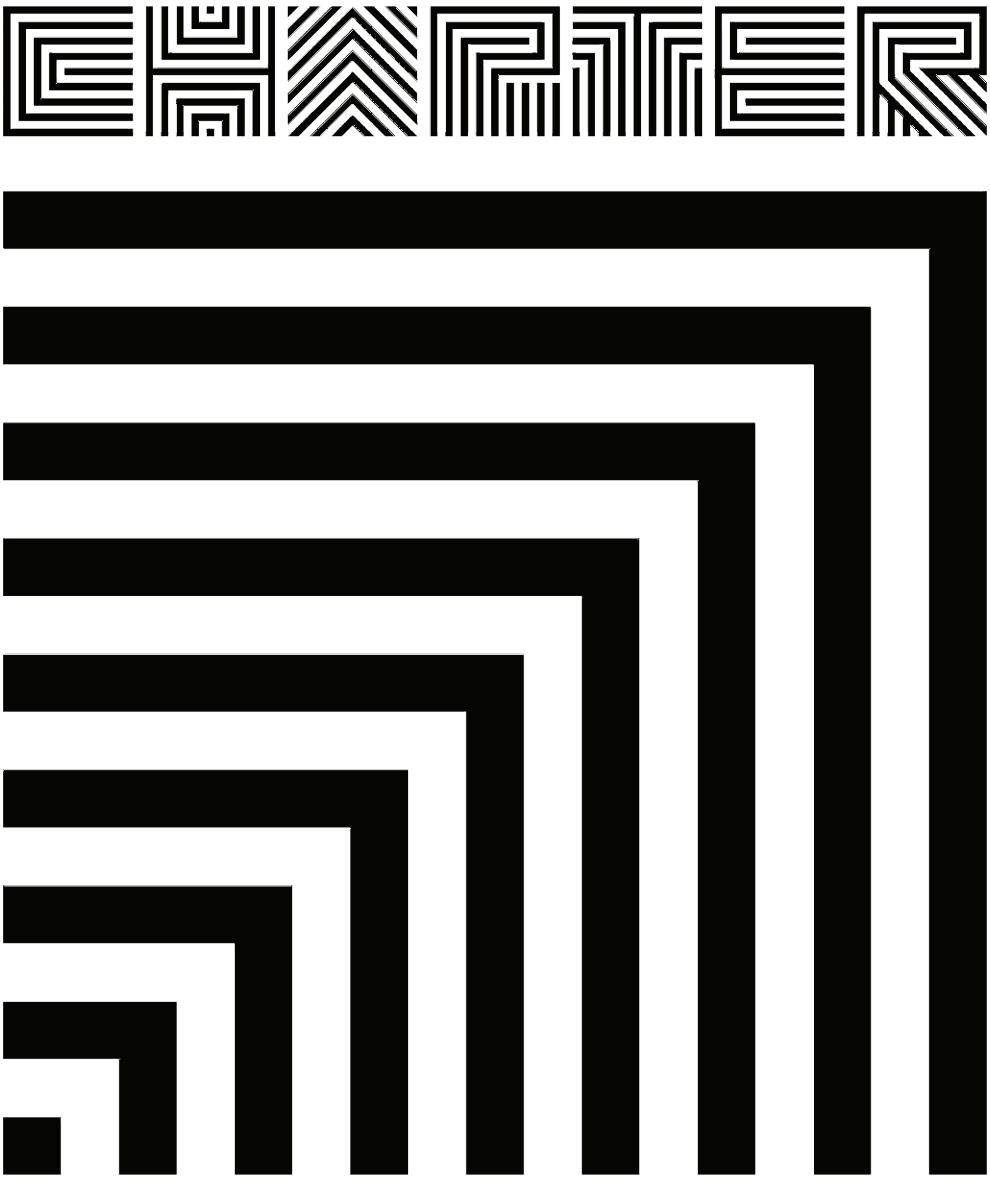

Summarizing Discussion

partially based on

“Transmission of influenza A/H5N1 viruses in mammals”

Virus Research, 2013

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The influenza A virus strain or subtype that will most likely cause the next influenza pandemic is a highly-debated topic in the influenza field. There are experts who believe that only influenza A viruses of the H1, H2, and H3 subtypes can cause pandemics, as no other virus subtypes are known to have caused pandemics in the past. If the number of subtypes of concern indeed was so low, the world would not need to be worried about zoonotic infections caused by avian influenza viruses including subtypes H5N1, H5N6, H7N7, H7N9, H9N2 or H10N8 that were associated with poultry outbreaks, beyond their direct impact on animal health and the limited numbers of human cases of infection (35, 54, 75, 252-255). In the opinion of some experts, avian influenza viruses are unlikely to be the direct source of pandemics and pigs are required to serve as an intermediate host to facilitate initial host adaptation or genetic mixing ("reassortment") between avian and mammalian viruses. Despite differences in opinions, most influenza experts do agree on one thing: there will be a future pandemic of unknown severity and the pandemic virus will likely be airborne transmissible, as this is a property shared by pandemic and epidemic influenza viruses that have been identified to date.

Influenza A viruses are well known for their genetic and phenotypic diversity in avian and mammalian hosts. Many determinants of influenza A virus cross-species transmission and pathogenicity have been identified in recent years with the advent of reverse genetics techniques. In sharp contrast, the determinants of airborne transmission of influenza viruses between humans or other mammals have still remained largely elusive. Recently, we (chapter 2) and others (125, 141, 142) reported important discoveries towards understanding airborne transmission of influenza A virus, using influenza H5N1 viruses and ferret and guinea pig transmission models. These discoveries have contributed to increased understanding of the genetic and phenotypic viral factors as well as host cell factors that drive transmission between mammals. This line of research may ultimately lead to enhanced predictability of influenza virus subtypes possibly causing future pandemics.

Pandemic preparedness comprises a wide range of aspects ranging from strategic stockpiling of antiviral drugs and potential pre-pandemic vaccines to medical emergency planning. To prevent a new influenza virus pandemic from happening in the first place, surveillance programs directed to the reservoir and spillover hosts in question and to the viruses that are expected to pose the biggest risk for public health are of great importance. Knowledge of the molecular changes that facilitate pandemic emergence may have added value in such surveillance programs.

To elucidate the molecular changes required for the generation of pandemic viruses, there are two main general approaches. Firstly, the examination of pandemic viruses in comparison to their counterparts in the reservoir species (e.g. avian vs human H2N2) and secondly, the adaptation of zoonotic viruses

to a mammalian host in a laboratory setting. Both approaches were used in this thesis. The detection and characterization of naturally selected substitutions that emerged during repeated passage of a highly pathogenic avian influenza (HPAI) H5N1 influenza virus in ferrets, and that ultimately conferred airborne-transmissibility between ferrets were characterized in chapters 2 and 3. Furthermore, human pandemic H2N2 influenza virus isolates were compared to avian (progenitor) H2 isolates in terms of their genetic and antigenic characteristics and capability to transmit between ferrets in chapters 4, 5 and 6.

Transmission and phenotypic studies on H5N1 viruses

The inefficient airborne transmission of HPAI H5N1 virus has been demonstrated in several mammalian models including mice, guinea pigs, and ferrets. Several studies conducted with either wild-type H5N1 viruses, H5N1 reassortants with H3N2 or pH1N1 viruses, H5N1 viruses adapted via repeated passage, or genetically modified H5N1 viruses have failed to yield H5 viruses that were airborne transmissible, but showed direct-contact transmission in some instances (89-92, 98, 126, 256). However, more recently, four studies have obtained H5N1 airborne transmissible viruses independently. A comparison of substitutions required in each of the viruses as well as the associated phenotypes may lead to a more comprehensive picture of the requirements for airborne transmission of H5N1. Several substitutions in the HA of H5N1 viruses, including Q222L, G224S, E186D, K189R, S223N, and N182K, resulted in decreased binding to α-2,3 linked sialic acids and/or increased binding to α-2,6 linked sialic acids (91, 103, 118, 257). However, none of the genetically modified H5N1 viruses displaying a receptor switch alone were capable to transmit via the air, despite preferentially binding α-2,6 linked sialic acids (91). Therefore, at least with respect to HPAI H5N1 viruses, human-type receptor recognition alone was not sufficient for airborne transmission among mammals (125, 140-142). Moreover, subtle differences in receptor preference beyond simple α-2,3 versus α-2,6 sialic acid specificity may be required for efficient transmission to occur. Hence, the tissue specific glycan composition in anatomical structures of the respiratory tract implicated in influenza A virus transmission should be elucidated in detail to understand the receptor requirements of transmissible viruses to the fullest extent (e.g. by shotgun glycan arrays, lectin stainings, mass spectrometry).

Interestingly, the loss of an N-linked glycosylation site at residues 158–160 in HA was shared by the transmissible H5 viruses identified in four independent studies (125, 140-142). Previous studies have shown that the disruption of this glycosylation site by substitution T156A results in enhanced binding of H5N1 viruses to human-type receptors (135, 258). Moreover, the loss of this glycosylation site has been reported to contribute to the efficient replication in the upper respiratory tract of ferrets of H5N1 viruses that also possess the

Q222L or Q222L/G224S substitutions (135). Furthermore, the glycosylation of the HA has implications on antibody-mediated recognition of viral epitopes, receptor avidity and T-cell mediated immunity (259). Increased virulence of heavily glycosylated H5N1 viruses mediated by an exacerbation of the host's immune system has recently been described (260). Glycosylation sites in the head domain of H5 HA appear to determine the degree of receptor binding and antibody recognition, a balance that might be important for airborne transmission to occur and perhaps for virus establishment in the human population.

Mutations near the receptor binding site often destabilize the HA molecule, reviewed in (261), and compensatory stabilizing substitutions elsewhere in the protein might be required for efficient airborne transmission. The switch in receptor binding preference was shown to be independent of HA stabilizing substitutions in airborne transmissible H5N1 viruses (150). The fact that substitution T315I as described by Imai et al. and H103Y identified by us had a similar stabilizing effect on the HA molecule suggests that this is an important phenotypic trait of airborne H5N1 influenza viruses. Similarly, substitutions N220K and Q222L were essential to alter receptor binding and contribute to airborne transmission in Imai et al., while we report the combination of mutations Q222L and G224S to have a similar effect. Varying combinations of substitutions in the head and stalk domain of HA, while considering differences in the structural makeup of individual HAs in diverse virus isolates, might ultimate lead to a switch in receptor preference, HA stability and in combination with a human adapted polymerase complex confer airborne transmission.

In order to predict the likelihood of emergence of a certain number of mutations in a single virus, Russell et al. (262) mapped the diversity of H5N1 viruses worldwide. Furthermore, (combinations of) substitutions in the two sets of substitutions (HA H103Y, T156A, Q222L, G224S in Herfst et al. and N154D, N220K, Q222L, T315I in Imai et al. in addition to PB2 E627K and PB1 H99Y) leading to airborne transmission were identified that were already present in naturally occurring isolates and the statistical chance was investigated for airborne transmissible H5N1 viruses to arise in nature. Two phenotypic changes were frequently found in H5N1 viruses (loss of glycosylation in the head-domain of HA (substitutions N154D or T156A) and increased polymerase activity due to E627K in PB2), reducing the number of substitutions required for a naturally occurring virus to become airborne transmissible between mammals. Other yet unidentified substitutions might have a similar effect and contribute to an airborne-transmissible phenotype as described by Herfst et al. (140) and Imai et al. (125). Clade 2.2.1 H5N1 influenza viruses with a preference for human-type receptors were especially prevalent in Egypt (263, 264) and may be relatively close to an airborne transmissible phenotype, since they harbor one or two substitutions

contributing to airborne transmission already (159). We estimated that the remaining mutations to yield airborne-transmissible H5N1 viruses between mammals based on the sets of substitutions identified by Imai et al. and us might arise within one mammalian host, although at very low frequency. However, it is impossible to make explicit predictions on the likelihood for these mutations to accumulate in a single virus, because the exact selection mechanisms resulting in fixation of individual substitutions are unknown. Richard et al. reported that only the combination of substitutions leading to enhanced polymerase activity, HA stability and altered receptor binding preference in H5N1, but not viruses with individual phenotypic alterations, lead to viral attenuation in chickens (265). This confirms that individual mutations can accumulate in viruses in their natural host without a detrimental effect on replication, before a zoonotic event allows further adaptation to increased replication and transmission between mammals.

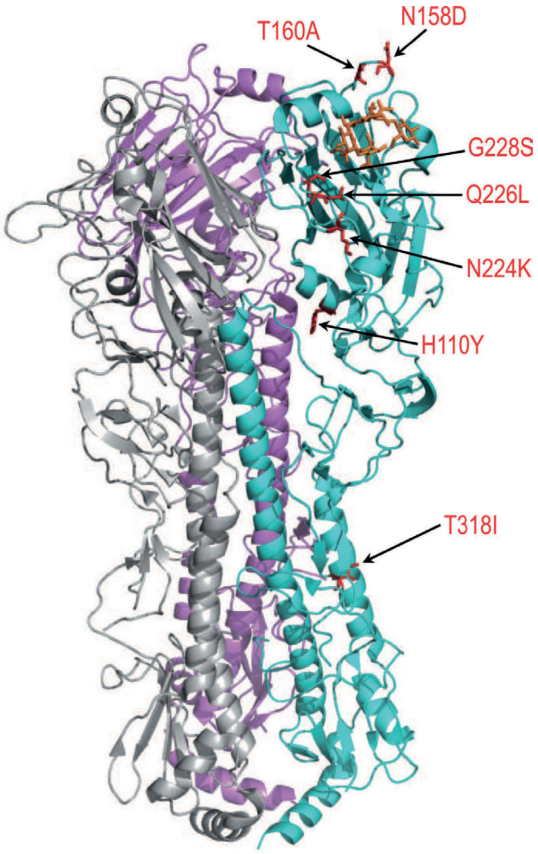


Figure 1: Localization of amino acid changes
identified in our studies and Imai et al. (125) on the three-dimensional structure of the trimer of A/Vietnam/1203/2004 (H5N1) HA (Protein data bank (PDB) code, 2FK0)
The mutations that support airborne transmission between ferrets are shown in red. All mutations are shown in H3 numbering. The human receptor analog (derived from its complex with H9 HA (PDB, 1JSI); shown in orange) is docked into the structure.

H5Nx and other potentially pandemic influenza A subtypes

While H5 viruses with an NA other than N1 are frequently detected in waterfowl, H5N1 remained the only HPAI H5 virus capable of causing human infection until 2014, when an H5N6 virus infected humans in China (266, 267). To date, there have been 23 confirmed human H5N6 cases including 7 deaths reported from China (266). H5N6 viruses have become predominant in poultry in China since, replacing H5N1 entirely (268). Strikingly, the internal genes of the H5N6 virus originated from avian H6N6, H7N9 and H9N2 viruses, suggesting multiple reassortment events in hosts other than humans (268). HPAI H5 viruses have since reassorted with viruses containing N2, N3, N5, N6 and N8 genes (269).

Influenza viruses of the H7 subtype accounted for the majority of zoonotic avian influenza virus infections before the emergence of HPAI H5N1 viruses. Infections with H7 influenza viruses were observed in people handling infected live poultry during outbreak events in the Netherlands (2003, H7N7), Canada (2004, H7N3), the USA (2007, H7N2), and the UK (2007, H7N2) (270, 271). H7N9, still a low pathogenic avian influenza virus by then, kept causing human infections in waves between 2013 and 2017. Zoonotic H7N9 viruses harbored a range of human adaptation markers and displayed limited airborne transmission between ferrets, in which transmission events occurred more often than with typical avian viruses, but less frequent than with human-adapted influenza viruses (41, 42, 166, 272, 273). Mutations in the HA of H7N9 have been described that confer high human type receptor affinity in an acid and thermostable HA (274, 275). Another study reported varying degrees of binding to and replication in cells of the upper respiratory tract of ferrets and monkeys, characteristics that are unusual for avian influenza viruses (276). In July 2016, a HPAI H7N9 virus variant that possessed multiple basic amino acids in cleavage site of HA was detected and in the subsequent year, HPAI H7N9 viruses were identified in humans (277). Mass vaccination of poultry with a bivalent H5/H7 vaccine has been introduced in Guandong Province, China in 2017 and has led to a substantial reduction of H7 viruses found in live poultry markets and observed human H7N9 cases (278). H7N9 has evolved into multiple genotypes with enhanced virulence in humans compared to early isolates (279). Influenza A virus subtypes H6N1, H9N2, H10N7, H10N8 have also been described to incidentally infect humans (280-283) and add to the pool of potentially pandemic viruses.

Our studies indicate that mutations in NA are not strictly required to lead to airborne H5N1 viruses. Nevertheless, the current detection of H5Nx viruses might imply the varying combinations of NAs and internal genes are not yet sufficient to improve the fitness of current H5 viruses to infect humans efficiently. In this context, it seems necessary to further characterize the functional balance between receptor affinity of HA and the enzymatic activity of NA for these substrates. H5N6 viruses have been described to possess

increased human receptor preference, but lack additional mammalian adaptation markers (284). They spread efficiently via direct contact, but cannot transmit via the airborne route between ferrets (284, 285). At this point it is impossible to predict which other phenotypic traits are still required, beyond receptor binding preference, HA stability and an optimal polymerase complex, before an H5Nx virus evolves that can spark a new pandemic. A fitness valley might exist that cannot be crossed by acquiring individual mutations, but reassortment with other viruses might allow the generation of H5 viruses that are fitter in terms of replication and transmission than A/Goose/Guangdong/1/96-derived H5 viruses.

Genetic, antigenic and transmission studies on H2N2 viruses

In chapters 4, 5 and 6, we describe the genetic, antigenic and transmission aspects of viruses that led to the emergence of human H2N2 viruses in or before 1957, and which caused the so-called 'Asian' influenza that originated in East Asia. The 'H2N2 Asian flu' was the first pandemic during which influenza virus isolates were obtained from infected patients and were preserved to date.

Genetic evidence strongly suggests viral divergence and multiple reassortment events before the introduction of the pandemic H2N2 virus into humans in 1957 (184, 186). Genetic comparisons of recent avian H2N2 virus isolates as a substitute for avian viruses circulating before 1957 and pandemic human H2N2 viruses revealed changes that were likely required during the evolution towards a human transmissible virus and are assessed in chapter 4. Eurasian avian, North American avian and human H2N2 influenza virus sequences each form a distinct clade in the maximum-clade credibility temporal tree indicating different regions of circulation and/or host populations. The time to the most common recent ancestor and nucleotide substitution rates indicate high variability of HA as well as augmented variation of avian origin NA and PB1 compared to human adapted gene segments. High ratios of nonsynonymous versus synonymous substitutions (dN/dS ratio) in HA further suggest increased evolutionary pressures. This variation possibly reflects the viral diversity in varying avian hosts and the expendability of additional adaptation to humans after zoonoses occurred.

The antigenic variation of H2N2 viruses during its time of circulation in humans between 1957 and 1968 is described in chapter 5. Nucleotide changes and resulting amino acid differences in the influenza virus genome are introduced randomly due to the lack of proofreading activity of the viral polymerases and are selected for in the host in which the mutation of the viral genome occurred and during repeated infections with the slightly altered virus. Characteristics that determine the fitness of a virus that can be targeted by natural selection are numerous and include replication speed, modes to evade the host's immune system including escape from preexisting antibodies, spread to

naïve hosts and others. Viral spread via the airborne route is considered a fitness advantage of respiratory viruses and is considered a prerequisite for pandemics to occur. Airborne transmissibility of human H2N2 viruses as an important feature of potential pandemic influenza viruses is assessed here in the ferret model. Six recent avian H2N2 virus isolates were found unable to spread via the airborne route, which is in line with a previous report (170). Airborne transmission between ferrets was only observed in one instance when a human virus isolate from 1958 was tested, that displays complete human receptor binding preference based on the amino acids at positions 226 and 228 of HA. Individual isolates from 1957 (A/Netherlands/M1/1957) and 1968 (A/Netherlands/B1/1968) representing the earliest and latest Dutch human isolates failed to transmit via the airborne route. This is in contrast to seasonal H1N1, seasonal H3N2 and pdm(H1N1)09 viruses, that are more readily transmissible via the air (95). In the case of H5N1, a possible scenario for adaptation is not unlike the findings for H2N2 in which a virus that can be transmitted via direct contact only ultimately obtains the capacity to spread via the air after the infection of a sufficient number of naïve hosts.

A complete preference for α-2,6 linked sialic acid might be required for H2N2 viruses to obtain airborne transmissibility. A/Netherlands/M1/1957 has only partial human receptor preference, suggesting that early human H2N2 virus transmission between humans may have occurred inefficiently, and perhaps primarily by direct contact transmission, as previously suggested by Pappas et al (70). A/Netherlands/B1/1968 appears to be fully adapted to humans based on its receptor binding preference and its circulation time in humans, but it lacks the capacity to transmit via the air. Furthermore, it displays a slightly delayed replication pattern in ferrets. In general, all H2N2 virus isolates show a rather low virus titer in directly inoculated animals compared to other human virus subtypes that were previously tested in ferrets. These characteristics might represent an only partially human- adapted phenotype and might have facilitated the successful establishment of an H3N2 virus after only 11 years of circulation of H2N2 viruses in humans. In part, these assumptions are supported by studies that found higher rates of adaptation in viruses that comprise a 1957 pandemic polymerase complex compared to viruses with PB2, PB1, PA, but not NP from viruses isolated after 1958 (286). Alternatively, the ferret model may be less permissive to H2N2 influenza viruses as compared to other human virus subtypes. Surveillance for known human adaptation markers in influenza virus isolates derived from avian species might further reduce the chance of reintroduction of H2N2 viruses and other virus subtypes into the human population.

To study the antigenic evolution of human H2N2 virus isolates spanning from 1957 to 1968, antigenic maps using ferret postinfection antisera were analyzed and revealed a directional evolution with increasing divergence of later isolates. A temporal directionality and the importance of substitutions

near the receptor binding site was also observed in similar studies on A(H1N1) pdm09, H3N2 and H5N1 (223, 224, 226). In particular, the study on A(H1N1) pdm09 suggest that the number of escape mutants is limited due to exposure to seasonal H1N1 viruses in large parts of the population, whereas the work on H3N2 and H5N1 establishes a range of mutations in the headdomain of HA that mediate major antigenic drift. In H3N2, one or two substitutions were sufficient to mediate transition from one antigenic cluster to the subsequent one. While no clusters were defined in the H2N2 dataset, the positioning of the antigens in the antigenic map and the fact that we find two mutation to be important determinants for antigenic drift, might suggest the presence of only two antigenically distinct groups of viruses. The rate of antigenic evolution of H2N2 viruses in years 1957 to 1968 is significantly lower than that of H3N2 (223, 233). This slower rate of antigenic evolution might be indicative of a restricted ability to tolerate substitutions without leading to a detrimental phenotype. A recent study described that individuals born before 1968 in the US and Hong Kong still had antibody titers that might reduce the frequency of transmission events and thereby the spread of an H2N2 virus (287).

General remarks on (potentially) pandemic influenza viruses and recent developments

The approach of serial passage of viruses was already used by famous Louis Pasteur in 1881 during the development of a rabies vaccine candidate by repeated virus inoculation of rabbits (288). Notably, repeated passage in laboratory animals is typically employed to generate viruses with decreased pathogenicity in the original host compared to the seed virus. The concept of host barriers, defining species related physiologic peculiarities that allow or prohibit a particular virus from replication in alternative hosts, is generally accepted (289). Multiple host barriers must be surmounted before a zoonotic influenza virus can establish in the human population (290). Mutations leading to these ‘host jumps’ might happen in the reservoir species by chance or might arise in the new host after ‘accidental’ initial infection and be selected for in subsequent rounds of replication.

As mentioned previously, there are several viral phenotypic prerequisites contributing to airborne transmission of influenza A viruses, including HA stability, increased replication in the upper respiratory tract, altered receptor binding and perhaps others. Only the sum of these changes allows a virus to spread via the air. Selection for viruses that transmitted via the air in a zoonotic event is likely to be stringent, thereby reducing in-host-variation after each transmission event. Varble et al. have quantified the genetic bottlenecks involved in different transmission routes including airborne transmission by molecularly tagged individual viruses in a mixture of otherwise identical viruses. The proportions of each of these viruses after

each transmission event was assessed and revealed a robust reduction in the number of viruses that were capable to transmit via the airborne route and cause infection in the recipient host (291). Another recent study describes low in-host variation of pandemic H1N1 viruses and genetic similarity of virus populations detected in household contacts and epidemiologically confirmed direct transmission cases (292). The intrahost variation of H5N1 viruses has been found to expand during replication in ferrets and to be drastically reduced upon respiratory droplet transmission, confirming the presence of a strong bottleneck effect during influenza virus transmission (293). In contrast, direct contact transmission of influenza viruses has been found to impose a rather loose bottleneck compared to airborne transmission (294) The effective bottleneck population sizes during transmission events of pandemic H1N1 and H3N2 was estimated at 192 and 196 viruses, with large variation between transmission pairs, confirming strong selection pressures during airborne transmission (295). A study investigating the transmission patterns and bottleneck sizes of an '1918-like' avian H1N1 virus in ferrets describes loose bottlenecks during the initial adaptation of the virus to the mammalian host and much more stringent selection during airborne transmission (296). The authors further describe the initial diversification of the HA by acquiring varying mutation, the subsequent reduction in the diversity of HAs and finally the occurrence of polymerase variants. This order of events leading to airborne transmissible influenza A viruses might be generalizable.

Knowledge of the phenotypic properties associated with a particular substitution is crucial, because this phenotype rather than the actual mutation can more easily be translated directly to other virus subtypes. Furthermore, this approach enables the identification of other substitutions leading to a similar virus phenotype (i.e. functionally equivalent mutations). As an example, the description of substitution E627K in PB2 only allows for the screening of presence or absence of this particular mutation, while the phenotypic description of this substitution (increased virus replication in mammalian hosts) permits the definition of a potential phenotypic prerequisite for pandemic viruses and the genome-wide screen for functionally equivalent mutations that increase viral replication (297-299). Many additional studies have identified functionally equivalent mutations that affect receptor binding of HA (103, 119, 300) and polymerase activity (301, 302). In addition, not all substitutions or combinations of mutations will have the exact same effect in all viruses, since interactions between proteins (e.g. the polymerase complex) are possibly playing a crucial role for all essential viral processes to be functional (e.g. receptor binding and release). Of note, pdm(H1N1)09 only occurred after the complex reassortment events between avian H1N1, classical swine H1N1, and seasonal human H3N2 and the sporadic infection of humans with swine reassortant viruses, that ultimately obtained the M and NA gene from Eurasian swine viruses to result in the pandemic virus

(67). The structural location of the two substitutions determining major antigenic drift in the HA of H2N2 as described in chapter 5 have equivalent mutations in the headdomain of H3N2 HA. Yamada et al. have structurally compared the substitutions found in H5N1 viruses isolated from humans and pdm(H1N1)09 and describe different amino acid substitution in the PB2 gene to result in similar phenotypic effect for both subtypes (303). Substitutions in different viral proteins might act concomitantly and interdepend. The recent resolution of the atomic structure of the heterotrimeric polymerase complex facilitates the localization of these adaptive substitutions in the viral polymerase complex and adds greatly to the understanding of influenza virus replication (304).

Influenza A viruses are pleomorphic and basically adopt two shapes; whereas filamentous influenza viruses have been observed mainly in clinical isolates, and are selected for in vivo, spherical particles have a fitness advantage in embryonated eggs (305, 306). Future research has to identify the requirements for airborne transmissibility of influenza A viruses with regards to the shape of its virions. High levels of virus replication in susceptible and permissive cells leading to increased virus titers are mainly associated with viral polymerase activity, but a combination of high titers of infectious virus and the possibility for incorporation of viruses in aerosols or respiratory droplets, which could be virion-shape dependent, might be required for airborne transmission to occur.

The stability of the HA protein was previously not recognized as a factor that contributes to airborne transmissibility. However, the pH of fusion of viral and cellular membranes was previously implicated with host range and adaptation though (145). The two experimental assays that we use to quantify the HA stability (pH-dependent cell-cell fusion assay and temperature stability assay) are indirect measures of the viral phenotype potentially required for airborne transmission of H5N1 virus. Whereas a lower pH of fusion might be implicated with adaptation to the cell for increased virus replication, HA stability might be required for the physical stability of the virion in the aerosol or respiratory droplet, or might hint us towards a yet to be identified viral phenotype with direct implications on airborne transmission. This newly identified phenotypic requirement has been the focus of research since. Batishchev et al. have described the possibility for disintegration of the viral M1 scaffold only at relatively low pH values, directly linking relative acidity to the disintegration of the viral core (307). Furthermore, in the case of pH1N1, a strong correlation of destabilizing substitutions leading to an increase in fusion pH and the loss of airborne transmission and decreased pathogenicity in the ferret model was established (308). Substitutions affecting HA stability have also been shown to be conserved between different influenza virus subtypes (309) and increased acid stability of H5 was found to boost viral replication in the upper tract of ferrets (158).

Other factors that might determine host range and modes of virus transmission are encoded by additional viral genes, including PB2 and NP that regulate the virus interaction with importins to facilitate virus genome delivery into the nucleus (310); NA, essential for receptor cleavage and budding of infectious virions that can then be incorporated in aerosols and respiratory droplets; the NS2 gene, also called NEP, that has a role in RNA replication by interaction with the PB1 and PB2 polymerase subunits (311); and the evasion of host immunity mediated by NS1. Furthermore, a single substitution can have more than one modulating effect, exemplified in the case of substitution E627K in PB2 that besides virus replication also affects virus recognition by cellular sensors (312).

We are using ferrets (mustela putorius furo) in our studies, because they display clinical symptoms and pathological changes similar to those observed in humans (249). Ferrets can be infected with a range of avian and human influenza viruses, whereby all avian virus isolates do not and all human-adapted viruses do have the ability to transmit via the airborne route (chapter 3). Buhnerkempe and colleagues have described a good correlation between secondary attack rates – defined as the probability of infection of a susceptible individual following contact with an infected individual – between humans and ferrets, underlining the importance of the ferret model as a substitute for humans (313). Efforts to study the physical requirements for airborne virus transmission have started in the 1970's and many special devices have been described to date (314). Air samplers can determine the size of particles in the air and have been used to determine that infectious influenza virus is mainly found in exhaled particles of less than 5 micrometre in diameter (315, 316). Impactors and impingers are used to collect particles from the air by either forcing the gas to make a sharp bend inside the device, creating centrifugal turbulences, or by electrostatic precipitation. Several devices are successfully employed to detect infectious virus in air samples (317, 318). Nebulizers generate aerosols by varying methods and can be used in infection studies with virus-laden droplets to mimic a more natural way of infection compared to standard methods (319). Another important aspect to the use of these devices is that they will ultimately reduce the number of laboratory animals employed in transmission studies and perhaps increase the statistical power of transmission research. Limitations of the transmission experiment as used throughout this thesis include mechanical parameters of the transmission setup, biological variation of the laboratory animals and their seronegative immune status for the influenza A subtypes tested. Although the genetic variation of outbred ferrets is bigger compared to often preferred inbred animal lines, they cannot reflect the genetic variation of humans worldwide by far. In the vast majority of studies, animals with no detectable antibody titers against currently circulating viruses and the subtype studied are used to obtain reproducible results and to ensure robust

infection after virus inoculation. For example, reduced virus replication and transmission between ferrets was observed when HA stalk antibodies were transferred to the infected ferret (320), emphasizing the role of immunity on transmission. In addition, the role of pre-existing (often heterosubtypic) immunity on excess morbidity and mortality in a pandemic scenario is poorly understood and needs to be addressed in dedicated studies (321). Experiments involving animals with pre-existing immunity against influenza viruses should therefore be conducted in the future to even closer mimic an outbreak scenario in humans. These studies have become even more important since Laurie et al. (322) have described the outcome of co-infection studies in ferrets with varying timing and order of virus infections to range from a complete block of secondary infection to delayed or simultaneous coinfection. In general, extrapolations from experiments involving laboratory animals with the aim to comment on potential Public Health threats should be done with the required care and prudence.

The addition of bats as hosts for influenza viruses (5), the detection of new variant lineages in dogs, horses, pigs and humans (323, 324), increased frequency of so-called reverse zoonoses (325), and the generation of influenza viruses of subtypes other than H5 and H7 with proven capacity for airborne transmission, that have not caused human infections so far (326) might further increase the possibility for the emergence of a pandemic influenza virus, and are a reminder to not ease pandemic preparedness efforts. Other groups of respiratory viruses including virus families Coronaviridae and Paramyxoviridae also pose the threat of large-scale outbreaks in humans (e.g. Middle East Respiratory Syndrome (MERS) coronavirus and Nipah virus). Many of these viruses are only poorly understood in terms of transmission modes and molecular determinants for efficient human-to-human spread.

Future perspectives

Pandemic preparedness is a multifactorial endeavor with the noble aim to prevent a future pandemic or at least mitigate the impact of it. Fundamental science is one of the least obvious, but possibly most influential aspect of these efforts. The studies on airborne transmission of H5N1 viruses urge the political decision makers to intensify surveillance efforts particularly in countries where H5N1 and other zoonotic influenza A viruses are endemic, as well as worldwide. Appropriate measures to prevent large-scale outbreaks of HPAI H5N1 virus in poultry such as effective vaccines and quarantine seem warranted. To prevent further virus spread in an outbreak scenario, timely preventive culling in a predetermined perimeter around the initial outbreak might be necessary as an ultima ratio. Since close contact to infected poultry is one of the main risk factors for human infection with avian influenza viruses, proper hygiene measures especially during slaughtering and separation of poultry housing and human homes should be the norm.

Viral sequences of H5N1 virus isolates can now be analyzed for substitutions that were shown to be required for airborne transmission between mammals. The ability to screen for potential adaptation markers early after the detection of a novel virus allows for the exclusive application of the more impactful control measures, which include culling, to outbreaks with an elevated risk for humans. Functionally equivalent mutations should be identified and be added to the repertoire of substitutions to be screened for. The relatively fast emergence of adaptive mutations during passaging of H5N1 viruses in ferrets hints towards the fact that these substitutions are non-detrimental for these viruses and can also be naturally acquired in alternative hosts. Replication of viruses in humans and transmission between them poses the risk of acquiring additional human-adaptive mutations. Substitution H103Y in HA is associated with the previously unrecognized phenotype of protein stability. Future studies have to prove if this change in viral characteristics is linked to timing of replication (especially membrane fusion of virus and endosome resulting in viral genome release into the cytoplasm), virus stability required for infectivity of virus-laden aerosols or respiratory droplets, a combination of these processes or another yet unidentified factor.

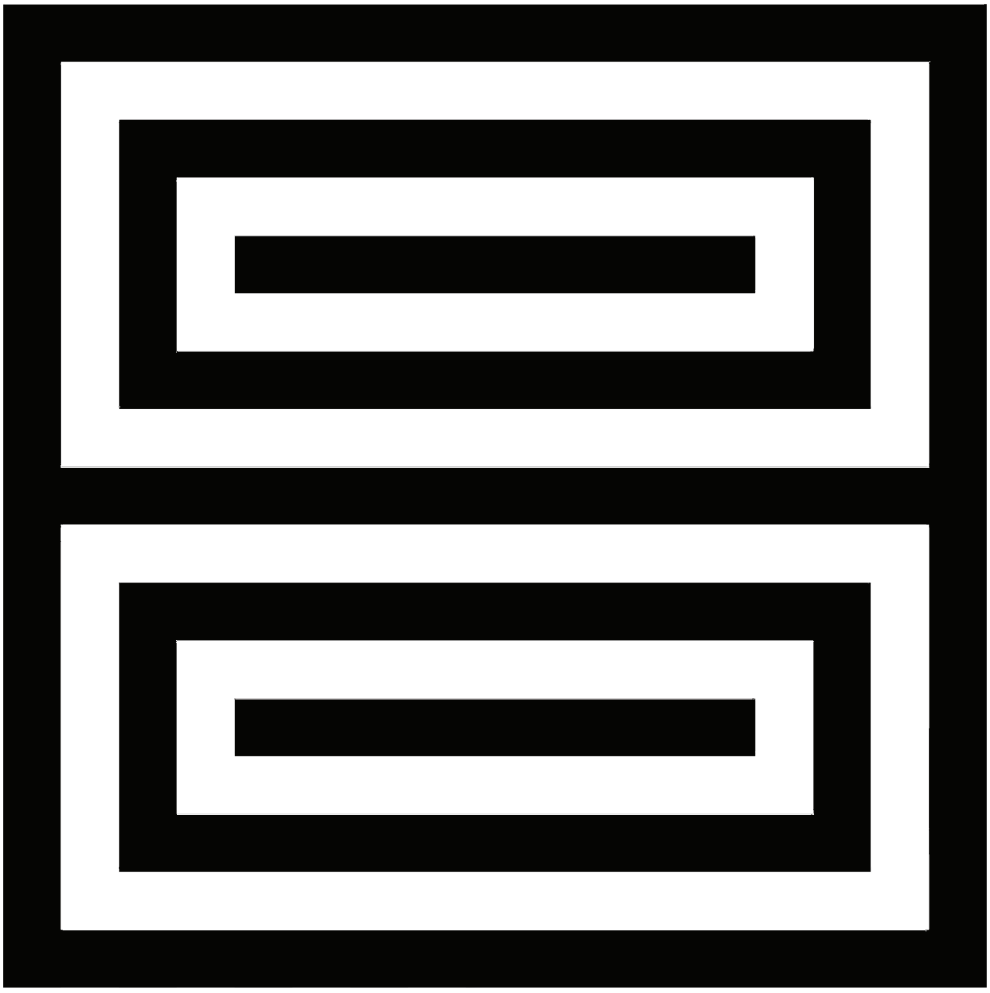
Amino acid positions that were identified to be under selective pressure during the host jump from avian to human in H2N2 viruses can be added to the screening repertoire. Currently circulating avian H2N2 influenza viruses are not directly transmissible between mammals, which is in line with the observation that reassortment was necessary for H2N2 viruses to become pandemic in humans. Furthermore, early human isolates have been found not to be readily transmissible via the airborne route between ferrets. These findings do not rule out the possibility of emergence of a new H2N2 pandemic virus, either with or without an intermediate host, but the imminent threat from avian H2N2 viruses to spark a pandemic seems moderate. Substantial levels of antibody-mediated immunity in large parts of the population directed against H1 and H3 virus isolates were reported (327). These antibodies might provide basic levels of protection, e.g. by binding conserved epitopes in the stalk region. Although, as exemplified by the 2009 H1N1 pandemic, this baseline antibody reactivity might not prevent human virus infection altogether, it might lower the spread of virus and alleviate morbidity in a pandemic scenario. More specific studies to assess pandemic potential and the close monitoring of human adaptation mutations in these viruses is thus warranted, because of the proven potential of H1, H2 and H3 viruses to cause pandemics.

In our studies, we have identified the viral traits that are required to render a fully avian H5N1 virus airborne-transmissible between ferrets. Additional studies need to demonstrate if these phenotypic changes are specific for the chosen virus isolate or the group of genetically similar viruses it belongs to (here clade 2.1.) and if they can be translated to other influenza virus

subtypes as well. Future research extending from these studies should thus be focused on the identification of other influenza subtypes with the potential to become pandemic in humans, the identification of functionally equivalent mutations leading to airborne transmissible viruses and the screening for these substitutions as well as their associated phenotypes in surveillance efforts. Further characterization of the events leading to the generation of 'new' pandemic viruses and the chance estimation for reintroduction of previously circulating pandemic viruses are required.

The political dimensions of our studies on airborne transmission of H5N1 virus were somewhat unexpected and unprecedented. All parties involved in research with the potential for misuse, also termed 'Dual-Use Research of Concern (DURC)' should identify their studies as such and collectively implement measures to minimize associated risks. A careful and meaningful exploration of potential benefits and risks in DURC studies seems highly needed, but appears particularly challenging. Efforts need to be made to allow and advance these important lines of research while preventing unintentional or intentional misuse. Only by knowing which pathogens mother nature is capable of producing, we might be able to better prepare for a prospective outbreak.

CHAMBER



Nederlandse Samenvatting

Deutsche Zusammenfassung

Griepvirussen, oftewel influenzavirussen, zijn een groep ziekteverwekkers die via de luchtwegen het menselijk lichaam kunnen binnendringen en zich daar vermenigvuldigen. Deze infectie veroorzaakt typische ziekteverschijnselen zoals koorts, keelpijn, hoesten, hoofdpijn en vermoeidheid. In ernstige gevallen kunnen griepvirussen een longontsteking veroorzaken, die zelfs tot de dood kan leiden. Influenzavirussen worden onderverdeeld in types A, B, C en D. Influenza A virussen worden verder ingedeeld in subtypes gebaseerd op de eigenschappen van de oppervlakte-eiwitten hemagglutinine (HA) en neuraminidase (NA). De combinatie van deze eiwitten wordt gebruikt in de naamgeving van de subtypes, en leidt tot afkortingen zoals H1N1 of H3N2. Er zijn 16 HA en 9 NA subtypes in watervogels, de gastheer voor de meeste influenzavirussen, en twee mogelijke HA en NA subtypes in vleermuizen beschreven. Voorbeelden van dieren die ook met influenzavirussen besmet kunnen worden zijn pluimvee en varkens, maar ook tijgers en zeehonden.
Als virussen zich vermenigvuldigen in een cel van de gastheer, ontstaan er continu mutaties in het virus genoom. Deze mutaties gebeuren spontaan en aspecifiek en zijn dus meestal ongunstig voor het virus. Echter, soms zijn de mutaties neutraal (geen effect) en alleen bij uitzondering voordelig. Uiteindelijk kunnen deze mutaties een groot effect hebben, bijvoorbeeld als zij de snelheid van vermenigvuldiging verhogen of het virus helpen om aan het afweersysteem van de gastheer te ontsnappen.
Het meestal bolvormige virusdeeltje bestaat uit een membraan dat onder andere de HA en NA eiwitten bevat. In deze ‘bol’ is het erfelijk materiaal - ook virus genoom genoemd - ingesloten. Het influenza A virus genoom bestaat uit acht negatief-strengs RNA segmenten, die in afnemende lengte afgekort worden tot PB2, PB1, PA, HA, NP, NA, M en NS. Mutaties in het influenzavirus genoom ontstaan vaak en kunnen leiden tot veranderingen in de gecodeerde eiwitten. Zulke mutaties kunnen er bijvoorbeeld voor zorgen dat het virusdeeltje niet meer herkend wordt door eerder opgebouwde afweer tegen dit virusdeeltje. Deze continue verandering van influenzavirussen is de reden waarom vaccins regelmatig geüpdatet moeten worden om effectief te blijven tegen circulerende influenzavirussen. Eén van de manieren waarop een individu de influenzavirus infectie kan proberen terug te dringen en een nieuwe infectie door hetzelfde type virus te voorkomen, is door antistoffen te produceren. Hierbij is het goed functioneren van het afweersysteem van groot belang. Mensen met een minder goed werkende afweer zijn daarom ook eerder vatbaar voor infectie. Dit zijn bijvoorbeeld jonge kinderen, ouderen, maar ook mensen met een onderliggende ziekte of die voorbereid worden voor een orgaantransplantatie.

Pandemieën, seizoensgriep en zoönoses

In de afgelopen eeuw zijn er vier influenzavirus pandemieën geweest, namelijk in 1918, 1957, 1968 en 2009. Pandemieën zijn uitbraken van ziekteverwekkers, die in korte tijd op meerdere continenten mensen infecteren. In het verleden hebben alleen griepvirussen van het type H1, H2, en H3 pandemieën veroorzaakt. Het aantal sterftegevallen is bij pandemische virussen vaak sterk verhoogd ten opzichte van niet-pandemische jaren, omdat nog niemand afweer heeft opgebouwd tegen het nieuwe virus.

Een H1N1 influenzavirus heeft in 1918 de tot nu toe meest ernstige pandemie veroorzaakt, met naar schatting ongeveer 50 miljoen doden in het eerste jaar. Deze pandemie wordt ook wel de 'Spaanse griep' genoemd en dit virus was waarschijnlijk direct afkomstig van vogels. Dit H1N1 virus werd in 1957 vervangen door een H2N2 virus, met de 'Aziatische grieppandemie' als gevolg, die tot 1-2 miljoen doden leidde. Dit H2N2 virus is ontstaan door menging van het genetisch materiaal (reassortering) van het voorgaande humane H1N1 virus en gensegmenten van een of meerdere virussen afkomstig van vogels. In 1968 is het H2N2 virus door een H3N2 virus afgelost, dat de 'Hong Kong grieppandemie' heeft veroorzaakt, met naar schatting één miljoen doden wereldwijd. Ook dit virus is ontstaan door reassortering, dit keer tussen het bestaande humane H2N2 virus en een H3 virus uit vogels. De meest recente pandemie is in 2009 ontstaan in Noord-Amerika. Dit pandemische H1N1 virus is in varkens ontstaan na reassortering van virussen die afkomstig zijn van zowel vogels, varkens en mensen. De sterftecijfers variëren tussen de 18.500 (laboratorium bevestigde infecties) en 300.000 doden (geschat) in het eerste jaar na de uitbraak.

De virussen die een pandemie hebben veroorzaakt, veroorzaken in de jaren na de pandemie als seizoensgriepvirus jaarlijks uitbraken (epidemieën) tijdens de wintermaanden. Dit zijn momenteel virussen van de subtype H1N1, H3N2 en influenza B virussen. Tegen deze virussen is echter een vaccin beschikbaar, maar desalniettemin komen naar schatting 250.000-500.000 mensen ieder jaar te overlijden als gevolg van infectie met seizoensgriepvirussen.

Incidenteel worden mensen blootgesteld aan een zoönotische infectie. De term 'zoönose' beschrijft de overdracht van een ziekteverwekker van dier op mens. Zo kunnen een aantal influenza A virus subtypes die in bijvoorbeeld vogels voorkomen bij uitzondering ook de mens infecteren, zoals bijvoorbeeld het H5N1 vogelgriepvirus. Vogelgriepvirussen worden naast de onderverdeling in subtypes, ook ingedeeld op basis van hun pathogeniteit (vermogen om ziekteverschijnselen te veroorzaken) in laag- of hoog pathogeen. Virussen van het subtype H5 en H7 kunnen laag- en hoog pathogeen zijn, terwijl alle andere subtypes uitsluitend laag pathogeen zijn. Deze indeling is gebaseerd op een verandering in het erfelijk materiaal die de vermenigvuldiging in - en daarmee de verspreiding naar - de meeste weefsels van het lichaam mogelijk

maakt. ‘Laag pathogeen’ betekent niet noodzakelijkerwijs dat deze virussen geen ziekteverschijnselen kunnen veroorzaken. Hoog pathogene virussen van het subtype H5 en H7 kunnen grote uitbraken in pluimvee veroorzaken, die gepaard gaan met hoge sterfte. Terwijl H5N1 en H7N9 vogelgriepvirussen alleen sporadisch in mensen worden gedetecteerd, veroorzaakten hoog pathogene H5 virussen, subtypes H5N5, H5N6 en H5N8, recentelijk grote uitbraken in pluimvee en enkele sterftegevallen in wilde vogels in Europa en elders.

Onderzoek aan H5N1 en H2N2 virussen

Hoog pathogene H5N1 virussen werden voor het eerst in watervogels gedetecteerd in 1996 in China en Hong Kong. Sinds 1997 infecteren H5N1 virussen sporadisch mensen, voornamelijk in landen in Zuidoost-Azië en het Midden-Oosten. In ongeveer de helft van de laboratorium-bevestigde gevallen hadden deze infecties een dodelijke afloop. Omdat het H5N1 virus - in tegenstelling tot virussen van het subtype H1, H2, en H3 - nooit op grote schaal mensen heeft geïnfecteerd, is de overgrote meerderheid van de bevolking niet beschermd tegen infectie met een virus van het H5 subtype. De inefficiënte virus overdracht van geïnfecteerd pluimvee naar individuen in hun directe omgeving leek lange tijd de enige optie voor verspreiding van H5N1 virussen. Ook was het niet bekend of een gemuteerd H5N1 virus wel effectief tussen mensen kon worden overgedragen. De twee hierboven beschreven ingrediënten, de afwezigheid van bestaande afweer tegen H5N1 virussen in de bevolking en een efficiënte virus overdracht van mens op mens via de lucht, zouden de basis kunnen vormen voor een nieuwe grieppandemie.

Om te bestuderen of een H5N1 virus ooit overdraagbaar zou kunnen worden van mens op mens, hebben wij in hoofdstuk 2 onderzocht of het mogelijk is om een hoog pathogeen H5N1 virus dusdanig aan te passen dat het via de lucht kan worden overgedragen tussen zoogdieren. Deze overdraagbaarheid kunnen we bestuderen door gebruik te maken van het frettenmodel. Fretten zijn kleine roofdieren, die tot de familie van marterachtigen behoren. In het onderzoek aan influenzavirussen worden vaak fretten gebruikt omdat zij ten eerste dezelfde soorten receptoren (structuren op de cellen van de luchtwegen waar virussen aan binden als eerste stap in het infectieproces) hebben, ten tweede over vergelijkbare anatomische en histologische structuren beschikken (opbouw van het lichaam en weefsels), ten derde met een groot aantal influenzavirussen besmet kunnen worden en ten vierde soortgelijke ziekteverschijnselen vertonen als mensen. Voor onze experimenten worden deze dieren in een zeer veilig en beveiligd laboratorium in twee kooien gehuisvest, die door twee metalen roosters op 10 centimeter afstand van elkaar gescheiden zijn. Daardoor kan de lucht van een geïnfecteerde ‘donor’ fret naar de ‘ontvanger’ fret waaien, maar direct

contact tussen de dieren is uitgesloten. De enige manier waarop het virus dus kan worden overgedragen is via de lucht. In dit dierenmodel kon een H5N1 virus, dat in 2005 uit een mens is geïsoleerd, niet van een donor fret op een ontvanger fret worden overgedragen via de lucht. Om te testen of dit H5N1 virus ooit via de lucht overdraagbaar zou kunnen worden, hebben wij in het laboratorium het virus zodanig veranderd, dat het beter aan cellen in de bovenste luchtwegen van zoogdieren kan binden en dat na infectie nieuwe virusdeeltjes sneller worden aangemaakt. Dit werd bereikt door een aantal mutaties (G222S en Q224L in HA en E627K in PB2), die al eerder waren beschreven in de wetenschappelijke literatuur, in het genoom van het virus aan te brengen. Helaas was ook dit virus (en een hele reeks andere virussen met verschillende mutaties) niet in staat om van fret op fret overgedragen te worden via de lucht. In een volgende poging om dit virus verder aan de bovenste luchtwegen van zoogdieren aan te passen - en daarmee hopelijk virusoverdracht via de lucht te bereiken - hebben wij het virus in fretten gepasseerd. Hiervoor werd een virusmonster dat was verkregen uit de bovenste luchtwegen van een geïnfecteerd dier, gebruikt om een andere fret in de neus te infecteren. Door adaptatie van het virus aan fretten tijdens tien van dergelijke passages, zijn uiteindelijk virussen ontstaan die via de lucht overgedragen konden worden tussen fretten. Dit was de eerste keer dat het was aangetoond dat een vogelgriepvirus, zonder reassortering met een humaan virus, overgedragen kan worden tussen zoogdieren via de lucht. Deze bevinding betekent ook dat, als we deze resultaten extrapoleren naar de situatie in de mens, het mogelijk is dat H5N1 virussen, of andere vogelgriepvirussen in de natuur kunnen ontstaan die tussen mensen kunnen worden overgedragen.

In ons vervolgonderzoek hebben we gekeken naar de genetische veranderingen en de bijbehorende functionele veranderingen, die er uiteindelijk voor gezorgd hadden dat het H5N1 virus overdraagbaar was via de lucht (hoofdstuk 3). Hiervoor hebben we de genetische code van de lucht-overdraagbare virussen bepaald (gesequenced) en we vonden daarin verschillende mutaties ten opzichte van het oorspronkelijke virus. Om te bepalen welke van deze mutaties strikt noodzakelijk zijn voor virus overdracht via de lucht, hebben wij in het laboratorium combinaties van mutaties in het H5N1 virus aangebracht, waarbij we in ieder te testen virus steeds één mutatie hebben weggelaten. Als zo'n virus nog steeds tussen fretten via de lucht kon worden overgedragen, dan was de mutatie niet per se noodzakelijk voor virusoverdracht, maar zou het wel aan een betere overdracht kunnen hebben bijgedragen. Op deze manier werden mutaties H103Y en T156A in HA en H99Y in PB1 geidentificeerd die samen met de eerder beschreven mutaties die we al hadden aangebracht (G222S of Q224L in HA en E627K in PB2), een minimale set van mutaties vormen, die tot virusoverdracht via de lucht kan leiden. Verder hebben wij aangetoond dat deze mutaties, die

tijdens het passeren van het virus in fretten zijn ontstaan, al in passage 3 - dus redelijk snel - aanwezig waren en dat hun aanwezigheid met ieder nieuwe passage toe nam. Deze minimale set van mutaties hebben wij vervolgens verder onderzocht om te kijken welk effect zij hebben op het functioneren van het virus. We hebben ontdekt dat de stabiliteit van het HA eiwit, de afwezigheid van een suikerketen in de buurt van de receptorbindingsplaats van het HA eiwit en een beter uitgebalanceerde vermenigvuldiging van het virus uitermate belangrijk zijn.

Naast het gevaar van pandemieën veroorzaakt door nieuwe zoönotische influenzavirussen, is het ook mogelijk dat virus subtypes die al eerder in mensen hebben gecirculeerd, weer opduiken en opnieuw een pandemie veroorzaken. De kans hierop is groter wanneer de bescherming tegen dit virus, door bestaande afweer in een groot deel van de bevolking, is verdwenen. Met behulp van fylogenetische computeranalyses (fylogenie - Grieks voor afkomst van een ras) kan de genetisch verwantschap tussen virussen worden bestudeerd. Met deze methode kan bijvoorbeeld worden bestudeerd of er in de loop der tijd mutaties zijn opgetreden in de genetische code van virussen als het gevolg van aanpassing aan een nieuwe gastheer. Deze sequenties beschrijven de volgorde van nucleotiden (‘letters’ in het genoom), die in aminozuren (‘bouwstenen’ van eiwitten) worden vertaald en uiteindelijk de vorm en functie van eiwitten bepalen. In alle bekende sequenties van het genetische materiaal van H2N2 influenzavirussen hebben wij in hoofdstuk 4 met fylogenetische methodes gekeken of er aminozuur posities zijn die vaker veranderen dan verwacht. De verhoogde frequentie van verandering op bepaalde posities kan een indicatie zijn voor evolutionaire druk; de noodzaak om te veranderen omdat dit tot een beter aangepast of ‘fitter’ virus leidt. Wij hebben vijf aminozuur posities in het H2N2 HA eiwit gevonden die onder een verhoogde evolutionaire druk lagen, waarvan twee zich in de receptorbindingsplaats bevonden. Ook zagen we dat over het algemeen meer veranderingen ontstonden in de humane H2N2 virussen dan in de H2N2 virussen uit vogels. Verder hebben wij in hoofdstuk 5 de antigene evolutie - mutaties die effect hebben op de virus herkenning door antistoffen - van humane H2N2 virussen in kaart gebracht met behulp van de antigene cartografie methode en vonden een langzamere antigene evolutie ten opzichte van H3N2 virussen.

Zoals hierboven beschreven, hebben tot op heden alleen virussen van het subtype H1, H2 en H3 pandemieën veroorzaakt. H1N1 en H3N2 virussen veroorzaken momenteel epidemieën in mensen. H2 virussen circuleren momenteel nog wel in vogels, maar zijn sinds 1968 niet meer in mensen aangetoond. Personen die na 1968 zijn geboren zijn dus nog nooit in aanraking gekomen met dit virussubtype, hebben daarom ook geen bestaande afweer tegen dit virus en zijn dus niet tegen dit virus beschermd. In hoofdstuk 6 hebben wij aangetoond dat een aantal H2 virussen die in de

afgelopen jaren in vogels zijn aangetroffen niet overdraagbaar zijn tussen fretten. Het lijkt dus onwaarschijnlijk dat een uit vogels afkomstig H2N2 virus direct op mensen kan worden overgedragen en vervolgens verspreid. Zelfs H2N2 virussen die in mensen circuleerden tussen 1957 en 1968 (dus aan het begin en aan het einde van de pandemie), kunnen niet tussen fretten via de lucht worden overgedragen. Alleen een humaan H2N2 virus uit 1958 was in staat om overgedragen te worden via de lucht. Hier valt uit af te leiden dat H2N2 virussen zich tijdens het eerste jaar van de pandemie nog verder moesten aanpassen om efficiënt tussen mensen overgedragen te kunnen worden via de lucht. Daarnaast heeft het H3N2 virus na de introductie in 1968 het H2N2 virus mogelijk kunnen verdringen omdat het H2N2 virus uit 1968 niet meer via de lucht overdraagbaar was.

Samengevat hebben we aangetoond dat H5N1 virussen in staat zijn om overgedragen te worden tussen zoogdieren via de lucht. Verder hebben wij drie eigenschappen beschreven die noodzakelijk zijn voor virusoverdracht via de lucht (hoofdstukken 2 en 3). Vervolgens hebben we laten zien dat H2N2 virussen afkomstig uit vogels geen directe pandemische dreiging vormen voor de mens en de genetische en antigene evolutie van H2N2 virussen in kaart gebracht (hoofdstukken 4, 5 en 6). Alleen de continue surveillance van virussen in vogels, mensen en andere zoogdieren in combinatie met het bestuderen van het effect van veranderingen in influenzavirussen kunnen leiden tot effectieve preventieve en therapeutische maatregelen om virusoverdracht te verminderen of voorkomen.

Grippeviren, auch Influenzaviren genannt, sind eine Gruppe von Krankheitserregern, die über die Luftwege in den Körper eindringen und sich dort vermehren können. Diese Infektion kann typische Krankheitszeichen wie Fieber, Halsschmerzen, Husten, Kopfschmerzen und Müdigkeit auslösen. In schwerwiegenden Fällen können Grippeviren eine Lungenentzündung verursachen, die schließlich zum Tode führen kann.
Influenzaviren werden eingeteilt in die Typen A, B, C und D. Influenza A Viren werden weiter aufgeteilt in Subtypen aufgrund der Eigenschaften ihrer Oberflächeneiweiße Hämagglutinin (HA) und Neuraminidase (NA). Die Kombination dieser Eiweiße führt wiederum zu Abkürzungen wie H1N1 oder H3N2. Bisher sind 16 HA und 9 NA Subtypen bekannt in Wasservögeln, dem Wirt der Mehrheit aller Influenzaviren und jeweils 2 potentielle Subtypen in Fledermäusen. Beispiele von Tierarten, die mit Influenzaviren infiziert werden können sind Geflügel und Schweine, aber auch Tiger und Seehunde.
Wenn Viren sich in einer Wirtszelle vermehren entstehen kontinuierlich Mutationen im Virusgenom. Diese Mutationen tauchen spontan und nicht zielgerichtet auf und sind daher auch meist nachteilig für das Virus. Jedoch können diese Mutationen in seltenen Fällen neutral (ohne Effekt) sein oder sich vereinzelt sogar positiv für das Virus auswirken. Auf lange Sicht können diese Mutationen einen großen Effekt haben, wenn sie zum Beispiel die Geschwindigkeit der Virusvervielfältigung erhöhen oder dem Virus ermöglichen nicht mehr durch das Abwehrsystem des Wirtes erkannt zu werden.
Das meist kugelförmige Viruspartikel besteht aus einer Membran, die unter anderem die Oberflächeneiweiße HA und NA trägt. Diese “Kugel” enthält das virale Erbgut, welches auch genetisches Material oder Genom genannt wird. Das Influenza A Virus-Genom besteht aus acht Segmenten eines Negativstrang RNA Erbgutes, die in abnehmender Länge mit PB2, PB1, PA, HA, NP, NA, M und NS abgekürzt werden. Mutationen im Erbgut von Influenzaviren finden häufig statt und können zu Veränderungen in den kodierten Eiweißen führen. Solche Mutationen können zum Beispiel dazu führen, dass das Virus nicht mehr durch die zuvor gegen das Virus aufgebaute Immunabwehr erkannt werden kann. Diese kontinuierlichen Veränderungen von Influenzaviren ist auch der Grund weshalb Impfstoffe regelmäßig angepasst werden müssen, um effektiv gegen momentan zirkulierende Influenzaviren zu sein. Der Körper von Säugetieren hat eine Reihe von Mechanismen entwickelt um eine Infektion zurückzudrängen und die erneute Ansteckung mit demselben Virustyp zu verhindern. Hierzu gehört zum Beispiel die Produktion von Antikörpern, für die ein gut funktionierendes Abwehr- oder Immunsystem sehr wichtig ist. Daher sind Menschen mit einem geschwächten Immunsystem, zu denen junge Kinder, Senioren, Patienten mit bestimmten Vorerkrankungen oder Menschen, die auf eine Organtransplantation vorbereitet werden, gehören, anfälliger für eine Virusinfektion.

Pandemien, saisonale Grippe und Zoonosen

Im letzten Jahrhundert haben vier Influenzaviruspandemien stattgefunden, in den Jahren 1918, 1957, 1968 und 2009. Pandemien sind Ausbrüche von Krankheitserregern, die in kurzer Zeit Menschen auf mehreren Kontinenten infizieren. In der Vergangenheit haben nur Viren der Subtypen H1, H2 und H3 Pandemien verursacht. Die Anzahl von Todesfällen aufgrund von Infektionen mit Influenzaviren ist in pandemischen Jahren oft stark erhöht im Vergleich zu nicht-pandemischen Jahren, da noch niemand eine spezifische Abwehr aufbauen konnte.

Ein H1N1 Influenzavirus hat im Jahr 1918 die bisher schwerste Pandemie verursacht mit schätzungsweise 50 Millionen Todesopfern im ersten Jahr. Diese Pandemie wird auch „Spanische Grippe“ genannt und das Virus wurde wahrscheinlich direkt von Vögeln auf den Menschen übertragen. Dieses H1N1 Influenzavirus wurde in 1957 abgelöst durch ein H2N2 Virus, das die „Asiatische Grippe“ mit rund 1 bis 2 Millionen Todesopfern zur Folge hatte. Dieses H2N2 Virus ist durch Mischung von genetischem Material (Reassortierung) des zuvor zirkulierenden humanen H1N1 Virus und Gensegmenten von einem oder mehreren Vogelviren entstanden. Im Jahr 1968 wurde das H2N2 Virus von einem H3N2 Virus verdrängt, welches die „Hong Kong Grippe“ mit schätzungsweise einer Million Todesopfern weltweit verursacht hat. Auch dieses Virus ist durch Reassortierung des vorhergehenden humanen H2N2 Virus mit einem Vogel-H3 Virus entstanden. Die rezenteste Influenzapandemie hat ihren Ursprung im Jahr 2009 in Nordamerika. Dieses pandemische H1N1 Virus ist in Schweinen durch Reassortierung von Vogel-, Menschen-, und Schweineviren entstanden. Die Anzahl der Toten schwankt zwischen 18.500 (laborbestätigte Infektionen) und 300.000 (Schätzungen) im ersten Jahr nach Ausbruch.

Die Viren, die Pandemien nach sich zogen, verursachen meist in den Wintermonaten der Folgejahre als saisonales Grippevirus Infektionsausbrüche (Epidemien). Zurzeit gehören Influenza A Viren H1N1, H3N2 und Influenza B Viren zu den saisonalen Grippeviren. Trotz eines wirksamen Impfstoffs gegen diese Viren sterben jährlich nach Schätzungen 250.000 bis 500.000 Menschen infolge einer Infektion mit saisonalen Grippeviren.

Gelegentlich können sich Menschen auch mit zoonotischen Viren infizieren. „Zoonose“ bedeutet Übertragung eines Krankheitserregers von einem Tier auf den Menschen. Einige Influenza A Subtypen, wie zum Beispiel das H5N1 Vogelgrippevirus, die normalerweise ausschließlich in Vögeln vorkommen, können vereinzelt auch Menschen infizieren.

Neben der Einteilung in Subtypen werden Vogelgrippeviren aufgrund ihrer Pathogenität (krankmachende Wirkung) weiterhin klassifiziert in hoch- und niedrigpathogen. Viren der Subtypen H5 und H7 können sowohl hoch- als auch niedrigpathogen sein; alle anderen Subtypen sind ausschließlich niedrigpathogen. Diese Einteilung basiert auf einer Veränderung im Erbgut

von Influenzaviren. Hochpathogene Viren können sich in fast allen Geweben des menschlichen Körpers vermehren. „Niedrigpathogen“ bedeutet nicht zwangsläufig, dass diese Viren keine Krankheitszeichen verursachen. Hochpathogene Viren vom Subtyp H5 und H7 können Ausbrüche in Geflügel mit hohen Sterblichkeitsraten verursachen. Während H5N1 und H7N9 Vogelgrippeviren nur sporadisch in Menschen gefunden werden, haben hochpathogene H5 Viren vom Subtyp H5N5, H5N6 und H5N8 vor kurzem Ausbrüche beim Hausgeflügel und Infektionen von Wildvögeln unter anderem in Europa verursacht.

Studien an H5N1 und H2N2 Viren

Hochpathogene H5N1 Viren wurden zuerst im Jahr 1996 bei Vögeln in China und Hong Kong entdeckt. Seit 1997 verursachen H5N1 Viren sporadische Infektionen bei Menschen vor allem in Ländern Südostasiens und des Mittleren Ostens. Ungefähr die Hälfte aller laborbestätigter Infektionen verlaufen tödlich. Da das H5N1 Virus - im Gegensatz zu Viren der Subtypen H1, H2, und H3 - nie in größerem Umfang Menschen infiziert hat, ist die übergroße Mehrheit der Bevölkerung nicht geschützt vor einer Infektion mit Viren des Subtyps H5. Lange Zeit schien die Virenübertragung von infiziertem Geflügel auf den Menschen in dessen direkter Umgebung die einzige Möglichkeit für die Verbreitung von H5N1 Viren. Weiterhin war nicht bekannt, ob ein mutiertes H5N1 Virus nach weiterer Anpassung an den Menschen eventuell über die Luft von einem Individuum zum anderen übertragen werden kann. Die beiden zuvor beschrieben Eigenschaften, nämlich das Fehlen einer effektiven Abwehr gegen H5N1 Viren in der Bevölkerung und die einfache Übertragung des Virus von Mensch zu Mensch, könnten die Basis für eine erneute Grippepandemie bilden.

Um zu untersuchen ob ein H5N1 Virus eventuell von Mensch zu Mensch übertragen werden kann, haben wir in Kapitel 2 versucht ein hochpathogenes H5N1 Virus so zu verändern, dass es durch die Luft zwischen Säugetieren übertragen werden kann. Diese Übertragbarkeit haben wir im Frettchenmodell getestet. Frettchen sind kleine Raubtiere, die zur Familie der Marderartigen gehören. Bei Untersuchungen mit Influenzaviren werden Frettchen oft eingesetzt, da sie zum einen dieselben Rezeptoren (Strukturen auf den Zellen des Atemwegtraktes, an die die Viren als erster Schritt einer Infektion binden können) und ähnliche anatomische und histologische Strukturen besitzen wie Menschen. Frettchen können außerdem mit einer Reihe verschiedener Influenzaviren infiziert werden und weisen ähnliche Krankheitszeichen auf, wie sie auch beim Menschen beobachtete werden. In unseren Studien werden diese Tiere in einem besonders gesicherten (Zugangssicherung) und sicheren (biologische Sicherheit) Labor in zwei Käfigen gehalten, die durch zwei Metallgitter mit ca. 10 cm Abstand voneinander getrennt sind. Dadurch kann die Luft von einem infizierten „Spenderfrettchen“

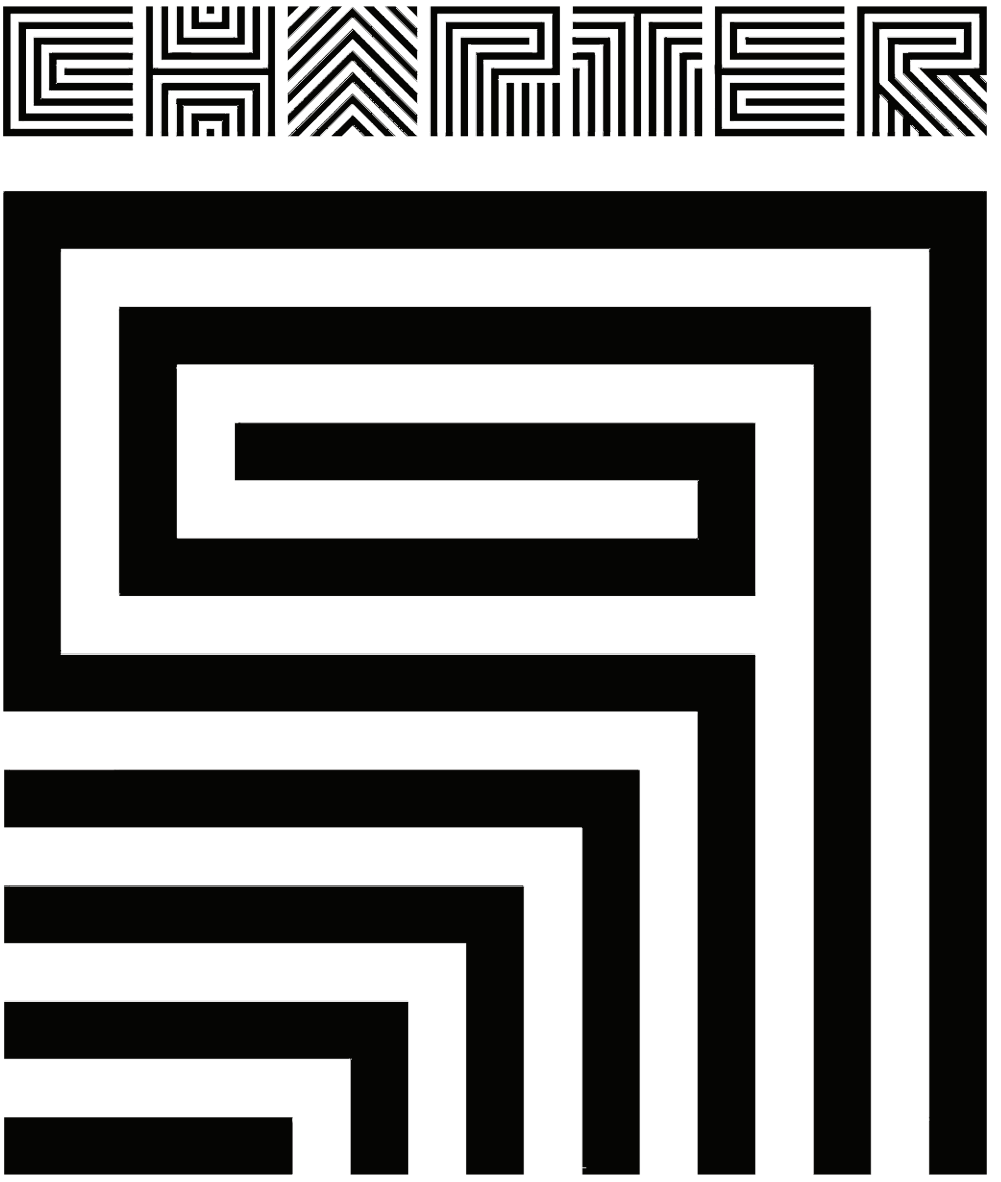
auf ein „Empfängerfrettchen“ strömen; der direkte Kontakt zwischen den zwei Frettchen ist jedoch ausgeschlossen. Der einzige Weg für eine Virusübertragung ist somit durch die Luft. In diesem Tiermodell konnte ein H5N1 Virus, das im Jahr 2005 von einem Menschen isoliert wurde, nicht durch die Luft von einem Spenderfrettchen auf ein Empfängerfrettchen übertragen werden. Um herauszufinden ob dieses H5N1 Virus durch die Luft übertragen werden kann, haben wir das Virus unter Laborbedingungen dahingehend verändert, dass es besser an Zellen der oberen Atemwege von Säugetieren binden kann und schneller neue Virusgenerationen produziert werden können. Dies wurde erreicht durch Mutationen (G222S und Q224L in HA, E627K in PB2), die zuvor in der Literatur beschrieben wurden, im Erbgut des Virus anzubringen. Leider war auch dieses Virus (genauso wie eine Reihe anderer Viren mit verschiedenen anderen Mutationen) nicht in der Lage um durch die Luft von Frettchen zu Frettchen übertragen zu werden. Ein weiterer Versuch um dieses Virus noch weiter an die Zellen der oberen Atemwege anzupassen – und so hoffentlich Virusübertragbarkeit durch die Luft zu erreichen – bestand im Passagieren des Virus in Frettchen. Hierzu wurde das Virus aus den oberen Atemwegen eines infizierten Frettchens isoliert um die Nasenschleimhaut eines weiteren Frettchens zu infizieren. Nach zehn solcher Durchgänge Viren, die durch die Luft von Frettchen zu Frettchen übertragbar waren, entstanden. Zum ersten Mal konnte gezeigt werden, dass ein Vogelgrippevirus durch die Luft zwischen Säugetieren übertragen werden kann, ohne zuvor mit eine menschlichen Virus zu reassortieren. Übertragen auf die Situation beim Menschen bedeutet dieses Resultat weiterhin, dass es wahrscheinlich ist, dass H5N1 Viren oder andere Vogelgrippeviren die von Mensch zu Mensch durch die Luft übertragbar sind, in der Natur entstehen können.

In weiteren Studien haben wir die genetischen Abweichungen und die daraus resultierenden funktionalen Veränderungen untersucht, die schlussendlich dazu geführt haben, dass das H5N1 Virus durch die Luft übertragbar wurde (Kapitel 3). Hierzu haben wir das Genom der luftübertragbaren Viren bestimmt (sequenziert) und dabei verschiedene Veränderungen gefunden im Vergleich zu dem urspünglichen Virus. Um festzustellen, welche dieser Mutationen unbedingt notwendig sind, haben wir im Labor Kombinationen von Mutationen in H5N1 Viren „angebracht“ und dabei jeweils eine Mutation weggelassen. Falls dieses Virus jetzt immer noch zwischen Frettchen durch die Luft übertragen werden konnten, war diese Mutation nicht strikt erforderlich für die Virusübertragung, könnte aber zu einer besseren Übertragung beigetragen haben. So konnten wir feststellen, dass Mutationen H103Y und T156A in HA sowie H99Y in PB1 in Kombination mit den zuvor beschriebenen Mutationen (G222S oder Q224L in HA, E627K in PB2) die minimale Anzahl Mutationen darstellen, die zu einer Virusübertragung durch die Luft führen kann. Weiterhin konnten wir zeigen, dass die Mutationen

die während der Passage in Frettchen entstanden sind schon in der dritten Passage – also relativ früh – feststellbar waren und dass der Anteil von Viren mit diesen Mutationen mit jeder weiteren Passage zunahm. Weiterhin haben wir den Effekt dieser minimalen Anzahl von Mutationen mit Bezug auf die Funktionen des Virus untersucht. Wir konnten nachweisen, dass die Stabilität des HA Eiweißes, das Nichtvorhandensein von bestimmten Zuckerketten in der Nähe der Rezeptorbindungsregion des HA Eiweißes und eine ausbalanciertere Vervielfältigung des Virus besonders wichtig sind.
Neben der Gefahr, dass Pandemien durch neue zoonotische Influenzaviren verursacht werden, besteht auch die Möglichkeit, dass Viren die zuvor in Menschen zirkuliert haben erneut in der Bevölkerung auftauchen und eine Pandemie verursachen, wenn der Schutz gegen dieses Virus in einem Großteil der Population nicht mehr vorhanden ist. Mithilfe von phylogenetischen Computeranalysen (Phylogenie: griechisch für Abstammung einer Rasse) kann die genetische Verwandtschaft – hier von Viren - untersucht werden. Mit dieser Methode kann zum Beispiel untersucht werden, ob im Laufe der Zeit Mutationen auftreten im Erbgut von Viren als Folge der Anpassung an einen neuen Wirt. Diese sogenannten Sequenzen bestehen aus der Aneinanderreihung von Nukleotiden („Buchstaben“ im Genom), die wiederum in Aminosäuren („Bausteine“ von Eiweißen) übersetzt werden und so die Struktur und Funktion von Eiweißen bestimmen. In allen bisher bekannten Sequenzen des genetischen Materials von H2N2 Influenzaviren haben wir mit phylogenetischen Methoden untersucht ob bestimmte Positionen häufiger verändert wurden als statistisch angenommen. Eine erhöhte Häufigkeit von Veränderungen an bestimmten Positionen kann auf evolutionären Druck hindeuten. Dies ist die Notwendigkeit sich zu verändern um sich zu einem Virus zu entwickeln mit Vorteilen gegenüber den Vorgängergenerationen. Wir haben fünf Aminosäurepositionen im HA Eiweiß von H2N2 Viren gefunden, die unter einem erhöhten evolutionären Druck stehen, zwei davon in der Rezeptorbindungsregion (Kapitel 4). Ebenso konnten wir feststellen, dass durchschnittlich mehr Veränderungen in menschlichen H2N2 Viren als in H2N2 Viren aus Vögeln stattfanden.
Weiterhin haben wir in Kapitel 5 die antigene Evolution – Mutationen, die sich auf die Erkennung des Virus durch Antikörper auswirken – von menschlichen H2N2 Viren aufgezeigt mit Hilfe der sogenannten „Antigenen Kartographie-Methode“ und haben eine langsamere antigene Entwicklung im Vergleich zu H3N2 Viren festgestellt.
Wie zuvor beschrieben, haben bis heute nur Viren der Subtypen H1, H2 und H3 Pandemien verursacht. H1N1 und H3N2 verursachen momentan Epidemien in Menschen. H2 Viren zirkulieren momentan zwar in Vögeln, sind aber seit 1968 nicht mehr in Menschen gefunden worden. Nach

1968 geborene Personen sind daher noch nie mit diesem Virus in Kontakt gekommen und haben daher keinen spezifischen Immunschutz aufbauen können. In Kapitel 6 haben wir gezeigt, dass einige H2 Influenzaviren, die in den vergangenen Jahren in Vögeln gefunden wurden, nicht zwischen Frettchen übertragbar sind. Es erscheint daher eher unwahrscheinlich, dass ein von Vögeln stammendes H2N2 Virus direkt auf den Menschen übertragen werden kann und sich dann weiter verbreitet. Sogar H2N2 Viren, die in den Jahren 1957 und 1968 in Menschen zirkulierten (also am Beginn und am Ende der Pandemie), können nicht von einem Frettchen auf ein anderes durch die Luft übertragen werden. Nur ein menschliches H2N2 Virus aus 1958 konnte durch die Luft übertragen werden. Hieraus können wir schließen, dass sich H2N2 Viren während des ersten Jahres der Pandemie noch weiter anpassen mussten um erfolgreich von Mensch zu Mensch übertragbar zu werden. Außerdem hat das H3N2 Virus nach seinem Auftauchen im Jahr 1968 das zuvor zirkulierende H2N2 Virus eventuell verdrängen können, da das H2N2 Virus aus 1968 nicht mehr in der Lage war durch die Luft übertragen zu werden.

Zusammenfassend haben wir gezeigt, dass H5N1 Viren in der Lage sind um durch die Luft übertragbar zu werden in Säugetieren. Weiterhin haben wir drei Eigenschaften der Viren beschrieben, die für die Übertragung dieser durch die Luft notwendig sind (Kapitel 2 und 3). Weiterhin haben wir neue Erkenntnisse gewonnen über die genetische und antigene Evolution von Viren und haben gezeigt, dass H2N2 Vogelviren keine direkte pandemische Bedrohung für den Menschen darstellen (Kapitel 4, 5 und 6). Allein durch kontinuierliche Überwachung von Viren in Vögeln, Menschen und anderen Säugetieren sowie weitere Analysen des Effekts von bestimmten Veränderungen in Influenzaviren, kann letztendlich zu effektiveren (präventiven und therapeutischen) Maßnahmen führen um Virusübertragungen zu vermindern oder zu verhindern.



REFERENCES

1. Sorrell EM, Schrauwen EJ, Linster M, De Graaf M, Herfst S, Fouchier RA. 2011. Predicting 'airborne' influenza viruses: (trans-) mission impossible? Curr Opin Virol 1:635-42.
2. Lefkowitz EJ, Dempsey DM, Hendrickson RC, Orton RJ, Siddell SG, Smith DB. 2017. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). Nucleic Acids Res doi:10.1093/nar/gkx932.
3. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79:2814-22.
4. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A 109:4269-74.
5. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO. 2013. New world bats harbor diverse influenza a viruses. PLoS pathogens 9:e1003657.
6. Perroncito E. 1878. Epizoozia tifoide nei gallinacei. Annali Accad Agri Torino:87-126.
7. Shope RE. 1931. Swine Influenza : I. Experimental Transmission and Pathology. J Exp Med 54:349-59.
8. Palese P, Shaw M. 2007. Fields virology. Orthomyxoviridae: The Viruses and Their Replication, 5th edn, Philadelphia, PA: Lippincott Williams & Wilkins, Wolters Kluwer Business:1647-1689.
9. Subbarao K, Joseph T. 2007. Scientific barriers to developing vaccines against avian influenza viruses. Nat Rev Immunol 7:267-78.
10. Lakadamyali M, Rust MJ, Zhuang X. 2004. Endocytosis of influenza viruses. Microbes Infect 6:929-36.
11. Neumann G, Noda T, Kawaoka Y. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459:931-9.
12. Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, Ruigrok RW. 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458:914-8.
13. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67:1761-4.
14. Klumpp K, Ruigrok RW, Baudin F. 1997. Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure. EMBO J 16:1248-57.
15. Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ, Wiley DC. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature 333:426-31.
16. Klenk HD, Garten W. 1994. Host cell proteases controlling virus pathogenicity. Trends Microbiol 2:39-43.
17. Schulze IT. 1997. Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. J Infect Dis 176 Suppl 1:S24-8.
18. Gambaryan AS, Robertson JS, Matrosovich MN. 1999. Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. Virology 258:232-9.

19. Naeve CW, Hinshaw VS, Webster RG. 1984. Mutations in the hemagglutinin receptor-binding site can change the biological properties of an influenza virus. J Virol 51:567-9.
20. Connor RJ, Kawaoka Y, Webster RG, Paulson JC. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205:17-23.
21. Suzuki Y, Ito T, Suzuki T, Holland RE, Jr., Chambers TM, Kiso M, Ishida H, Kawaoka Y. 2000. Sialic acid species as a determinant of the host range of influenza A viruses. J Virol 74:11825-31.
22. Palese P, Shaw, ML. 2013. Fields Virology.
23. Widdowson MA, Bresee JS, Jernigan DB. 2017. The Global Threat of Animal Influenza Viruses of Zoonotic Concern: Then and Now. J Infect Dis 216:S493-S498.
24. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr., Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, et al. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197-201.
25. Scholtissek C. 1995. Molecular evolution of influenza viruses. Virus Genes 11:209-15.
26. Scholtissek C. 1990. Pigs as ‘mixing vessels’ for the creation of new pandemic influenza A viruses. Medical Principles and Practice 2:65-71.
27. Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, Kodihalli S, Krauss S, Markwell D, Murti KG, Norwood M, Senne D, Sims L, Takada A, Webster RG. 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252:331-42.
28. de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. 1997. A pandemic warning? Nature 389:554.
29. Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE. 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 72:6678-88.
30. WHO. 2018. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO. http://www.who.int/influenza/human_animal_interface/2018_03_02_tableH5N1.pdf?ua=1. Accessed 1/Dec/2019
31. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza AV, Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM. 2008. Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med 358:261-73.
32. Yu H, Wu JT, Cowling BJ, Liao Q, Fang VJ, Zhou S, Wu P, Zhou H, Lau EH, Guo D, Ni MY, Peng Z, Feng L, Jiang H, Luo H, Li Q, Feng Z, Wang Y, Yang W, Leung GM. 2014. Effect of closure of live poultry markets on poultry-to-person transmission of avian influenza A H7N9 virus: an ecological study. Lancet 383:541-8.
33. WHO. 2015. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO [Online].
34. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A 101:1356-61.

35. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, Meijer A, van Steenbergen J, Fouchier R, Osterhaus A, Bosman A. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 363:587-93.
36. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 368:1888-97.
37. FAO. 2018. H7N9 situation update. http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation_update.html. Accessed 1/Dec/2019
38. Yang Y, Guo F, Zhao W, Gu Q, Huang M, Cao Q, Shi Y, Li J, Chen J, Yan J, Jin Z, Wang X, Deng Y, Sun L, Cai H, Huang J, Zheng Y, Li W, Liu A, Chen B, Zhou M, Qiu H, Slutsky AS. 2015. Novel avian-origin influenza A (H7N9) in critically ill patients in China*. Crit Care Med 43:339-45.
39. Belser JA, Gustin KM, Pearce MB, Maines TR, Zeng H, Pappas C, Sun X, Carney PJ, Villanueva JM, Stevens J, Katz JM, Tumpey TM. 2013. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. Nature 501:556-9.
40. Su S, Gu M, Liu D, Cui J, Gao GF, Zhou J, Liu X. 2017. Epidemiology, Evolution, and Pathogenesis of H7N9 Influenza Viruses in Five Epidemic Waves since 2013 in China. Trends Microbiol 25:713-728.
41. Richard M, Schrauwen EJ, de Graaf M, Bestebroer TM, Spronken MI, van Boheemen S, de Meulder D, Lexmond P, Linster M, Herfst S, Smith DJ, van den Brand JM, Burke DF, Kuiken T, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2013. Limited airborne transmission of H7N9 influenza A virus between ferrets. Nature 501:560-3.
42. Zhang Q, Shi J, Deng G, Guo J, Zeng X, He X, Kong H, Gu C, Li X, Liu J, Wang G, Chen Y, Liu L, Liang L, Li Y, Fan J, Wang J, Li W, Guan L, Li Q, Yang H, Chen P, Jiang L, Guan Y, Xin X, Jiang Y, Tian G, Wang X, Qiao C, Li C, Bu Z, Chen H. 2013. H7N9 influenza viruses are transmissible in ferrets by respiratory droplet. Science 341:410-4.
43. Zhou L, Ren R, Yang L, Bao C, Wu J, Wang D, Li C, Xiang N, Wang Y, Li D, Sui H, Shu Y, Feng Z, Li Q, Ni D. 2017. Sudden increase in human infection with avian influenza A(H7N9) virus in China, September-December 2016. Western Pac Surveill Response J 8:6-14.
44. WHO. 2017. World Health Organization. Human infection with avian influenza A(H7N9) virus – China. http://www.who.int/csr/don/17-january-2017-ah7n9-china/en/. Accessed 1/Dec/2019
45. Kang M, Lau EHY, Guan W, Yang Y, Song T, Cowling BJ, Wu J, Peiris M, He J, Mok CKP. 2017. Epidemiology of human infections with highly pathogenic avian influenza A(H7N9) virus in Guangdong, 2016 to 2017. Euro Surveill 22.
46. Ostrowsky B, Huang A, Terry W, Anton D, Brunagel B, Traynor L, Abid S, Johnson G, Kacica M, Katz J, Edwards L, Lindstrom S, Klimov A, Uyeki TM. 2012. Low pathogenic avian influenza A (H7N2) virus infection in immunocompromised adult, New York, USA, 2003. Emerg Infect Dis 18:1128-31.
47. Tweed SA, Skowronski DM, David ST, Larder A, Petric M, Lees W, Li Y, Katz J, Krajden M, Tellier R, Halpert C, Hirst M, Astell C, Lawrence D, Mak A. 2004. Human illness from avian influenza H7N3, British Columbia. Emerg Infect Dis 10:2196-9.
48. Yuan J, Zhang L, Kan X, Jiang L, Yang J, Guo Z, Ren Q. 2013. Origin and molecular characteristics of a novel 2013 avian influenza A(H6N1) virus causing human infection in Taiwan. Clin Infect Dis 57:1367-8.

49. Gillim-Ross L, Santos C, Chen Z, Aspelund A, Yang CF, Ye D, Jin H, Kemble G, Subbarao K. 2008. Avian influenza h6 viruses productively infect and cause illness in mice and ferrets. J Virol 82:10854-63.
50. Nam JH, Kim EH, Song D, Choi YK, Kim JK, Poo H. 2011. Emergence of mammalian species-infectious and -pathogenic avian influenza H6N5 virus with no evidence of adaptation. J Virol 85:13271-7.
51. Wang G, Deng G, Shi J, Luo W, Zhang G, Zhang Q, Liu L, Jiang Y, Li C, Sriwilaijaroen N, Hiramatsu H, Suzuki Y, Kawaoka Y, Chen H. 2014. H6 influenza viruses pose a potential threat to human health. J Virol 88:3953-64.
52. Liu D, Shi W, Gao GF. 2014. Poultry carrying H9N2 act as incubators for novel human avian influenza viruses. Lancet 383:869.
53. Sorrell EM, Wan H, Araya Y, Song H, Perez DR. 2009. Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc Natl Acad Sci U S A 106:7565-70.
54. Chen H, Yuan H, Gao R, Zhang J, Wang D, Xiong Y, Fan G, Yang F, Li X, Zhou J, Zou S, Yang L, Chen T, Dong L, Bo H, Zhao X, Zhang Y, Lan Y, Bai T, Dong J, Li Q, Wang S, Zhang Y, Li H, Gong T, Shi Y, Ni X, Li J, Zhou J, Fan J, Wu J, Zhou X, Hu M, Wan J, Yang W, Li D, Wu G, Feng Z, Gao GF, Wang Y, Jin Q, Liu M, Shu Y. 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. Lancet 383:714-21.
55. Mulder J, Masurel N. 1958. Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands. Lancet 1:810-4.
56. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. 2005. Characterization of the 1918 influenza virus polymerase genes. Nature 437:889-93.
57. Reid AH, Taubenberger JK. 2003. The origin of the 1918 pandemic influenza virus: a continuing enigma. J Gen Virol 84:2285-92.
58. Taubenberger JK, Morens DM. 2006. 1918 Influenza: the mother of all pandemics. Emerg Infect Dis 12:15-22.
59. Simonsen L, Clarke MJ, Schonberger LB, Arden NH, Cox NJ, Fukuda K. 1998. Pandemic versus epidemic influenza mortality: a pattern of changing age distribution. J Infect Dis 178:53-60.
60. Schafer JR, Kawaoka Y, Bean WJ, Suss J, Senne D, Webster RG. 1993. Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology 194:781-8.
61. Scholtissek C, Rohde W, Von Hoyningen V, Rott R. 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87:13-20.
62. Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, Gauger PC, Patnayak DP, Webby RJ, Richt JA. 2007. Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci U S A 104:20949-54.
63. Makarova NV, Kaverin NV, Krauss S, Senne D, Webster RG. 1999. Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. J Gen Virol 80 (Pt 12):3167-71.
64. Liu JH, Okazaki K, Bai GR, Shi WM, Mweene A, Kida H. 2004. Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. Virus Genes 29:81-6.
65. Ottis K, Sidoli L, Bachmann PA, Webster RG, Kaplan MM. 1982. Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. Arch Virol 73:103-8.
66. Cockburn WC, Delon PJ, Ferreira W. 1969. Origin and progress of the 1968-69 Hong Kong influenza epidemic. Bull World Health Organ 41:345-8.

67. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-5.
68. Novel Swine-Origin Influenza AVIT, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM. 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360:2605-15.
69. Kuiken T, Taubenberger JK. 2008. Pathology of human influenza revisited. Vaccine 26 Suppl 4:D59-66.
70. Pappas C, Viswanathan K, Chandrasekaran A, Raman R, Katz JM, Sasisekharan R, Tumpey TM. 2010. Receptor specificity and transmission of H2N2 subtype viruses isolated from the pandemic of 1957. PLoS One 5:e11158.
71. Roberts KL, Shelton H, Scull M, Pickles R, Barclay WS. 2011. Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding but rather a lower infectivity in vivo. J Gen Virol 92:1822-31.
72. Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A. 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 315:655-9.
73. Osterholm MT, Kelley NS, Sommer A, Belongia EA. 2012. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis 12:36-44.
74. Monto AS. 2003. The role of antivirals in the control of influenza. Vaccine 21:1796-800.
75. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev 56:152-79.
76. Shaw ML, Palese P. 2007. Fields Virology. Lippincott Williams & Wilkins, Philadelphia.
77. Wright PF, Neumann G, Kawaoka Y. 2007. Fields Virology, vol 3. Lippincott Williams & Wilkins, Philadelphia.
78. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW. 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7:1306-12.
79. Conenello GM, Palese P. 2007. Influenza A virus PB1-F2: a small protein with a big punch. Cell Host Microbe 2:207-9.
80. Alexander DJ. 2000. A review of avian influenza in different bird species. Vet Microbiol 74:3-13.
81. Alexander DJ, Brown IH. 2009. History of highly pathogenic avian influenza. Rev Sci Tech 28:19-38.
82. Webster RG, Rott R. 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell 50:665-6.
83. WHO. 2014. www.who.int/influenza/human_animal_interface/en/. Accessed 1/Dec/2019
84. Kandun IN, Wibisono H, Sedyaningsih ER, Yusharmen, Hadisoedarsuno W, Purba W, Santoso H, Septiawati C, Tresnaningsih E, Heriyanto B, Yuwono D, Harun S, Soeroso S, Giriputra S, Blair PJ, Jeremijenko A, Kosasih H, Putnam SD, Samaan G, Silitonga M, Chan KH, Poon LL, Lim W, Klimov A, Lindstrom S, Guan Y, Donis R, Katz J, Cox N, Peiris M, Uyeki TM. 2006. Three Indonesian clusters of H5N1 virus infection in 2005. N Engl J Med 355:2186-94.

85. Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, Uiprasertkul M, Boonnak K, Pittayawonganon C, Cox NJ, Zaki SR, Thawatsupha P, Chittaganpitch M, Khontong R, Simmerman JM, Chunsutthiwat S. 2005. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med 352:333-40.

86. Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, Huai Y, Dong J, Bao C, Wen L, Wang H, Yang P, Zhao W, Dong L, Zhou M, Liao Q, Yang H, Wang M, Lu X, Shi Z, Wang W, Gu L, Zhu F, Li Q, Yin W, Yang W, Li D, Uyeki TM, Wang Y. 2008. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. Lancet 371:1427-34.

87. de Wit E, Kawaoka Y, de Jong MD, Fouchier RA. 2008. Pathogenicity of highly pathogenic avian influenza virus in mammals. Vaccine 26 Suppl 4:D54-8.

88. Tscherne DM, Garcia-Sastre A. 2011. Virulence determinants of pandemic influenza viruses. J Clin Invest 121:6-13.

89. Jackson S, Van Hoeven N, Chen LM, Maines TR, Cox NJ, Katz JM, Donis RO. 2009. Reassortment between avian H5N1 and human H3N2 influenza viruses in ferrets: a public health risk assessment. J Virol 83:8131-40.

90. Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, Ortin J, Falcon A, Nguyen TH, Mai le Q, Sedyaningsih ER, Harun S, Tumpey TM, Donis RO, Cox NJ, Subbarao K, Katz JM. 2006. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A 103:12121-6.

91. Maines TR, Chen LM, Van Hoeven N, Tumpey TM, Blixt O, Belser JA, Gustin KM, Pearce MB, Pappas C, Stevens J, Cox NJ, Paulson JC, Raman R, Sasisekharan R, Katz JM, Donis RO. 2011. Effect of receptor binding domain mutations on receptor binding and transmissibility of avian influenza H5N1 viruses. Virology 413:139-47.

92. Yen HL, Lipatov AS, Ilyushina NA, Govorkova EA, Franks J, Yilmaz N, Douglas A, Hay A, Krauss S, Rehg JE, Hoffmann E, Webster RG. 2007. Inefficient transmission of H5N1 influenza viruses in a ferret contact model. J Virol 81:6890-8.

93. Smith W, Andrewes CH, Laidlaw PP. 1933. A virus obtained from Influenza patients. Lancet 222:66-68.

94. Maher JA, DeStefano J. 2004. The ferret: an animal model to study influenza virus. Lab Anim (NY) 33:50-3.

95. Munster VJ, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, van de Vijver D, Boucher CA, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA. 2009. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science 325:481-3.

96. Costa T, Chaves AJ, Valle R, Darji A, van Riel D, Kuiken T, Majo N, Ramis A. 2012. Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. Vet Res 43:28.

97. Mehle A, Doudna JA. 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. Proc Natl Acad Sci U S A 106:21312-6.

98. Steel J, Lowen AC, Mubareka S, Palese P. 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog 5:e1000252.

99. Van Hoeven N, Pappas C, Belser JA, Maines TR, Zeng H, Garcia-Sastre A, Sasisekharan R, Katz JM, Tumpey TM. 2009. Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A 106:3366-71.

100. de Wit E, Spronken MI, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2004. Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. Virus Res 103:155-61.

101. KNAW. 2007. www.knaw.nl/Content/Internet_KNAW/publicaties/pdf/20071092.pdf. Accessed 1/Dec/2019
102. Fouchier RA, Herfst S, Osterhaus AD. 2012. Public health and biosecurity. Restricted data on influenza H5N1 virus transmission. Science 335:662-3.
103. Chutinimitkul S, van Riel D, Munster VJ, van den Brand JM, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA, de Wit E. 2010. In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. J Virol 84:6825-33.
104. Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y. 2006. Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 444:378-82.
105. Nowak MA. 1992. What is a quasispecies? Trends Ecol Evol 7:118-21.
106. Bodewes R, Kreijtz JH, van Amerongen G, Fouchier RA, Osterhaus AD, Rimmelzwaan GF, Kuiken T. 2011. Pathogenesis of Influenza A/H5N1 virus infection in ferrets differs between intranasal and intratracheal routes of inoculation. Am J Pathol 179:30-6.
107. Schrauwen EJ, Herfst S, Leijten LM, van Run P, Bestebroer TM, Linster M, Bodewes R, Kreijtz JH, Rimmelzwaan GF, Osterhaus AD, Fouchier RA, Kuiken T, van Riel D. 2012. The multibasic cleavage site in H5N1 virus is critical for systemic spread along the olfactory and hematogenous routes in ferrets. J Virol 86:3975-84.
108. Epstein SL. 2006. Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. J Infect Dis 193:49-53.
109. McMichael AJ, Gotch FM, Noble GR, Beare PA. 1983. Cytotoxic T-cell immunity to influenza. N Engl J Med 309:13-7.
110. Bodewes R, Kreijtz JH, Geelhoed-Mieras MM, van Amerongen G, Verburgh RJ, van Trierum SE, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. 2011. Vaccination against seasonal influenza A/H3N2 virus reduces the induction of heterosubtypic immunity against influenza A/H5N1 virus infection in ferrets. J Virol 85:2695-702.
111. Kreijtz JH, Bodewes R, van den Brand JM, de Mutsert G, Baas C, van Amerongen G, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. 2009. Infection of mice with a human influenza A/H3N2 virus induces protective immunity against lethal infection with influenza A/H5N1 virus. Vaccine 27:4983-9.
112. van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, Munster V, Bestebroer T, Fouchier RA, Kuiken T, Osterhaus AD. 2010. Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. J Infect Dis 201:993-9.
113. Ha Y, Stevens DJ, Skehel JJ, Wiley DC. 2001. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc Natl Acad Sci U S A 98:11181-6.
114. Rogers GN, Daniels RS, Skehel JJ, Wiley DC, Wang XF, Higa HH, Paulson JC. 1985. Host-mediated selection of influenza virus receptor variants. Sialic acid-alpha 2,6Gal-specific clones of A/duck/Ukraine/1/63 revert to sialic acid-alpha 2,3Gal-specific wild type in ovo. J Biol Chem 260:7362-7.
115. Deom CM, Caton AJ, Schulze IT. 1986. Host cell-mediated selection of a mutant influenza A virus that has lost a complex oligosaccharide from the tip of the hemagglutinin. Proc Natl Acad Sci U S A 83:3771-5.
116. Mir-Shekari SY, Ashford DA, Harvey DJ, Dwek RA, Schulze IT. 1997. The glycosylation of the influenza A virus hemagglutinin by mammalian cells. A site-specific study. J Biol Chem 272:4027-36.

117. Rudneva IA, Ilyushina NA, Timofeeva TA, Webster RG, Kaverin NV. 2005. Restoration of virulence of escape mutants of H5 and H9 influenza viruses by their readaptation to mice. J Gen Virol 86:2831-8.
118. Stevens J, Blixt O, Chen LM, Donis RO, Paulson JC, Wilson IA. 2008. Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity. J Mol Biol 381:1382-94.
119. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y. 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 74:8502-12.
120. Bao Y, Bolotov P, Dernovoy D, Kiryutin B, Zaslavsky L, Tatusova T, Ostell J, Lipman D. 2008. The influenza virus resource at the National Center for Biotechnology Information. J Virol 82:596-601.
121. FAO. 2013. www.fao.org/avianflu/en/qanda.html. Accessed 1/Dec/2019
122. Bataille A, van der Meer F, Stegeman A, Koch G. 2011. Evolutionary analysis of inter-farm transmission dynamics in a highly pathogenic avian influenza epidemic. PLoS Pathog 7:e1002094.
123. de Wit E, Munster VJ, van Riel D, Beyer WE, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA. 2010. Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. J Virol 84:1597-606.
124. Jonges M, Bataille A, Enserink R, Meijer A, Fouchier RA, Stegeman A, Koch G, Koopmans M. 2011. Comparative analysis of avian influenza virus diversity in poultry and humans during a highly pathogenic avian influenza A (H7N7) virus outbreak. J Virol 85:10598-604.
125. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486:420-8.
126. Lowen AC, Mubareka S, Tumpey TM, Garcia-Sastre A, Palese P. 2006. The guinea pig as a transmission model for human influenza viruses. Proc Natl Acad Sci U S A 103:9988-92.
127. WHO/OIE/FAO. H5N1 Evolution Working Group. 2015. https://www.who.int/influenza/gisrs_laboratory/h5_nomenclature_clade2344/en/. Accessed 1/Dec/2019
128. Webster RG. 2012. Mammalian-transmissible H5N1 influenza: the dilemma of dual-use research. MBio 3.
129. COGEM. 2007. www.cogem.net/index.cfm/nl/publicaties/publicatie/pathogenese-en-transmissie-van- influenzavirussen. Accessed 1/Dec/2019
130. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97:6108-13.
131. Karber G. 1931. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. Exp Pathol Pharmakol 162.
132. Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, Aly MM, Brown IH, Couacy-Hymann E, De Mia GM, Dung do H, Guercio A, Joannis T, Maken Ali AS, Osmani A, Padalino I, Saad MD, Savic V, Sengamalay NA, Yingst S, Zaborsky J, Zorman-Rojs O, Ghedin E, Capua I. 2007. Genome analysis linking recent European and African influenza (H5N1) viruses. Emerg Infect Dis 13:713-8.
133. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146:2275-89.

134. Ha Y, Stevens DJ, Skehel JJ, Wiley DC. 2002. H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J 21:865-75.
135. Wang W, Lu B, Zhou H, Suguitan AL, Jr., Cheng X, Subbarao K, Kemble G, Jin H. 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. J Virol 84:6570-7.
136. van der Vries E, Veldhuis Kroeze EJ, Stittelaar KJ, Linster M, Van der Linden A, Schrauwen EJ, Leijten LM, van Amerongen G, Schutten M, Kuiken T, Osterhaus AD, Fouchier RA, Boucher CA, Herfst S. 2011. Multidrug resistant 2009 A/H1N1 influenza clinical isolate with a neuraminidase I223R mutation retains its virulence and transmissibility in ferrets. PLoS Pathog 7:e1002276.
137. Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2004. Mapping the antigenic and genetic evolution of influenza virus. Science 305:371-6.
138. Nobusawa E, Ishihara H, Morishita T, Sato K, Nakajima K. 2000. Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. Virology 278:587-96.
139. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351:472-7.
140. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336:1534-41.
141. Chen LM, Blixt O, Stevens J, Lipatov AS, Davis CT, Collins BE, Cox NJ, Paulson JC, Donis RO. 2012. In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity. Virology 422:105-13.
142. Zhang Y, Zhang Q, Kong H, Jiang Y, Gao Y, Deng G, Shi J, Tian G, Liu L, Liu J, Guan Y, Bu Z, Chen H. 2013. H5N1 hybrid viruses bearing 2009/H1N1 virus genes transmit in guinea pigs by respiratory droplet. Science 340:1459-63.
143. Aggarwal S, Dewhurst S, Takimoto T, Kim B. 2011. Biochemical impact of the host adaptation-associated PB2 E627K mutation on the temperature-dependent RNA synthesis kinetics of influenza A virus polymerase complex. J Biol Chem 286:34504-13.
144. Matrosovich MN, Gambaryan AS. 2012. Solid-phase assays of receptor-binding specificity. Methods Mol Biol 865:71-94.
145. Galloway SE, Reed ML, Russell CJ, Steinhauer DA. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. PLoS Pathog 9:e1003151.
146. Carr CM, Chaudhry C, Kim PS. 1997. Influenza hemagglutinin is spring-loaded by a metastable native conformation. Proc Natl Acad Sci U S A 94:14306-13.
147. Belser JA, Maines TR, Katz JM, Tumpey TM. 2013. Considerations regarding appropriate sample size for conducting ferret transmission experiments. Future Microbiol 8:961-5.
148. Russel WMS, Burch RL. 1959. The principles of humane experimental technique. London: Methuen.
149. Nishiura H, Yen HL, Cowling BJ. 2013. Sample size considerations for one-to-one animal transmission studies of the influenza A viruses. PLoS One 8:e55358.

150. de Vries RP, Zhu X, McBride R, Rigter A, Hanson A, Zhong G, Hatta M, Xu R, Yu W, Kawaoka Y, de Haan CA, Wilson IA, Paulson JC. 2014. Hemagglutinin receptor specificity and structural analyses of respiratory droplet-transmissible H5N1 viruses. J Virol 88:768-73.
151. Lu X, Shi Y, Zhang W, Zhang Y, Qi J, Gao GF. 2013. Structure and receptor-binding properties of an airborne transmissible avian influenza A virus hemagglutinin H5 (VN1203mut). Protein Cell 4:502-11.
152. Xiong X, Coombs PJ, Martin SR, Liu J, Xiao H, McCauley JW, Locher K, Walker PA, Collins PJ, Kawaoka Y, Skehel JJ, Gamblin SJ. 2013. Receptor binding by a ferret-transmissible H5 avian influenza virus. Nature 497:392-6.
153. Zhang W, Shi Y, Lu X, Shu Y, Qi J, Gao GF. 2013. An airborne transmissible avian influenza H5 hemagglutinin seen at the atomic level. Science 340:1463-7.
154. Liu J, Stevens DJ, Haire LF, Walker PA, Coombs PJ, Russell RJ, Gamblin SJ, Skehel JJ. 2009. Structures of receptor complexes formed by hemagglutinins from the Asian Influenza pandemic of 1957. Proc Natl Acad Sci U S A 106:17175-80.
155. Skehel JJ, Wiley DC. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69:531-69.
156. Krenn BM, Egorov A, Romanovskaya-Romanko E, Wolschek M, Nakowitsch S, Ruthsatz T, Kiefmann B, Morokutti A, Humer J, Geiler J, Cinatl J, Michaelis M, Wressnigg N, Sturlan S, Ferko B, Batishchev OV, Indenbom AV, Zhu R, Kastner M, Hinterdorfer P, Kiselev O, Muster T, Romanova J. 2011. Single HA2 mutation increases the infectivity and immunogenicity of a live attenuated H5N1 intranasal influenza vaccine candidate lacking NS1. PLoS One 6:e18577.
157. Shelton H, Roberts KL, Molesti E, Temperton N, Barclay WS. 2013. Mutations in haemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. J Gen Virol 94:1220-9.
158. Zaraket H, Bridges OA, Duan S, Baranovich T, Yoon SW, Reed ML, Salomon R, Webby RJ, Webster RG, Russell CJ. 2013. Increased acid stability of the hemagglutinin protein enhances H5N1 influenza virus growth in the upper respiratory tract but is insufficient for transmission in ferrets. J Virol 87:9911-22.
159. Neumann G, Macken CA, Karasin AI, Fouchier RA, Kawaoka Y. 2012. Egyptian H5N1 influenza viruses-cause for concern? PLoS Pathog 8:e1002932.
160. Tharakaraman K, Raman R, Viswanathan K, Stebbins NW, Jayaraman A, Krishnan A, Sasisekharan V, Sasisekharan R. 2013. Structural determinants for naturally evolving H5N1 hemagglutinin to switch its receptor specificity. Cell 153:1475-85.
161. Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, Bi Y, Wu Y, Li X, Yan J, Liu W, Zhao G, Yang W, Wang Y, Ma J, Shu Y, Lei F, Gao GF. 2013. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet 381:1926-32.
162. Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, Saito T, Kawaoka Y, Tashiro M. 2013. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. Euro Surveill 18:20453.
163. van Riel D, Leijten LM, de Graaf M, Siegers JY, Short KR, Spronken MI, Schrauwen EJ, Fouchier RA, Osterhaus AD, Kuiken T. 2013. Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans. Am J Pathol 183:1137-43.
164. Lam TT, Wang J, Shen Y, Zhou B, Duan L, Cheung CL, Ma C, Lycett SJ, Leung CY, Chen X, Li L, Hong W, Chai Y, Zhou L, Liang H, Ou Z, Liu Y, Farooqui A, Kelvin DJ, Poon LL, Smith DK, Pybus OG, Leung GM, Shu Y, Webster RG, Webby RJ, Peiris JS, Rambaut A, Zhu H, Guan Y. 2013. The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature 502:241-4.

165. Shi Y, Zhang W, Wang F, Qi J, Wu Y, Song H, Gao F, Bi Y, Zhang Y, Fan Z, Qin C, Sun H, Liu J, Haywood J, Liu W, Gong W, Wang D, Shu Y, Wang Y, Yan J, Gao GF. 2013. Structures and receptor binding of hemagglutinins from human-infecting H7N9 influenza viruses. Science 342:243-7.

166. Zhu H, Wang D, Kelvin DJ, Li L, Zheng Z, Yoon SW, Wong SS, Farooqui A, Wang J, Banner D, Chen R, Zheng R, Zhou J, Zhang Y, Hong W, Dong W, Cai Q, Roehrl MH, Huang SS, Kelvin AA, Yao T, Zhou B, Chen X, Leung GM, Poon LL, Webster RG, Webby RJ, Peiris JS, Guan Y, Shu Y. 2013. Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. Science 341:183-6.

167. Kuiken T, van den Hoogen BG, van Riel DA, Laman JD, van Amerongen G, Sprong L, Fouchier RA, Osterhaus AD. 2004. Experimental human metapneumovirus infection of cynomolgus macaques (Macaca fascicularis) results in virus replication in ciliated epithelial cells and pneumocytes with associated lesions throughout the respiratory tract. Am J Pathol 164:1893-900.

168. Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, van Riel D, Schrauwen EJ, Bestebroer TM, Koel B, Burke DF, Sutherland-Cash KH, Whittleston CS, Russell CA, Wales DJ, Smith DJ, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Garcia-Sastre A, Perez DR, Fouchier RA. 2010. Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. J Virol 84:11802-13.

169. Matrosovich M, Zhou N, Kawaoka Y, Webster R. 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 73:1146-55.

170. Jones JC, Baranovich T, Marathe BM, Danner AF, Seiler JP, Franks J, Govorkova EA, Krauss S, Webster RG. 2014. Risk assessment of H2N2 influenza viruses from the avian reservoir. J Virol 88:1175-88.

171. Kimble JB, Angel M, Wan H, Sutton TC, Finch C, Perez DR. 2014. Alternative reassortment events leading to transmissible H9N1 influenza viruses in the ferret model. J Virol 88:66-71.

172. WHO. 2002. WHO manual on animal influenza diagnosis and surveillance. http://www.wpro.who.int/emerging_diseases/documents/docs/manualonanimalaidiagnosisandsurveillance.pdf. Accessed 1/Dec/2019

173. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T. 2007. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 171:1215-23.

174. Herfst S, Mas V, Ver LS, Wierda RJ, Osterhaus AD, Fouchier RA, Melero JA. 2008. Low-pH-induced membrane fusion mediated by human metapneumovirus F protein is a rare, strain-dependent phenomenon. J Virol 82:8891-5.

175. Dunn JJ, Krippl B, Bernstein KE, Westphal H, Studier FW. 1988. Targeting bacteriophage T7 RNA polymerase to the mammalian cell nucleus. Gene 68:259-66.

176. Matrosovich M, Matrosovich T, Garten W, Klenk HD. 2006. New low-viscosity overlay medium for viral plaque assays. Virol J 3:63.

177. Turrell L, Lyall JW, Tiley LS, Fodor E, Vreede FT. 2013. The role and assembly mechanism of nucleoprotein in influenza A virus ribonucleoprotein complexes. Nat Commun 4:1591.

178. Depew J, Zhou B, McCorrison JM, Wentworth DE, Purushe J, Koroleva G, Fouts DE. 2013. Sequencing viral genomes from a single isolated plaque. Virol J 10:181.

179. Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, Afonso C, Zhang X, Anderson NG, Ghedin E, Spiro DJ. 2008. Viral genome sequencing by random priming methods. BMC Genomics 9:5.

180. Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, Wentworth DE. 2009. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. J Virol 83:10309-13.
181. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059-66.
182. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490.
183. Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29:1969-73.
184. Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, Webster RG, Peiris JS, Guan Y. 2009. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A 106:11709-12.
185. Klimov AI, Bender CA, Hall HE, Cox NJ. 1996. Evolution of human influenza A (H2N2) viruses.546-552.
186. Lindstrom SE, Cox NJ, Klimov A. 2004. Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957-1972: evidence for genetic divergence and multiple reassortment events. Virology 328:101-19.
187. Bahl J, Vijaykrishna D, Holmes EC, Smith GJ, Guan Y. 2009. Gene flow and competitive exclusion of avian influenza A virus in natural reservoir hosts. Virology 390:289-97.
188. Kilbourne ED. 2006. Influenza pandemics of the 20th century. Emerg Infect Dis 12:9-14.
189. Bahl J, Krauss S, Kuhnert D, Fourment M, Raven G, Pryor SP, Niles LJ, Danner A, Walker D, Mendenhall IH, Su YC, Dugan VG, Halpin RA, Stockwell TB, Webby RJ, Wentworth DE, Drummond AJ, Smith GJ, Webster RG. 2013. Influenza a virus migration and persistence in North American wild birds. PLoS Pathog 9:e1003570.
190. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. 2008. The genomic and epidemiological dynamics of human influenza A virus. Nature 453:615-9.
191. Delport W, Poon AF, Frost SD, Kosakovsky Pond SL. 2010. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 26:2455-7.
192. Kosakovsky Pond SL, Frost SD. 2005. Not so different after all: a comparison of methods for detecting amino acid sites under selection. Mol Biol Evol 22:1208-22.
193. Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol 24:1586-91.
194. Pybus OG, Rambaut A, Belshaw R, Freckleton RP, Drummond AJ, Holmes EC. 2007. Phylogenetic evidence for deleterious mutation load in RNA viruses and its contribution to viral evolution. Mol Biol Evol 24:845-52.
195. Herlocher ML, Bucher D, Webster RG. 1992. Host range determination and functional mapping of the nucleoprotein and matrix genes of influenza viruses using monoclonal antibodies. Virus Res 22:281-93.
196. Tsuchiya E, Sugawara K, Hongo S, Matsuzaki Y, Muraki Y, Li ZN, Nakamura K. 2001. Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus. J Gen Virol 82:2475-84.
197. Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Kosakovsky Pond SL. 2012. Detecting individual sites subject to episodic diversifying selection. PLoS Genet 8:e1002764.
198. Tamuri AU, Dos Reis M, Hay AJ, Goldstein RA. 2009. Identifying changes in selective constraints: host shifts in influenza. PLoS Comput Biol 5:e1000564.

199. Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, Kawaoka Y. 1998. The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J Virol 72:7626-31.
200. Medina RA, Garcia-Sastre A. 2011. Influenza A viruses: new research developments. Nat Rev Microbiol 9:590-603.
201. Greenbaum BD, Levine AJ, Bhanot G, Rabadan R. 2008. Patterns of evolution and host gene mimicry in influenza and other RNA viruses. PLoS Pathog 4:e1000079.
202. Worobey M, Han GZ, Rambaut A. 2014. A synchronized global sweep of the internal genes of modern avian influenza virus. Nature 508:254-7.
203. Zhou JP, Ge FF, Liu J, Ju HB, Yang DQ, Wang J, Zhang WY, Liu PH. 2012. Epidemiological survey and genetic evolution of H9 subtype influenza viruses in Shanghai, China, from 2006 to 2010. Arch Virol 157:1193-8.
204. Djikeng A, Kuzmickas R, Anderson NG, Spiro DJ. 2009. Metagenomic analysis of RNA viruses in a fresh water lake. PLoS One 4:e7264.
205. Langmuir AD, Pizzi M, Trotter WY, Dunn FL. 1958. [Asian influenza surveillance]. Public Health Rep 73:114-20.
206. Dunn FL. 1958. Pandemic influenza in 1957; review of international spread of new Asian strain. J Am Med Assoc 166:1140-8.
207. Fukumi H. 1959. Summary report on the Asian influenza epidemic in Japan, 1957. Bull World Health Organ 20:187-98.
208. 2013 RCT.
209. Gardiner-Garden M, Frommer M. 1987. CpG islands in vertebrate genomes. J Mol Biol 196:261-82.
210. Rabadan R, Levine AJ, Robins H. 2006. Comparison of avian and human influenza A viruses reveals a mutational bias on the viral genomes. J Virol 80:11887-91.
211. Blumenfeld HL, Kilbourne ED, Louria DB, Rogers DE. 1959. Studies on influenza in the pandemic of 1957-1958. I. An epidemiologic, clinical and serologic investigation of an intrahospital epidemic, with a note on vaccination efficacy. J Clin Invest 38:199-212.
212. Viboud C, Simonsen L, Fuentes R, Flores J, Miller MA, Chowell G. 2016. Global Mortality Impact of the 1957-1959 Influenza Pandemic. J Infect Dis 213:738-45.
213. Cobos AJ, Nelson CG, Jehn M, Viboud C, Chowell G. 2016. Mortality and transmissibility patterns of the 1957 influenza pandemic in Maricopa County, Arizona. BMC Infect Dis 16:405.
214. Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 3:e61.
215. Wu H, Peng X, Peng X, Cheng L, Wu N. 2016. Genetic and molecular characterization of a novel reassortant H2N8 subtype avian influenza virus isolated from a domestic duck in Zhejiang Province in China. Virus Genes 52:863-866.
216. Chen GL, Lamirande EW, Jin H, Kemble G, Subbarao K. 2010. Safety, immunogencity, and efficacy of a cold-adapted A/Ann Arbor/6/60 (H2N2) vaccine in mice and ferrets. Virology 398:109-14.
217. Hehme N, Engelmann H, Kunzel W, Neumeier E, Sanger R. 2002. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. Med Microbiol Immunol 191:203-8.
218. Isakova-Sivak I, de Jonge J, Smolonogina T, Rekstin A, van Amerongen G, van Dijken H, Mouthaan J, Roholl P, Kuznetsova V, Doroshenko E, Tsvetnitsky V, Rudenko L. 2014. Development and pre-clinical evaluation of two LAIV strains against potentially pandemic H2N2 influenza virus. PLoS One 9:e102339.
219. Nabel GJ, Wei CJ, Ledgerwood JE. 2011. Vaccinate for the next H2N2 pandemic now. Nature 471:157-8.

220. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417-27.
221. Wiley DC, Wilson IA, Skehel JJ. 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 289:373-8.
222. Wilson IA, Cox NJ. 1990. Structural basis of immune recognition of influenza virus hemagglutinin. Annu Rev Immunol 8:737-71.
223. Koel BF, Burke DF, Bestebroer TM, van der Vliet S, Zondag GC, Vervaet G, Skepner E, Lewis NS, Spronken MI, Russell CA, Eropkin MY, Hurt AC, Barr IG, de Jong JC, Rimmelzwaan GF, Osterhaus AD, Fouchier RA, Smith DJ. 2013. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science 342:976-9.
224. Koel BF, van der Vliet S, Burke DF, Bestebroer TM, Bharoto EE, Yasa IW, Herliana I, Laksono BM, Xu K, Skepner E, Russell CA, Rimmelzwaan GF, Perez DR, Osterhaus AD, Smith DJ, Prajitno TY, Fouchier RA. 2014. Antigenic variation of clade 2.1 H5N1 virus is determined by a few amino acid substitutions immediately adjacent to the receptor binding site. MBio 5:e01070-14.
225. Guarnaccia T, Carolan LA, Maurer-Stroh S, Lee RT, Job E, Reading PC, Petrie S, McCaw JM, McVernon J, Hurt AC, Kelso A, Mosse J, Barr IG, Laurie KL. 2013. Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. PLoS Pathog 9:e1003354.
226. Koel BF, Mogling R, Chutinimitkul S, Fraaij PL, Burke DF, van der Vliet S, de Wit E, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Smith DJ, Fouchier RA, de Graaf M. 2015. Identification of amino acid substitutions supporting antigenic change of influenza A(H1N1)pdm09 viruses. J Virol 89:3763-75.
227. Li Y, Myers JL, Bostick DL, Sullivan CB, Madara J, Linderman SL, Liu Q, Carter DM, Wrammert J, Esposito S, Principi N, Plotkin JB, Ross TM, Ahmed R, Wilson PC, Hensley SE. 2013. Immune history shapes specificity of pandemic H1N1 influenza antibody responses. J Exp Med 210:1493-500.
228. Lewis NS, Daly JM, Russell CA, Horton DL, Skepner E, Bryant NA, Burke DF, Rash AS, Wood JL, Chambers TM, Fouchier RA, Mumford JA, Elton DM, Smith DJ. 2011. Antigenic and genetic evolution of equine influenza A (H3N8) virus from 1968 to 2007. J Virol 85:12742-9.
229. Lewis NS, Russell CA, Langat P, Anderson TK, Berger K, Bielejec F, Burke DF, Dudas G, Fonville JM, Fouchier RA, Kellam P, Koel BF, Lemey P, Nguyen T, Nuansrichy B, Peiris JM, Saito T, Simon G, Skepner E, Takemae N, consortium E, Webby RJ, Van Reeth K, Brookes SM, Larsen L, Watson SJ, Brown IH, Vincent AL. 2016. The global antigenic diversity of swine influenza A viruses. Elife 5:e12217.
230. Both GW, Sleigh MJ, Cox NJ, Kendal AP. 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol 48:52-60.
231. Rota PA, Hemphill ML, Whistler T, Regnery HL, Kendal AP. 1992. Antigenic and genetic characterization of the haemagglutinins of recent cocirculating strains of influenza B virus. J Gen Virol 73 (Pt 10):2737-42.
232. Burke DF, Smith DJ. 2014. A recommended numbering scheme for influenza A HA subtypes. PLoS One 9:e112302.
233. Westgeest KB, de Graaf M, Fourment M, Bestebroer TM, van Beek R, Spronken MI, de Jong JC, Rimmelzwaan GF, Russell CA, Osterhaus AD, Smith GJ, Smith DJ, Fouchier RA. 2012. Genetic evolution of the neuraminidase of influenza A (H3N2) viruses from 1968 to 2009 and its correspondence to haemagglutinin evolution. J Gen Virol 93:1996-2007.
234. Al Khatib HA, Al Thani AA, Yassine HM. 2018. Evolution and dynamics of the pandemic H1N1 influenza hemagglutinin protein from 2009 to 2017. Arch Virol 163:3035-3049.

235. Su YCF, Bahl J, Joseph U, Butt KM, Peck HA, Koay ESC, Oon LLE, Barr IG, Vijaykrishna D, Smith GJD. 2015. Phylodynamics of H1N1/2009 influenza reveals the transition from host adaptation to immune-driven selection. Nat Commun 6:7952.
236. Neher RA, Bedford T, Daniels RS, Russell CA, Shraiman BI. 2016. Prediction, dynamics, and visualization of antigenic phenotypes of seasonal influenza viruses. Proc Natl Acad Sci U S A 113:E1701-9.
237. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307-21.
238. Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214.
239. Shapiro B, Rambaut A, Drummond AJ. 2006. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Mol Biol Evol 23:7-9.
240. Minin VN, Bloomquist EW, Suchard MA. 2008. Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. Mol Biol Evol 25:1459-71.
241. Choppin PW, Tamm I. 1960. Studies of two kinds of virus particles which comprise influenza A2 virus strains. II. Reactivity with virus inhibitors in normal sera. J Exp Med 112:921-44.
242. Webby RJ, Webster RG. 2003. Are we ready for pandemic influenza? Science 302:1519-22.
243. Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, Zeng H, Gustin KM, Pearce MB, Viswanathan K, Shriver ZH, Raman R, Cox NJ, Sasisekharan R, Katz JM, Tumpey TM. 2009. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science 325:484-7.
244. Schrauwen EJ, Herfst S, Chutinimitkul S, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Kuiken T, Fouchier RA. 2011. Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment. Emerg Infect Dis 17:200-8.
245. Schrauwen EJ, Bestebroer TM, Munster VJ, de Wit E, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2011. Insertion of a multibasic cleavage site in the haemagglutinin of human influenza H3N2 virus does not increase pathogenicity in ferrets. J Gen Virol 92:1410-5.
246. Gambaryan AS, Tuzikov AB, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Bovin NV, Matrosovich MN. 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyllactosamine). Virology 232:345-50.
247. Robertson JS. 1993. Clinical influenza virus and the embryonated Hen's egg. Reviews in Medical Virology 3:97-106.
248. Pappas C, Yang H, Carney PJ, Pearce MB, Katz JM, Stevens J, Tumpey TM. 2015. Assessment of transmission, pathogenesis and adaptation of H2 subtype influenza viruses in ferrets. Virology 477:61-71.
249. Belser JA, Barclay W, Barr I, Fouchier RAM, Matsuyama R, Nishiura H, Peiris M, Russell CJ, Subbarao K, Zhu H, Yen HL. 2018. Ferrets as Models for Influenza Virus Transmission Studies and Pandemic Risk Assessments. Emerg Infect Dis 24:965-971.
250. Reed LMH. 1938. A simple method for estimating fifty percent endpoints. The American Journal of Hygiene 27:493-497.

251. Imai M, Herfst S, Sorrell EM, Schrauwen EJ, Linster M, De Graaf M, Fouchier RA, Kawaoka Y. 2013. Transmission of influenza A/H5N1 viruses in mammals. Virus Res 178:15-20.

252. Pan M, Gao R, Lv Q, Huang S, Zhou Z, Yang L, Li X, Zhao X, Zou X, Tong W, Mao S, Zou S, Bo H, Zhu X, Liu L, Yuan H, Zhang M, Wang D, Li Z, Zhao W, Ma M, Li Y, Li T, Yang H, Xu J, Zhou L, Zhou X, Tang W, Song Y, Chen T, Bai T, Zhou J, Wang D, Wu G, Li D, Feng Z, Gao GF, Wang Y, He S, Shu Y. 2016. Human infection with a novel, highly pathogenic avian influenza A (H5N6) virus: Virological and clinical findings. J Infect 72:52-9.

253. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, Lochindarat S, Nguyen TK, Nguyen TH, Tran TH, Nicoll A, Touch S, Yuen KY, Writing Committee of the World Health Organization Consultation on Human Influenza AH. 2005. Avian influenza A (H5N1) infection in humans. N Engl J Med 353:1374-85.

254. Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, Xiang N, Chen E, Tang F, Wang D, Meng L, Hong Z, Tu W, Cao Y, Li L, Ding F, Liu B, Wang M, Xie R, Gao R, Li X, Bai T, Zou S, He J, Hu J, Xu Y, Chai C, Wang S, Gao Y, Jin L, Zhang Y, Luo H, Yu H, He J, Li Q, Wang X, Gao L, Pang X, Liu G, Yan Y, Yuan H, Shu Y, Yang W, Wang Y, Wu F, Uyeki TM, Feng Z. 2014. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med 370:520-32.

255. Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, Subbarao K, Guan Y, Krauss S, Shortridge K, Webster R, Cox N, Hay A. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci U S A 97:9654-8.

256. Schrauwen EJ, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA, Herfst S. 2013. Reassortment between Avian H5N1 and human influenza viruses is mainly restricted to the matrix and neuraminidase gene segments. PLoS One 8:e59889.

257. Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. 2006. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 312:404-10.

258. Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, Li Z, Guan Y, Tian G, Li Y, Shi J, Liu L, Zeng X, Bu Z, Xia X, Kawaoka Y, Chen H. 2009. Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog 5:e1000709.

259. Tate MD, Job ER, Deng YM, Gunalan V, Maurer-Stroh S, Reading PC. 2014. Playing hide and seek: how glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. Viruses 6:1294-316.

260. Zhao D, Liang L, Wang S, Nakao T, Li Y, Liu L, Guan Y, Fukuyama S, Bu Z, Kawaoka Y, Chen H. 2017. Glycosylation of the Hemagglutinin Protein of H5N1 Influenza Virus Increases Its Virulence in Mice by Exacerbating the Host Immune Response. J Virol 91.

261. Russell CJ, Hu M, Okda FA. 2018. Influenza Hemagglutinin Protein Stability, Activation, and Pandemic Risk. Trends Microbiol 26:841-853.

262. Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, Herfst S, van Boheemen S, Linster M, Schrauwen EJ, Katzelnick L, Mosterin A, Kuiken T, Maher E, Neumann G, Osterhaus AD, Kawaoka Y, Fouchier RA, Smith DJ. 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science 336:1541-7.

263. Watanabe Y, Ibrahim MS, Ellakany HF, Kawashita N, Mizuike R, Hiramatsu H, Sriwilaijaroen N, Takagi T, Suzuki Y, Ikuta K. 2011. Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. PLoS Pathog 7:e1002068.

264. Abdelwhab EM, Hassan MK, Abdel-Moneim AS, Naguib MM, Mostafa A, Hussein ITM, Arafa A, Erfan AM, Kilany WH, Agour MG, El-Kanawati Z, Hussein HA, Selim AA, Kholousy S, El-Naggar H, El-Zoghby EF, Samy A, Iqbal M, Eid A, Ibraheem EM, Pleschka S, Veits J, Nasef SA, Beer M, Mettenleiter TC, Grund C, Ali MM, Harder TC, Hafez HM. 2016. Introduction and enzootic of A/H5N1 in Egypt: Virus evolution, pathogenicity and vaccine efficacy ten years on. Infect Genet Evol 40:80-90.

265. Richard M, Herfst S, van den Brand JMA, de Meulder D, Lexmond P, Bestebroer TM, Fouchier RAM. 2017. Mutations Driving Airborne Transmission of A/H5N1 Virus in Mammals Cause Substantial Attenuation in Chickens only when combined. Sci Rep 7:7187.

266. WHO. 2019. Avian Influenza Weekly Update Number 674. https://www.who.int/docs/default-source/wpro---documents/emergency/surveillance/avian-influenza/ai-20190201.pdf?sfvrsn=223ca73f_6. . Accessed 1/Dec/2019

267. WHO. 2019. Avian Influenza Weekly Update Number 691. https://www.who.int/docs/default-source/wpro---documents/emergency/surveillance/avian-influenza/ai-20190531.pdf?sfvrsn=c0382d50_18. Accessed 1/Dec/2019

268. Bi Y, Chen Q, Wang Q, Chen J, Jin T, Wong G, Quan C, Liu J, Wu J, Yin R, Zhao L, Li M, Ding Z, Zou R, Xu W, Li H, Wang H, Tian K, Fu G, Huang Y, Shestopalov A, Li S, Xu B, Yu H, Luo T, Lu L, Xu X, Luo Y, Liu Y, Shi W, Liu D, Gao GF. 2016. Genesis, Evolution and Prevalence of H5N6 Avian Influenza Viruses in China. Cell Host Microbe 20:810-821.

269. Taubenberger JK MD. 2017. H5Nx Panzootic Bird Flu—Influenza's Newest Worldwide Evolutionary Tour. . Emerg Infect Dis 23(2):340–342.

270. Freidl GS, Meijer A, de Bruin E, de Nardi M, Munoz O, Capua I, Breed AC, Harris K, Hill A, Kosmider R, Banks J, von Dobschuetz S, Stark K, Wieland B, Stevens K, van der Werf S, Enouf V, van der Meulen K, Van Reeth K, Dauphin G, Koopmans M. 2014. Influenza at the animal-human interface: a review of the literature for virological evidence of human infection with swine or avian influenza viruses other than A(H5N1). Euro Surveill 19.

271. Belser JA, Bridges CB, Katz JM, Tumpey TM. 2009. Past, present, and possible future human infection with influenza virus A subtype H7. Emerg Infect Dis 15:859-65.

272. Belser JA, Creager HM, Sun X, Gustin KM, Jones T, Shieh WJ, Maines TR, Tumpey TM. 2016. Mammalian Pathogenesis and Transmission of H7N9 Influenza Viruses from Three Waves, 2013-2015. J Virol 90:4647-4657.

273. Sun X, Belser JA, Pappas C, Pulit-Penaloza JA, Brock N, Zeng H, Creager HM, Le S, Wilson M, Lewis A, Stark TJ, Shieh WJ, Barnes J, Tumpey TM, Maines TR. 2019. Risk Assessment of Fifth-Wave H7N9 Influenza A Viruses in Mammalian Models. J Virol 93.

274. Schrauwen EJ, Richard M, Burke DF, Rimmelzwaan GF, Herfst S, Fouchier RA. 2016. Amino Acid Substitutions That Affect Receptor Binding and Stability of the Hemagglutinin of Influenza A/H7N9 Virus. J Virol 90:3794-9.

275. de Vries RP, Peng W, Grant OC, Thompson AJ, Zhu X, Bouwman KM, de la Pena ATT, van Breemen MJ, Ambepitiya Wickramasinghe IN, de Haan CAM, Yu W, McBride R, Sanders RW, Woods RJ, Verheije MH, Wilson IA, Paulson JC. 2017. Three mutations switch H7N9 influenza to human-type receptor specificity. PLoS Pathog 13:e1006390.

276. Watanabe T, Kiso M, Fukuyama S, Nakajima N, Imai M, Yamada S, Murakami S, Yamayoshi S, Iwatsuki-Horimoto K, Sakoda Y, Takashita E, McBride R, Noda T, Hatta M, Imai H, Zhao D, Kishida N, Shirakura M, de Vries RP, Shichinohe S, Okamatsu M, Tamura T, Tomita Y, Fujimoto N, Goto K, Katsura H, Kawakami E, Ishikawa I, Watanabe S, Ito M, Sakai-Tagawa Y, Sugita Y, Uraki R, Yamaji R, Eisfeld AJ, Zhong G, Fan S, Ping J, Maher EA, Hanson A, Uchida Y, Saito T, Ozawa M, Neumann G, Kida H, Odagiri T, Paulson JC, Hasegawa H, Tashiro M, Kawaoka Y. 2013. Characterization of H7N9 influenza A viruses isolated from humans. Nature 501:551-5.

277. WHO. 2013. Avian Influena A(H7N9) Virus. http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/index.html. Accessed 1/Dec/2019

278. Wu J, Ke C, Lau EHY, Song Y, Cheng KL, Zou L, Kang M, Song T, Peiris M, Yen HL. 2019. Influenza H5/H7 Virus Vaccination in Poultry and Reduction of Zoonotic Infections, Guangdong Province, China, 2017-18. Emerg Infect Dis 25:116-118.

279. Ma MJ, Yang Y, Fang LQ. 2019. Highly Pathogenic Avian H7N9 Influenza Viruses: Recent Challenges. Trends Microbiol 27:93-95.

280. Matrosovich MN, Krauss S, Webster RG. 2001. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281:156-62.

281. Arzey GG, Kirkland PD, Arzey KE, Frost M, Maywood P, Conaty S, Hurt AC, Deng YM, Iannello P, Barr I, Dwyer DE, Ratnamohan M, McPhie K, Selleck P. 2012. Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia. Emerg Infect Dis 18:814-6.

282. Liu M, Li X, Yuan H, Zhou J, Wu J, Bo H, Xia W, Xiong Y, Yang L, Gao R, Guo J, Huang W, Zhang Y, Zhao X, Zou X, Chen T, Wang D, Li Q, Wang S, Chen S, Hu M, Ni X, Gong T, Shi Y, Li J, Zhou J, Cai J, Xiao Z, Zhang W, Sun J, Li D, Wu G, Feng Z, Wang Y, Chen H, Shu Y. 2015. Genetic diversity of avian influenza A (H10N8) virus in live poultry markets and its association with human infections in China. Sci Rep 5:7632.

283. Wei SH, Yang JR, Wu HS, Chang MC, Lin JS, Lin CY, Liu YL, Lo YC, Yang CH, Chuang JH, Lin MC, Chung WC, Liao CH, Lee MS, Huang WT, Chen PJ, Liu MT, Chang FY. 2013. Human infection with avian influenza A H6N1 virus: an epidemiological analysis. Lancet Respir Med 1:771-8.

284. Sun H, Pu J, Wei Y, Sun Y, Hu J, Liu L, Xu G, Gao W, Li C, Zhang X, Huang Y, Chang KC, Liu X, Liu J. 2016. Highly Pathogenic Avian Influenza H5N6 Viruses Exhibit Enhanced Affinity for Human Type Sialic Acid Receptor and In-Contact Transmission in Model Ferrets. J Virol 90:6235-6243.

285. Herfst S, Mok CKP, van den Brand JMA, van der Vliet S, Rosu ME, Spronken MI, Yang Z, de Meulder D, Lexmond P, Bestebroer TM, Peiris JSM, Fouchier RAM, Richard M. 2018. Human Clade 2.3.4.4 A/H5N6 Influenza Virus Lacks Mammalian Adaptation Markers and Does Not Transmit via the Airborne Route between Ferrets. mSphere 3.

286. Chin AW, Yen HL, Krauss S, Webby RJ, Poon LL. 2016. Recombinant influenza virus with a pandemic H2N2 polymerase complex has a higher adaptive potential than one with seasonal H2N2 polymerase complex. J Gen Virol 97:611-9.

287. Babu TM, Perera R, Wu JT, Fitzgerald T, Nolan C, Cowling BJ, Krauss S, Treanor JJ, Peiris M. 2018. Population Serologic Immunity to Human and Avian H2N2 Viruses in the United States and Hong Kong for Pandemic Risk Assessment. J Infect Dis 218:1054-1060.

288. Pasteur L, Roux E, Chamberland C, Thuillier L. 1881. Note sur la rage. C R Acad Sci 92.

289. Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS, Grenfell BT. 2006. Host species barriers to influenza virus infections. Science 312:394-7.

290. Reperant LA, Kuiken T, Osterhaus AD. 2012. Adaptive pathways of zoonotic influenza viruses: from exposure to establishment in humans. Vaccine 30:4419-34.
291. Varble A, Albrecht RA, Backes S, Crumiller M, Bouvier NM, Sachs D, Garcia-Sastre A, tenOever BR. 2014. Influenza A virus transmission bottlenecks are defined by infection route and recipient host. Cell Host Microbe 16:691-700.
292. Poon LL, Song T, Rosenfeld R, Lin X, Rogers MB, Zhou B, Sebra R, Halpin RA, Guan Y, Twaddle A, DePasse JV, Stockwell TB, Wentworth DE, Holmes EC, Greenbaum B, Peiris JS, Cowling BJ, Ghedin E. 2016. Quantifying influenza virus diversity and transmission in humans. Nat Genet 48:195-200.
293. Wilker PR, Dinis JM, Starrett G, Imai M, Hatta M, Nelson CW, O'Connor DH, Hughes AL, Neumann G, Kawaoka Y, Friedrich TC. 2013. Selection on haemagglutinin imposes a bottleneck during mammalian transmission of reassortant H5N1 influenza viruses. Nat Commun 4:2636.
294. Frise R, Bradley K, van Doremalen N, Galiano M, Elderfield RA, Stilwell P, Ashcroft JW, Fernandez-Alonso M, Miah S, Lackenby A, Roberts KL, Donnelly CA, Barclay WS. 2016. Contact transmission of influenza virus between ferrets imposes a looser bottleneck than respiratory droplet transmission allowing propagation of antiviral resistance. Sci Rep 6:29793.
295. Sobel Leonard A, Weissman DB, Greenbaum B, Ghedin E, Koelle K. 2017. Transmission Bottleneck Size Estimation from Pathogen Deep-Sequencing Data, with an Application to Human Influenza A Virus. J Virol 91.
296. Moncla LH, Zhong G, Nelson CW, Dinis JM, Mutschler J, Hughes AL, Watanabe T, Kawaoka Y, Friedrich TC. 2016. Selective Bottlenecks Shape Evolutionary Pathways Taken during Mammalian Adaptation of a 1918-like Avian Influenza Virus. Cell Host Microbe 19:169-80.
297. Manz B, Schwemmle M, Brunotte L. 2013. Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier. J Virol 87:7200-9.
298. Song W, Wang P, Mok BW, Lau SY, Huang X, Wu WL, Zheng M, Wen X, Yang S, Chen Y, Li L, Yuen KY, Chen H. 2014. The K526R substitution in viral protein PB2 enhances the effects of E627K on influenza virus replication. Nat Commun 5:5509.
299. Yamaji R, Yamada S, Le MQ, Li C, Chen H, Qurnianingsih E, Nidom CA, Ito M, Sakai-Tagawa Y, Kawaoka Y. 2015. Identification of PB2 mutations responsible for the efficient replication of H5N1 influenza viruses in human lung epithelial cells. J Virol 89:3947-56.
300. Wu NC, Xie J, Zheng T, Nycholat CM, Grande G, Paulson JC, Lerner RA, Wilson IA. 2017. Diversity of Functionally Permissive Sequences in the Receptor-Binding Site of Influenza Hemagglutinin. Cell Host Microbe 21:742-753.e8.
301. Czudai-Matwich V, Otte A, Matrosovich M, Gabriel G, Klenk HD. 2014. PB2 mutations D701N and S714R promote adaptation of an influenza H5N1 virus to a mammalian host. J Virol 88:8735-42.
302. Manz B, de Graaf M, Mogling R, Richard M, Bestebroer TM, Rimmelzwaan GF, Fouchier RAM. 2016. Multiple Natural Substitutions in Avian Influenza A Virus PB2 Facilitate Efficient Replication in Human Cells. J Virol 90:5928-5938.
303. Yamada S, Hatta M, Staker BL, Watanabe S, Imai M, Shinya K, Sakai-Tagawa Y, Ito M, Ozawa M, Watanabe T, Sakabe S, Li C, Kim JH, Myler PJ, Phan I, Raymond A, Smith E, Stacy R, Nidom CA, Lank SM, Wiseman RW, Bimber BN, O'Connor DH, Neumann G, Stewart LJ, Kawaoka Y. 2010. Biological and structural characterization of a host-adapting amino acid in influenza virus. PLoS Pathog 6:e1001034.
304. Pflug A, Guilligay D, Reich S, Cusack S. 2014. Structure of influenza A polymerase bound to the viral RNA promoter. Nature 516:355-60.
305. Roberts PC, Lamb RA, Compans RW. 1998. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology 240:127-37.

306. Seladi-Schulman J, Steel J, Lowen AC. 2013. Spherical influenza viruses have a fitness advantage in embryonated eggs, while filament-producing strains are selected in vivo. J Virol 87:13343-53.
307. Batishchev OV, Shilova LA, Kachala MV, Tashkin VY, Sokolov VS, Fedorova NV, Baratova LA, Knyazev DG, Zimmerberg J, Chizmadzhev YA. 2016. pH-Dependent Formation and Disintegration of the Influenza A Virus Protein Scaffold To Provide Tension for Membrane Fusion. J Virol 90:575-85.
308. Russier M, Yang G, Rehg JE, Wong SS, Mostafa HH, Fabrizio TP, Barman S, Krauss S, Webster RG, Webby RJ, Russell CJ. 2016. Molecular requirements for a pandemic influenza virus: An acid-stable hemagglutinin protein. Proc Natl Acad Sci U S A 113:1636-41.
309. Byrd-Leotis L, Galloway SE, Agbogu E, Steinhauer DA. 2015. Influenza hemagglutinin (HA) stem region mutations that stabilize or destabilize the structure of multiple HA subtypes. J Virol 89:4504-16.
310. Gabriel G, Herwig A, Klenk HD. 2008. Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathog 4:e11.
311. Manz B, Brunotte L, Reuther P, Schwemmle M. 2012. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. Nat Commun 3:802.
312. Weber M, Sediri H, Felgenhauer U, Binzen I, Banfer S, Jacob R, Brunotte L, Garcia-Sastre A, Schmid-Burgk JL, Schmidt T, Hornung V, Kochs G, Schwemmle M, Klenk HD, Weber F. 2015. Influenza virus adaptation PB2-627K modulates nucleocapsid inhibition by the pathogen sensor RIG-I. Cell Host Microbe 17:309-19.
313. Buhnerkempe MG, Gostic K, Park M, Ahsan P, Belser JA, Lloyd-Smith JO. 2015. Mapping influenza transmission in the ferret model to transmission in humans. Elife 4.
314. Gerone PJ, Couch RB, Keefer GV, Douglas RG, Derrenbacher EB, Knight V. 1966. Assessment of experimental and natural viral aerosols. Bacteriol Rev 30:576-88.
315. Milton DK, Fabian MP, Cowling BJ, Grantham ML, McDevitt JJ. 2013. Influenza virus aerosols in human exhaled breath: particle size, culturability, and effect of surgical masks. PLoS Pathog 9:e1003205.
316. Verreault D, Moineau S, Duchaine C. 2008. Methods for sampling of airborne viruses. Microbiol Mol Biol Rev 72:413-44.
317. Haig CW, Mackay WG, Walker JT, Williams C. 2016. Bioaerosol sampling: sampling mechanisms, bioefficiency and field studies. J Hosp Infect 93:242-55.
318. Nikitin N, Petrova E, Trifonova E, Karpova O. 2014. Influenza virus aerosols in the air and their infectiousness. Adv Virol 2014:859090.
319. Mumford JA, Hannant D, Jessett DM. 1990. Experimental infection of ponies with equine influenza (H3N8) viruses by intranasal inoculation or exposure to aerosols. Equine Vet J 22:93-8.
320. Nachbagauer R, Miller MS, Hai R, Ryder AB, Rose JK, Palese P, Garcia-Sastre A, Krammer F, Albrecht RA. 2015. Hemagglutinin Stalk Immunity Reduces Influenza Virus Replication and Transmission in Ferrets. J Virol 90:3268-73.
321. Gagnon A, Acosta E, Hallman S, Bourbeau R, Dillon LY, Ouellette N, Earn DJD, Herring DA, Inwood K, Madrenas J, Miller MS. 2018. Pandemic Paradox: Early Life H2N2 Pandemic Influenza Infection Enhanced Susceptibility to Death during the 2009 H1N1 Pandemic. MBio 9.
322. Laurie KL, Guarnaccia TA, Carolan LA, Yan AW, Aban M, Petrie S, Cao P, Heffernan JM, McVernon J, Mosse J, Kelso A, McCaw JM, Barr IG. 2015. Interval Between Infections and Viral Hierarchy Are Determinants of Viral Interference Following Influenza Virus Infection in a Ferret Model. J Infect Dis 212:1701-10.

323. Parrish CR, Murcia PR, Holmes EC. 2015. Influenza virus reservoirs and intermediate hosts: dogs, horses, and new possibilities for influenza virus exposure of humans. J Virol 89:2990-4.
324. Epperson S, Jhung M, Richards S, Quinlisk P, Ball L, Moll M, Boulton R, Haddy L, Biggerstaff M, Brammer L, Trock S, Burns E, Gomez T, Wong KK, Katz J, Lindstrom S, Klimov A, Bresee JS, Jernigan DB, Cox N, Finelli L, Influenza AvVIT. 2013. Human infections with influenza A(H3N2) variant virus in the United States, 2011-2012. Clin Infect Dis 57 Suppl 1:S4-S11.
325. Nelson MI, Vincent AL. 2015. Reverse zoonosis of influenza to swine: new perspectives on the human-animal interface. Trends Microbiol 23:142-53.
326. Sutton TC, Finch C, Shao H, Angel M, Chen H, Capua I, Cattoli G, Monne I, Perez DR. 2014. Airborne transmission of highly pathogenic H7N1 influenza virus in ferrets. J Virol 88:6623-35.
327. Te Beest DE, de Bruin E, Imholz S, Koopmans M, van Boven M. 2017. Heterosubtypic cross-reactivity of HA1 antibodies to influenza A, with emphasis on nonhuman subtypes (H5N1, H7N7, H9N2). PLoS One 12:e0181093.