Reassortments and Mutations Modulating Virulence and Transmission of Influenza A Virus

Eefje Schrauwen

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Reassortments and Mutations Modulating Virulence and Transmission of Influenza A Virus

Reassorteringen en mutaties bepalend voor virulentie en transmissie van influenza A virus

Proefschrift

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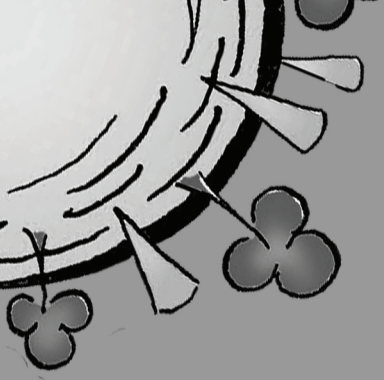
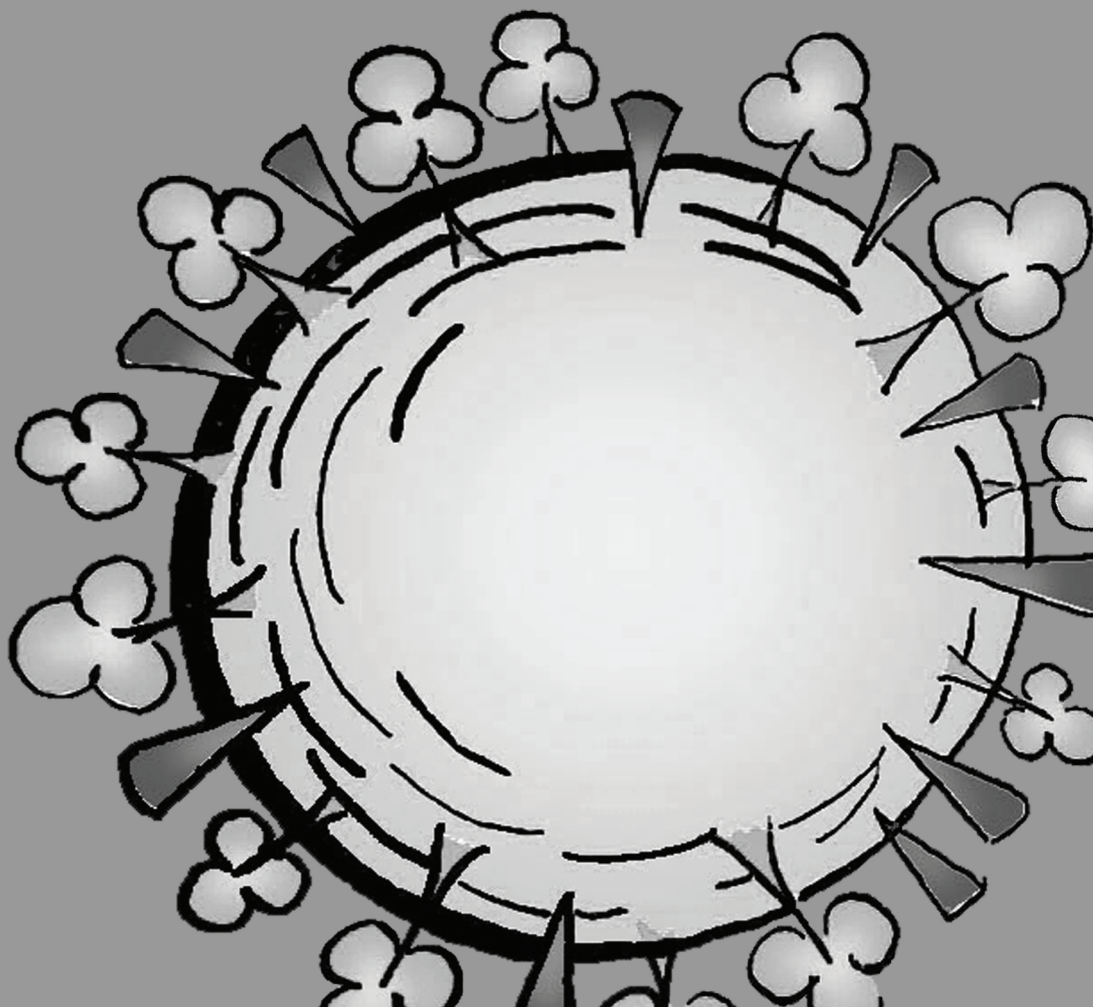
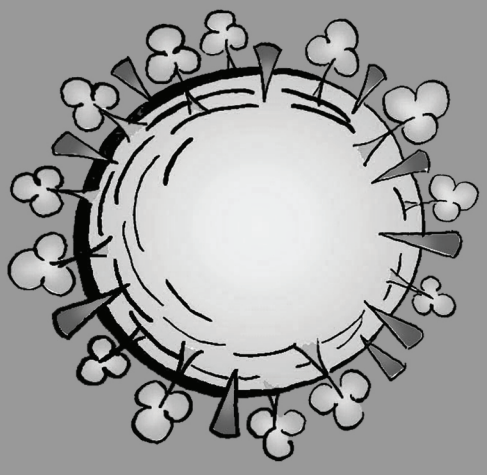




CHAPTER 1
GENERAL INTRODUCTION



INTRODUCTION

Influenza A virus

Influenza A virus is a member of the Orthomyxoviridae family. The influenza A viruses are classified on the basis of antigenic properties of the glycoproteins hemagglutinin (HA) and neuraminidase (NA) into 17 HA subtypes (H1-H17) and 10 NA subtypes (N1-N10) [1-3]. These different subtypes of HA and NA are used in influenza A virus nomenclature, to discriminate viruses based on the surface glycoproteins (e.g. H5N1, H3N2). All subtypes with the exception of H17N10 circulate in wild birds, which are considered their natural reservoir [4]. The H17N10 subtype has recently been isolated from bats, raising the possibility that birds are not the exclusive influenza virus reservoir.

Influenza A virus is a single-stranded negative-sense segmented RNA virus, with a genome consisting of eight gene segments, that can encode up to 16 proteins [5-9] (Fig 1a). Influenza A virus particles are enveloped with sizes ranging from 80 to 120 nm. The HA glycoprotein is composed of two subunits, the globular surface subunit HA1 and the stalk-like transmembrane subunit HA2. 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 cellular proteases [10]. The sequence of the cleavage site determines by which cellular proteases HA0 can be cleaved. Cleavage of HA by cellular proteases is a crucial step in the replication cycle; virus particles with uncleaved HAs are not infectious. In the initial stage of virus replication, the HA glycoprotein binds to specific sialic acid (SA) receptors on the surface of susceptible cells (Fig 1b). Human influenza viruses preferentially bind to α2,6-linked SA receptors which are mainly expressed on epithelial cells in the human upper respiratory tract (URT), whereas avian influenza viruses bind to α2,3-linked SA receptors, which are abundantly present on epithelial cells in the intestine of birds and in the lower respiratory tract (LRT) of humans [11-14]. After binding to these SA receptors, virus particles enter the cell through receptor-mediated endocytosis. Subsequently, the increasingly acidic pH in the endosome triggers a conformational change in HA2, which exposes the fusion peptide that mediates fusion of the viral membrane with the endosomal membrane. The viral ribonucleoproteins (vRNPs), which consist of the viral RNA (vRNA), the polymerase complex and the nucleocapsid protein (NP) (Fig 1c), are subsequently released into the host cell cytoplasm. The RNA-dependent RNA polymerase complex consists of three polymerase subunits; polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). The vRNPs are then transported to the nucleus where the viral RNA-dependent RNA polymerase transcribes and replicates the negative sense vRNA. The newly transcribed messenger RNAs (mRNAs) are transported to the cytoplasm for translation after which the viral proteins required for transcription and replication are translocated back to the nucleus. Newly synthesized vRNPs are transported to the cytoplasm by the nuclear export protein (NEP) and the matrix protein (M1).

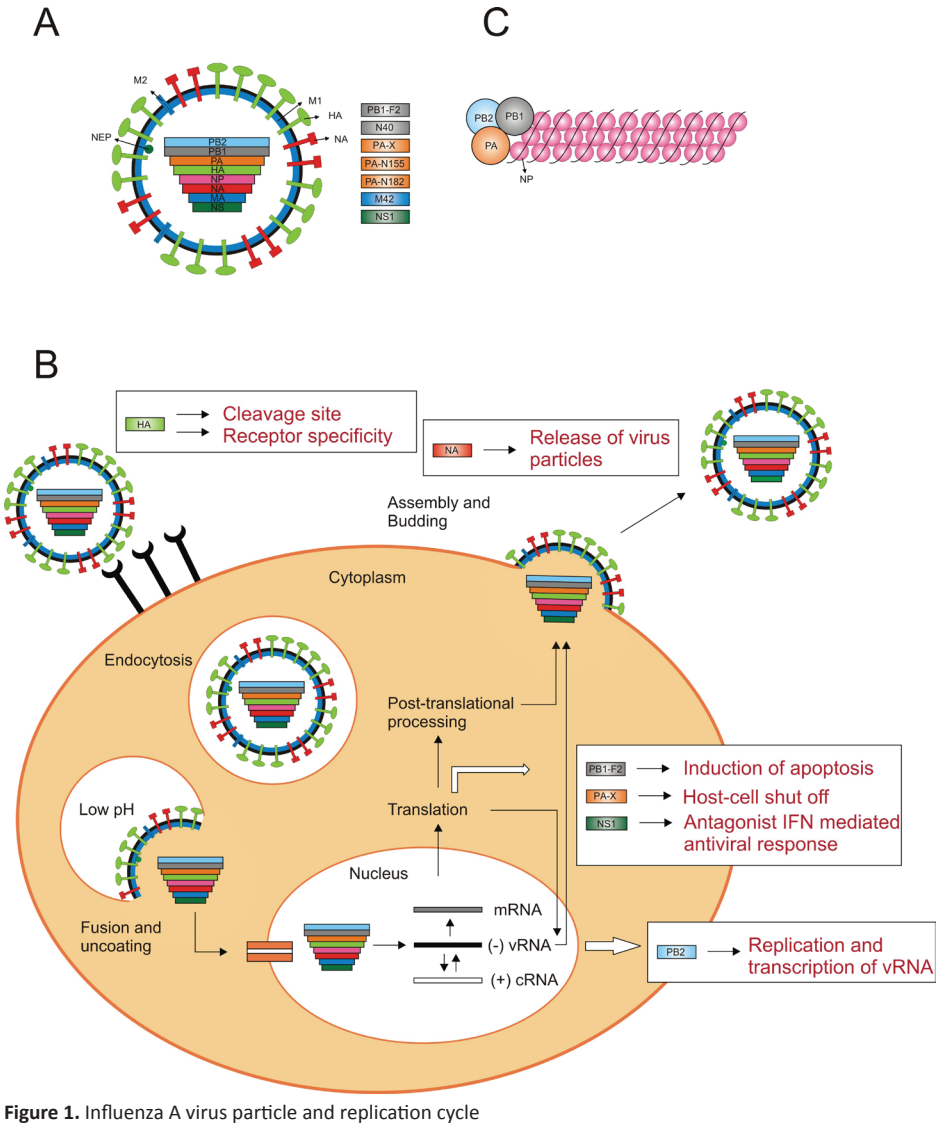


Figure 1. Influenza A virus particle and replication cycle
A) Schematic representation of influenza A particle and gene segments. The influenza genome consists of eight single-stranded RNAs. The non-structural proteins and/or newly identified proteins with unknown function are depicted in rectangles. B) Schematic representation of an influenza A virus replication cycle. Several influenza A proteins have been shown to be important determinants of virulence. These viral proteins are indicated and discussed in the main text. C) Schematic illustration of the vRNP structure. The viral RNA is wrapped around NP, and this structure is bound to the polymerase complex (PB2, PB1 and PA).

Assembly of new virions takes place at the plasma membrane, a process known as budding. The NA protein of influenza A viruses has sialidase activity and subsequently cleaves SA off the cellular surface receptors, resulting in the release of virus progeny from infected cells.

The influenza virus genome encodes additional proteins that are either not directly involved in virus replication or for which the function has not yet been elucidated.

The polymerase subunit PB1 can encode two more proteins. The PB1-F2 protein, that is encoded by the +1 alternate open reading frame of the PB1 gene, is present in most human and avian influenza viruses, and contributes to the virulence in a host- and strain-dependent manner [15]. PB1 N40 is a truncated version of PB1 that lacks transcriptase function but nevertheless interacts with PB2 and the polymerase complex in the cellular environment [7]. Similarly, the influenza virus PA gene contains a second open reading frame that is accessed via ribosomal frameshifting. This newly identified protein; PA-X, is a fusion protein that incorporates the N-terminal endonuclease domain of the PA protein with a short C-terminal domain and modulates the host response to infection by repressing cellular gene expression [8]. The NS1 protein functions as an antagonist to block the type 1 interferon (IFN) mediated host antiviral response [16,17]. M42 has only been discovered recently and encodes a novel M2-like protein with a variant extracellular domain. M42 can compensate for the loss of M2 in tissue culture cells and animals [6]. Currently, the identification of novel influenza virus proteins is receiving considerable interest and influenza segment PA has now been shown to encode as many as four proteins. Two recently identified PA-related proteins are translated from the eleventh and thirteenth in-frame AUG start codons in PA; PA-N155 and PA-N182 [9]. These proteins do not have polymerase activity, however, when viruses lacking the N-truncated PAs were investigated in mice, they demonstrated lower pathogenicity than the wildtype virus.

Influenza epidemics and pandemics

Influenza A viruses are the cause of recurrent epidemics and occasional pandemics. The annual epidemics result in about three to five million cases of severe illness, and about 250000 to 500000 deaths worldwide [18]. Infection with influenza A virus results in protective immunity against the viral surface glycoproteins HA and NA. However, the accumulation of point mutations in HA and to a lesser extent NA, allows the virus to escape the host immunity. This phenomenon, known as antigenic drift, explains the occurrence of seasonal influenza epidemics. As a result of this antigenic drift, the vaccine composition has to be updated almost annually [19].

Antigenic shift refers to a major change to produce a new influenza A virus subtype in humans that was not currently circulating among people. Antigenic shift can be caused by direct introduction of an influenza A virus from the animal reservoir via interspecies transmission or by reassortment, i.e. the mixing of genes from two (or more) influenza A viruses, between animal and human influenza A viruses. While influenza viruses are continuously changing by antigenic drift, antigenic shift happens only occasionally.

Influenza viruses with pandemic potential may arise in pigs upon reassortment [20], as pig cells express both human and avian influenza receptors, therewith providing an opportunity for replication of avian and human influenza viruses in the same cell [21]. In the human population, co-infections with different influenza strains have

also been observed [22-24]. In 2001, an H1N2 virus was identified which was the result of reassortment between contemporary circulating H1N1 and H3N2 viruses. Fortunately, this virus did not persist in the human population [25,26]. Reassortment is also an important factor to increase genetic variation of influenza viruses as multiple reassortment events have been shown to occur between different lineages of the H3N2 virus [27,28].

In the last century, four human influenza A virus pandemics have emerged, at least three of which resulted from reassortment of human and animal influenza A viruses [29-31] (Fig 2).

Spanish H1N1 pandemic

The 'Spanish' influenza H1N1 pandemic of 1918 is known as the deadliest single event recorded in human history, killing approximately as many as 50 million people worldwide [29]. The mortality rates were unusually high among young adults, a phenomenon which remains poorly understood to this day. Although its origin has not been fully resolved [29,32], the avian influenza virus-like genome of the 1918 pandemic H1N1 virus suggests that it evolved from an avian virus around 1910, with or without adaptation in an intermediate host [33]. Reverse genetics allowed the reconstruction of the 1918 pandemic H1N1 virus [34] and its pathogenesis was studied in mice, ferrets and non-human primates. The 1918 pandemic H1N1 virus caused efficient viral infection and disease in mice without prior adaptation, which is uncommon for human influenza viruses, and elicited an aberrant innate immune response [35]. In non-human primates this unusual innate immune response was also demonstrated [36]. There is no obvious single virulence factor in the 1918 pandemic H1N1 virus. However, HA, NS1, PB1-F2 and the polymerase complex proteins are known to play an important role in its pathogenesis [37-43] (see paragraph on virulence factors).

Asian H2N2 pandemic

The 'Asian' pandemic H2N2 virus of 1957 was responsible for approximately 2 million deaths globally [44]. This H2N2 virus affected predominantly very young and very old individuals. The H2N2 virus emerged after the introduction of a novel subtype in humans through reassortment between human and avian influenza viruses. The HA, NA and PB1 genes originated from an avian H2N2 virus and the remaining gene segments from the H1N1 virus that circulated prior to 1957 [30,45].

Hong Kong H3N2 pandemic

In 1968, the circulating H2N2 virus was replaced by the 'Hong Kong' H3N2 virus, which has continued to circulate in humans to date. The pandemic in 1968 killed an estimated one million people worldwide [44]. The pandemic H3N2 virus was also the result of reassortment between avian and human influenza viruses, this time the HA and PB1 segments of H2N2 were replaced by those of an avian H3 virus [30,45]. This pandemic was mild compared to the earlier pandemics, possibly as a result of prior-immunity in the human population against the N2 subtype.

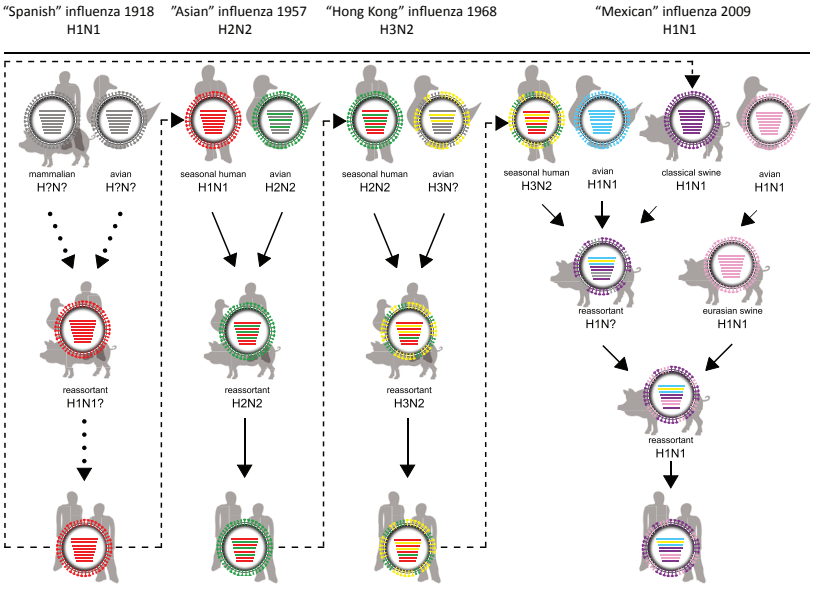


Figure 2. Reassortment and adaptation events of pandemic influenza A viruses
For the 1918 H1N1 "Spanish influenza" pandemic, evidence for two mutually exclusive scenarios has been presented: the gradual adaptation of avian genes to the human host and a reassortment event between avian and mammalian viruses. After 1918, the same H1N1 virus caused seasonal epidemics until 1957, when the H2N2 virus emerged upon reassortment between the seasonal H1N1 and an avian H2N2 virus, introducing the avian HA, NA, and PB1 genes. This H2N2 virus circulated in humans until 1968, when reassortment of the H2N2 with an avian H3 virus resulted in exchange of the H3 HA and PB1 genes to yield a new pandemic virus of subtype H3N2. The 2009 H1N1 pandemic virus included the NA and M genes of the Eurasian swine lineage, and the other genes of a "triple reassortant" swine influenza virus that had previously acquired its genes upon reassortment between human, avian, and (classical) swine viruses. Grey colour in virus particles indicates uncertainty of viral gene segment origin or lack of data. Dotted arrows indicate uncertain scenarios and solid arrows indicate events that are supported by scientific evidence. Dashed arrows represent pandemic viruses circulating in subsequent influenza seasons.

Re-emergence of H1N1

In 1977, an influenza outbreak was detected that affected predominantly young adults. This outbreak was caused by the reintroduction of an H1N1 virus also known as the 'Russian' H1N1 virus. This virus was genetically very similar to viruses circulating in the early 1950s and lacked years of genetic evolution. This suggests that the 1977 H1N1 virus was accidentally released from a laboratory [46]. The reintroduced H1N1 virus did not replace the circulating H3N2 virus and subsequently these subtypes co-circulated in humans until the 'Russian' H1N1 virus was replaced in 2009 by the pandemic H1N1 influenza virus [47].

H1N1 pandemic

The H1N1 virus (pH1N1) caused the first influenza pandemic in this century and continues to be detected worldwide [47,48]. In spring 2009, pH1N1 emerged in Mexico, after which it spread around the world in only a few months. In terms of mortality, the pH1N1 virus was relatively mild and preferentially affected young adults. People

over 65 years of age experienced a relatively mild infection because at least in part of cross-reactive antibodies to the pH1N1 virus as this virus was antigenically similar to H1N1 viruses circulating before 1957 [49].
The pH1N1 virus is a reassortant virus that contains the NA and M genes from the Eurasian swine influenza virus lineage, while other genes originate from a 'triple reassortant' swine influenza virus that had previously acquired its gene constellation upon reassortment between human, avian, and (classical) swine influenza viruses [50]. The fact that the pH1N1 virus emerged after reassortment in swine, supports the hypothesis that swine can serve as a mixing vessel for the generation of new human influenza viruses [20].
Since the start of the H1N1 pandemic there have been concerns that the virus may mutate or reassort with contemporary influenza viruses and give rise to a more pathogenic virus. Especially since the pH1N1 virus does not contain any of the known virulence factors (further information on virulence factors p. 6) and retained some host-range markers typical for avian influenza viruses [31]. However, the introduction of several known virulence factors in pH1N1 by reverse genetics did not affect its virulence in animal models [51-53]. In humans, the only reported mutation associated with increased virulence is D222G in HA, which was related to fatal and severe cases of infection [54-57].

Pandemic threats

Historically, influenza viruses of three HA subtypes (H1, H2 and H3) have acquired the ability to be transmitted efficiently between humans. To date, influenza viruses of the H1 and H3 subtype co-circulate in humans, however influenza viruses of the H2, H5, H6, H7 and H9 subtype are also considered to have pandemic potential.
In 1997, a large outbreak of highly pathogenic avian influenza (HPAI) H5N1 virus in poultry in Hong Kong resulted in the first documented cases of direct transmission of HPAI H5N1 virus from poultry to humans, with a fatal outcome in 6 out of 18 cases [58]. As a result, this outbreak warranted the mass culling of 1.5 million chickens. HPAI H5N1 viruses have spread throughout Asia, Europe and Africa since, causing severe disease outbreaks in poultry. Furthermore, HPAI H5N1 viruses have been isolated from mammals on numerous occasions. Since 2003, over 600 cases of human HPAI H5N1 infections have been reported, more than half of which were fatal [59]. Although rare cases of H5N1 virus transmission between humans have been reported, sustained human-to-human transmission of HPAI H5N1 virus has not been detected yet [60-62]. It is this absence of efficient human-to-human transmission that has prevented an H5N1 pandemic to occur. With the sporadic introduction of HPAI H5N1 virus in the human population, it is feared that these avian H5N1 viruses may mutate or reassort with contemporary human influenza viruses, possibly resulting in adaptation to humans. Fortunately, at present, reassortment of HPAI H5 virus with contemporary human influenza viruses has not been detected in nature. However, co-infections of avian and human influenza viruses in humans or

pigs may provide new opportunities for reassortment [63-65]. Due to the enzootic nature of HPAI H5N1, the large host reservoir (over 20 different mammalian species [66]) and the accumulation of mammalian adaptation mutations, this virus is currently considered to be the largest pandemic threat to humans.

Several outbreaks of HPAI H7 viruses in poultry have resulted in transmission to humans. In 2003, a large outbreak of an HPAI H7N7 virus in poultry in the Netherlands resulted in 89 cases of human infections, one of which was fatal [67,68]. HPAI H7N7 virus displayed an unusual tissue tropism; the virus targeted the conjunctiva, resulting in conjunctivitis, a symptom rarely reported for other influenza virus subtypes [69]. Recently, a novel H7N9 virus emerged in China, which is responsible for 108 confirmed human cases that resulted in death of 22 cases up to now [70]. Influenza viruses of the H7 subtype have the ability to infect humans, which underlines its pandemic potential.

Since the mid-1990's, H9N2 viruses have become endemic in poultry populations throughout Eurasia. The first human case of infection with an avian H9N2 virus was documented in 1999 in Hong Kong [71] and sporadically human infections have been described since [72]. Furthermore, this subtype has been isolated from pigs and numerous reassortment events between H9N2 virus and other influenza virus subtypes (i.e. H5N1 and H6N2) [65,73] have been reported. H9N2 viruses with either avian and human or human receptor specificity [74] are now prevalent in many Eurasian countries, thereby increasing the possibility of this virus to infect humans.

Human infections with H6 viruses have not been reported, however, some H6 viruses were found to replicate in mice and ferrets without prior adaptation [75]. In addition, given the high prevalence and frequent reassortment of H6 viruses in birds, concerns have risen about the possible emergence of a pandemic H6 virus [76,77].

Influenza viruses of the H2 subtype have not circulated in humans since 1968 and therefore a large proportion of the current world population is likely to be susceptible for infection with H2 viruses if they would re-emerge. As H2 viruses continue to circulate in swine and several avian species [78-81], they remain a potential pandemic threat.

The pH1N1 virus in 2009 illustrated that a new pandemic does not necessarily require the introduction of a virus with an HA subtype that is new to the human population. A novel influenza A (H3N2) variant virus (H3N2v) containing seven gene segments of swine influenza origin and the pH1N1 M segment was isolated from 12 humans in 2011 [82-84]. Although there is a low level of cross-reactive antibodies with a human H3N2 virus that circulated in the 1990's [85,86], the H3N2v is antigenically different from the currently circulating seasonal H3N2 viruses and can thus potentially infect a large proportion of the human population.

With the continuous pandemic threat of avian influenza A viruses of different subtypes, bird surveillance is essential to allow the early detection of influenza viruses with pandemic potential. Moreover, after the pH1N1 virus introduction from swine, surveillance studies in swine populations were expanded. Such surveillance studies are crucial for preparedness.

Influenza A virulence factors

HA

Amino acid substitutions in HA that affect the receptor binding preference can influence the cellular host range and tissue tropism which may alter virulence. Specific amino acid residues in HA regulate the receptor binding specificity of human and avian influenza viruses and, these specific residues differ among subtypes. Binding patterns of pandemic influenza A viruses of the H1 subtype are modulated by amino acids at position 190 and 225 in HA (H3 numbering) [87,88]. For influenza viruses of the H2 and H3 subtypes, positions 226 and 228 are important for receptor binding specificity [89]. Since the HA proteins of the 1918, 1957, and 1968 pandemic strains were derived from avian influenza viruses, adaptation of avian HA proteins to the human receptor is considered to be a prerequisite for efficient human-to-human transmission [89]. Numerous studies have described amino acid substitutions in HA of HPAI H5N1 viruses that change and/or increase binding to human α2,6-linked SA receptors that are present in the human URT [90-93]. The majority of these amino acid substitutions are located in or near the receptor binding site of HA. However, a potential N-glycosylation motif at amino acid position 154–156 of HA, which is proximal but not immediately adjacent to the receptor-binding site, may also affect binding preference and virulence [94,95].

One of the best-known virulence determinants of HPAI viruses is the multi basic cleavage site (MBCS) in HA. The sequence of this cleavage site determines by which cellular proteases HA0 is cleaved and consequently determines viral tropism and virulence (Fig 3). The HA of low pathogenic avian influenza (LPAI) viruses and human influenza viruses harbors a monobasic cleavage site that is cleaved by trypsin-like proteases. These trypsin-like proteases are only present in the respiratory tract of humans and the respiratory and/or intestinal tract of birds, thereby restricting

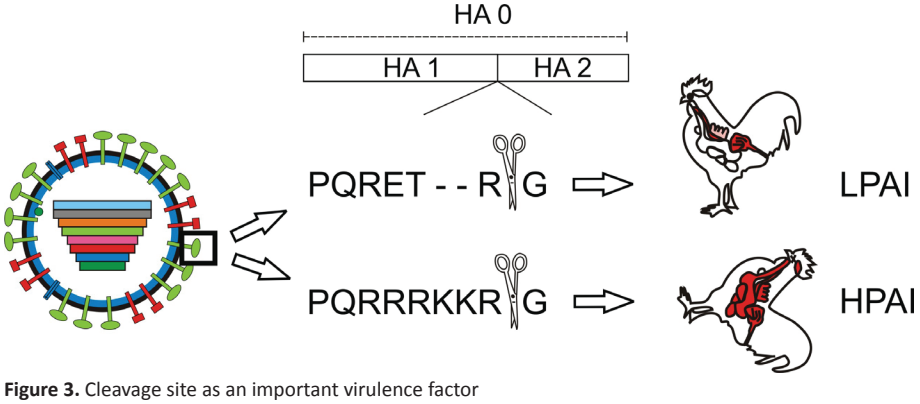


Figure 3. Cleavage site as an important virulence factor
The HA0 is cleaved into two subunits HA1 and HA2 by cellular proteases which recognize either a mono basic or multi basic cleavage site. HA0 of LPAI viruses, which harbor a mono basic cleavage site, are cleaved by trypsin-like proteases only, limiting replication of these viruses to sites where these enzymes are expressed; i.e. respiratory and intestinal tract. The HA0 of HPAI viruses of the H5 and H7 subtype can be cleaved by ubiquitously expressed furin-like proteases, facilitating systemic replication in chickens.

virus replication in these tissues [96]. Cleavage of the human influenza virus HA is thought to occur by trypsin-like serine proteases of the type II transmembrane serine protease (TTSP) family that are present at the plasma membrane, or by extracellular proteases present in the respiratory tract [10].

Influenza viruses of the H5 and H7 subtype may become highly pathogenic after circulation in poultry. The switch from a low pathogenic to a high pathogenic phenotype of these H5 and H7 influenza viruses is caused by the introduction of basic amino acid residues into the HA0 cleavage site by insertion or recombination [97-99]. This HA0 can subsequently be cleaved by ubiquitously expressed pro-protein convertases of the subtilisin family, like furin or PC5/6, thereby facilitating systemic replication in chickens [10,100,101].

In mammals, the association between the presence of an MBCS and systemic spread is less obvious. The presence of a MBCS in H5 HA does not always induce systemic spread in mammals since the inoculation of cynomolgus macaques with HPAI H5N1 resulted in respiratory infection only [102]. However, in mice, deletion of the MBCS of an HPAI H5N1 virus results in a virus that only causes respiratory tract infection in contrast to the systemic spread of the wildtype HPAI H5N1 virus, indicating that the MBCS is a major virulence factor in mice [103].

Polymerase proteins

The influenza virus polymerase proteins, and in particular PB2, have been shown to be important determinants of virulence. Amino acid substitution lysine (K) to glutamic acid (E) at position 627 in PB2 has been studied extensively in the context of mammalian adaptation [104]. This substitution is suggested to occur in order to adapt to physiological constraints, e.g. differences in body temperature. Most avian influenza A viruses, which preferentially replicate at a relatively high temperature of around 41 degrees Celsius in the digestive tract of birds, have a E residue at position 627. In contrast, human influenza A viruses replicate at the lower temperature of around 33 degrees Celsius, which is the temperature in the human URT. These viruses typically have a K residue at this position. The E627K substitution was acquired rapidly when avian viruses were experimentally passaged in mice [105,106]. Moreover, this mutation has been correlated with increased virulence of human HPAI H5N1 virus isolates and was found in a fatal human case of infection with HPAI H7N7 [95,103]. In the absence of the E627K mutation, an aspartate (D) to asparagine (N) substitution at position 701 of H5N1 PB2 was found to increase virulence and to expand the host range of avian H5N1 virus to mice and humans [105,107,108]. The adaptive mutation D701N induces enhancement of binding of PB2 to importin alpha1 in mammalian cells resulting in increased transport of PB2 into the nucleus [109]. Noteworthy, unlike other human influenza viruses, pH1N1 does not contain the mammalian adaptation residues 627K and/or 701N. When the mammalian adaptation substitutions E627K or D701N were introduced in pH1N1, no increase in virulence was observed [51]. However, the absence of these mammalian adaptation markers in PB2 have been compensated to some extent by a

serine (S) and arginine (R) substitution at position 590 and 591 that my affect the protein's interaction with viral and/or cellular factors and hence its ability to support virus replication in mammals [110,111]. Besides the extensively studied adaption mutations, E627K and D701N, a mutation close to the cap-binding region of PB2, T271A, may also enhance viral polymerase activity in mammalian cells [112]. During several adaptation experiments in mice, substitutions in PA have been identified that contribute to increased polymerase activity and virulence [113-117]. Furthermore, the substitution of an avian PA by that of pH1N1, has also been identified as a potential mechanism to increase viral replication and to potentially cross the species barrier [118]. In addition, mutations in PB1 and NP have also been implicated as determinants of virulence [109,119].

PB1-F2

PB1-F2 contributes to the virulence of influenza A viruses by inducing apoptosis of infected cells [15]. Moreover, PB1-F2 promotes and increases severity of secondary pneumonia [40]. The PB1-F2 protein was demonstrated to contribute to the virulence of HPAI H5N1 and the 1918 H1N1, 1957 H2N2, 1968 H3N2 pandemic strains [37,120,121]. In particular, an N to S substitution at position 66 (N66S) of HPAI H5N1 and 1918 pandemic influenza PB1-F2, is partly responsible for the high virulence of these viruses [37]. The PB1-F2 N66S variant reduces the production of IFN, which is part of the innate immune response [122]. In contrast to previous pandemic influenza viruses, pH1N1 does not encode a PB1-F2, because of three premature stop codons. Surprisingly, when the pH1N1 PB1-F2's coding capacity was restored, the virulence was only modestly affected in mice and ferrets [53,123].

PA-X

The PA gene encodes a newly identified protein PA-X [8]. This protein modulates the host response by repressing cellular gene expression, i.e. host-cell shut off. PA-X deficient influenza viruses cause more severe disease in mice, as a result of an accelerated host response. Moreover, influenza viruses lacking PA-X differ in host-cell shutoff compared to wildtype virus. A truncation of PA-X protein appears to be associated with influenza virus lineages circulating in particular hosts, indicating that there may be some species specificity to the evolution of PA-X [124].

NS1

In response to the presence of pathogens in the host, interferons (IFNs) are secreted by cells and 'interfere' with viral replication. To establish productive infection, influenza viruses have mechanisms to evade host immune responses, including the type-I IFN response. The influenza virus NS1 protein has several ways to act as an IFN antagonist; (1) NS1 prevents activation of the RIG-I-dependent pathway by blocking recognition of influenza pathogen associated molecular patterns, (2) Downregulation of IFN response by blocking nuclear export of cellular mRNA and (3) NS1 inhibits transcription of IFN and interferon stimulated genes by blocking mRNA processing via interaction with the cellular 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30) [125-127]. Binding to CPSF30 is a

property maintained in circulating human influenza viruses [128].
NS1 has been studied extensively as a molecular determinant of virulence. Influenza viruses lacking the IFN antagonist NS1 are only able to replicate in cells or mice that have a compromised IFN response [17,129]. H5N1 viruses, unlike other human, avian and swine influenza viruses, are relatively resistant to the antiviral effects of interferons which result in increased levels of pro-inflammatory cytokines [130-132]. This effect requires a glutamic acid (E) at amino acid position 92 of the NS1 molecule and allows virus replication in the presence of IFN; this mutation was a determinant of virulence in pigs [133]. Furthermore, a glutamic acid, instead of an aspartic acid, at this position increased virulence of HPAI H5N1 in mice. Similarly, the P42S substitution in NS1 in HPAI H5N1 resulted in increased virulence in mice [134]. Considering the binding of CPSF30 by H5N1 NS1 proteins, L103F and I106M play important roles [128].
Large-scale genome sequence analysis of avian influenza virus isolates indicated that four C-terminal residues of the NS1 protein form a PDZ (postsynaptic density protein 95, Drosophila disc large tumor suppressor, and zonula occludens 1 protein) ligand domain of the X-S/T-X-V type [135]. This carboxyterminal portion of NS1 has also been shown to influence influenza virus virulence. Pandemic 1918 H1N1 and H5N1 HPAI viruses contain 4 carboxyterminal amino acids encoding a PDZ ligand domain motif which increases virulence when introduced into a mouse-adapted influenza strain [136]. This demonstrates that NS1 can modulate virulence through different mechanisms.
pH1N1 lacks the ability to block host gene expression in both human and swine cell lines [137], which is partially due to mutations that block CPSF30-binding. Additionally, pH1N1 has a truncated NS1 protein with a 11 amino acid deletion at its C-terminus, and therefore lacks the PDZ-binding domain [31]. However, even when these functions are restored for pH1N1, they do not appear to have a significant effect on replication, virulence or transmission of pH1N1 in various animal models [52,137].

NA

Considering that HA binds to SA receptors and NA cleaves SA from cellular receptors, the balance between HA and NA activity is critical for virus replication and transmission [138,139].
Two influenza viruses are known that have developed additional mechanisms that promote cleavage of HA. The NA of the neurovirulent laboratory H1N1 strain A/WSN/33 recruits plasminogen which, when converted to plasmin, cleaves HA in the absence of trypsin [140]. On the other hand, the 1918 H1N1 NA gene enables the virus to replicate in the absence of trypsin. Additionally, this NA protein was shown to play a critical role in the high virulence of the 1918 pandemic H1N1 in mice [34].
In 2003, an outbreak of HPAI H7N7 virus in poultry in the Netherlands resulted in the death of one person and 89 human cases of conjunctivitis. When the sequence of the virus obtained from the fatal case was compared to a virus isolated from a patient with conjunctivitis, four amino acid substitutions in the NA gene were identi-

fied [67]. These mutations contributed to an increased NA activity, resulting in more efficient replication in mammalian cells most likely by preventing the formation of virus aggregates [95].
When avian influenza viruses are transmitted from wild birds to poultry, genetic changes as a result of adaptation to the new host frequently occur. One example of such a change is a deletion in the stalk region of the NA that has been reported in several viruses isolated from unrelated poultry outbreaks [141-143]. This shortened NA stalk region is frequently detected upon transmission of avian influenza viruses from waterfowl to domestic poultry and is associated with increased virulence [144,145]. It is not yet clear how this shortened NA stalk region influences virulence, however, deletion in the NA stalk does not enhance the release of progeny viruses but probably affects an earlier step of the viral cycle.

Transmission

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. Efficient and sustained human-to-human transmission is critical for the circulation of seasonal and pandemic influenza viruses in the human population. Transmission has been studied extensively in mammalian models, in particular the ferret and guinea pig [146]. Ferrets are naturally susceptible to both human and avian influenza viruses and upon infection develop similar symptoms and pulmonary pathology as humans. In addition, cells of the ferret respiratory tract express predominantly α2,6-linked SA receptors in the URT and α2,3-linked SA receptors in the LRT similar to humans [147,148]. Avian influenza viruses do not transmit via the airborne route in the ferret model [149,150]. Therefore, the ferret model is a valuable tool to study viral traits for influenza virus transmission in mammals. This is highly relevant as it is currently unclear what exactly determines transmission of influenza viruses in mammals via aerosols or respiratory droplets. For this reason, the ferret model was used extensively to compare the transmissibility of pH1N1 with the contemporary seasonal H1N1 virus, when it first emerged in humans [150]. In addition, transmission of the 1918 H1N1 virus was studied in ferrets. These studies showed that changes in the HA receptor-binding domain and PB2 were critical to initiate transmission of an avian-derived influenza virus [88,151]. Similar genetic changes were required for the Asian H2N2 virus. An early H2N2 virus (1957) failed to transmit to naïve ferrets. However a glutamine to leucine at position 226 in HA was sufficient to change its preference from avian to human receptor, subsequently resulting in transmission between ferrets [152]. Overall, amino acid substitutions in HA and polymerase proteins can affect host range and transmission of influenza viruses [108,110,151].
As described above, avian influenza viruses of the H5, H7, and H9 subtypes are considered a potential pandemic threat. However, the requirements for a virus to become pandemic (i.e. transmissible between humans) are poorly understood. In

order to study the determinants that could lead to a pandemic virus, a avian H9N2 virus was genetically engineered hereby potentially creating an transmissible avian influenza virus. An avian H9N2 virus that harbored the internal genes from a human H3N2 virus could be transmitted efficiently between ferrets via respiratory droplets after adaptation of this reassortant virus by serial passage in ferrets [153]. This indicates that avian H9N2 viruses may acquire the ability to be transmitted between humans. Furthermore, this demonstrates that airborne transmission is not restricted to human influenza viruses of the subtype H1, H2, and H3.

The lack of sustained airborne transmission of HPAI H5N1 virus between humans has been confirmed in guinea pigs and ferrets. Attempts to create airborne-transmissible H5 viruses by generating reassortant viruses between H5N1 and human influenza viruses did not result in H5 viruses that could be transmitted between mammals via the airborne route [92,154]. However, based on evidence from previous influenza pandemics, it has been hypothesized that a switch of receptor binding preference from avian α2,3-linked to human α2,6-linked SA receptors is required for an avian virus to become transmissible between humans. Nevertheless, changing the receptor binding preference alone was not sufficient to confer airborne transmission of H5N1 virus, indicating that additional adaptive changes are required for H5N1 viruses to become transmissible [92].

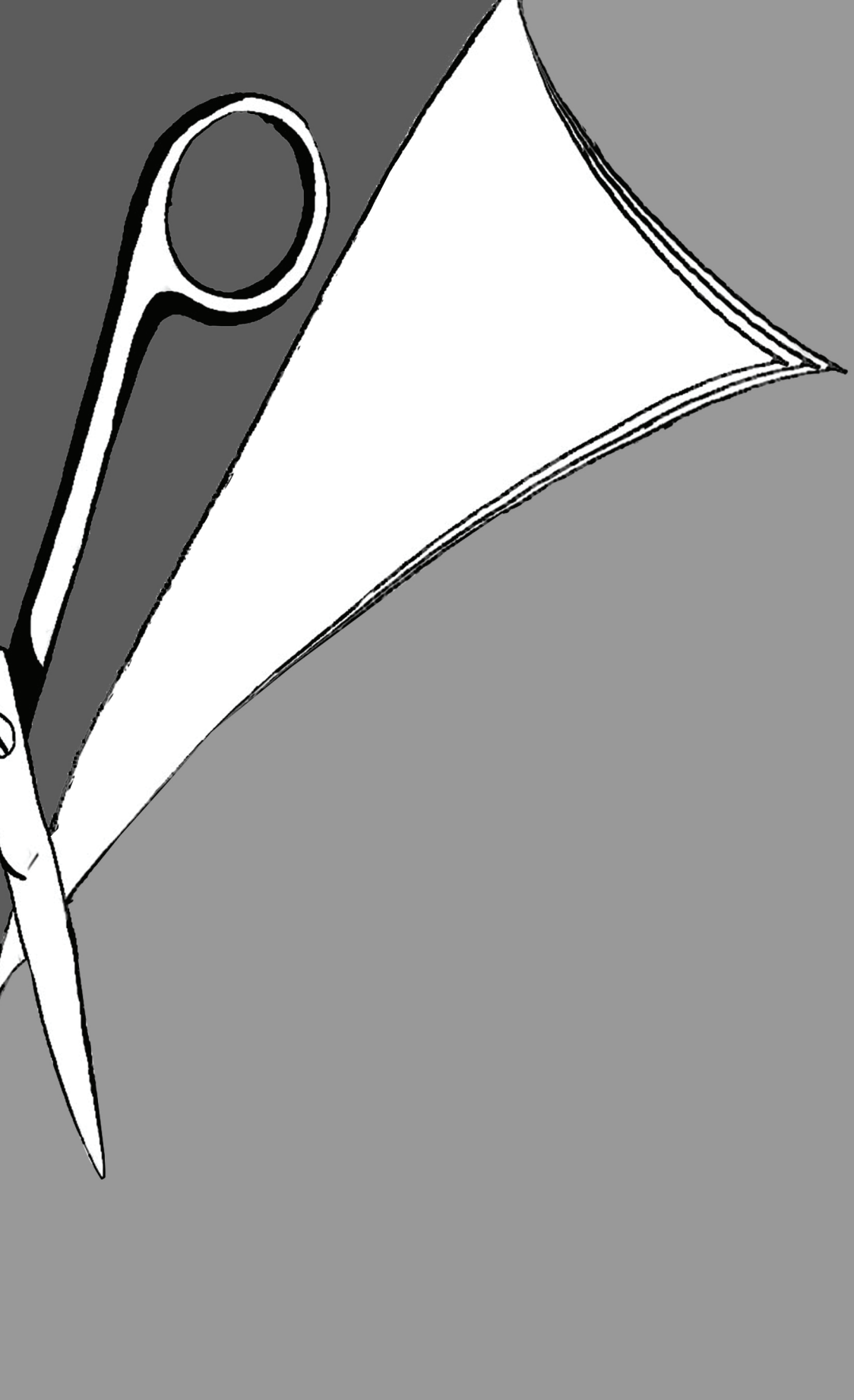
OUTLINE OF THIS THESIS

HPAI H5N1 viruses remain a scourge for the poultry industry. The high lethality and virulence in poultry and mammals is mainly determined by the acquisition of an MBCS in the HA glycoprotein. The presence of an MBCS is only observed in influenza viruses of the H5 and H7 subtype. In chapter 2.1 we investigated whether an LPAI H6N1 virus can convert into a highly pathogenic phenotype by the introduction of an MBCS. In chapter 2.2 we determined the effect of the MBCS on virulence in mammals in the context of a human H3N2 virus. In chapter 2.3 the role of the MBCS in HPAI H5N1 virus in systemic replication in ferrets was studied. In addition, we describe the spread of HPAI H5N1 virus in ferrets over time, including along the olfactory route.

Virus reassortment has played an important role for influenza A viruses in the generation of new strains with pandemic potential. In chapter 3.1 we used an in vitro selection method to generate reassortant viruses between pH1N1 and the seasonal H1N1 or H3N2 viruses. These viruses were tested for their replication kinetics in tissue culture and pathogenicity, tissue tropism and transmissibility was investigated in ferrets. In chapter 3.2 we used the same in vitro selection method to produce reassortant viruses between HPAI H5N1 and pH1N1, seasonal H1N1 or seasonal H3N2 viruses. These viruses were evaluated for their replication kinetics in (primary) tissue cultures. Subsequently, a mixture of reassortants between HPAI H5N1 and pH1N1 was tested in the ferret transmission model to screen for reassortants that might have gained the ability to be transmitted via the airborne route.

In chapter 4.1, knowledge about pandemic, zoonotic and epidemic influenza viruses was used to formulate hypotheses about the minimal requirements for efficient transmission of an animal influenza virus between humans. Chapter 4.2 addresses the question whether HPAI H5N1 can acquire the ability to be transmitted via the airborne route between mammals. For this purpose, a genetically modified HPAI H5N1 virus was serially passaged in the URT of ferrets, followed by extensive transmissibility studies in ferrets.

In chapter 5, the results of the previous chapters and implications of these studies are evaluated in a summarizing discussion.



CHAPTER 2
Multi basic cleavage site as a virulence factor of influenza A viruses

2.1
Insertion of a multi basic cleavage motif into the hemagglutinin of a low pathogenic avian influenza H6N1 virus induces a highly pathogenic phenotype

Vincent J. Munster*, Eefje J. A. Schrauwen*, Emmie de Wit, Judith M. A. van den Brand, Theo M. Bestebroer, Sander Herfst, Guus F. Rimmelzwaan, Albert D. M. E. Osterhaus and Ron. A. M. Fouchier

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*Both authors contributed equally

ABSTRACT

The highly pathogenic avian influenza (HPAI) virus phenotype is restricted to influenza A viruses of the H5 and H7 hemagglutinin (HA) subtypes. To obtain more information on the apparent subtype specific nature of the HPAI phenotype, a low pathogenic avian influenza (LPAI) H6N1 virus was generated containing an HPAI H5 R-R-R-K-K-R↓G multi basic cleavage site (MBCS) motif in HA (the downward arrow indicates the site of cleavage). This insertion converted the LPAI virus phenotype into an HPAI virus phenotype in vitro and in vivo. The H6N1 virus with an MBCS displayed in vitro characteristics similar to those of HPAI H5 viruses, such as cleavage of HA0 (the HA protein of influenza A virus initially synthesized as a single plypeptide precursor) and virus replication in the absence of exogenous trypsin. Studies of chickens confirmed the HPAI phenotype of the H6N1 virus with an MBCS, with an intravenous pathogenicity index of 1.4 and systemic virus replication upon intranasal inoculation, the hallmarks of HPAI viruses. This study provides evidence that the subtype specific nature of the emergence of HPAI viruses is not at the molecular, structural or functional level, since the introduction of an MBCS resulted in a fully functional virus with an HPAI genotype and phenotype.

INTRODUCTION

Wild birds represent the natural reservoir of avian influenza A viruses in nature [2]. Influenza A viruses are classified on the basis of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. In wild birds throughout the world, influenza A viruses representing 16 HA and 9 NA antigenic subtypes have been found in numerous combinations (also called subtypes, e.g., H1N1, H6N1) [1]. Besides classification based on the antigenic properties of HA and NA, avian influenza A viruses can also be classified based on their pathogenic phenotype in chickens. Highly pathogenic avian influenza (HPAI), an acute generalised disease of poultry in which mortality may be as high as 100%, is restricted to subtypes H5 and H7. Other avian influenza A virus subtypes are generally low pathogenic avian influenza (LPAI) viruses that cause much milder, primarily respiratory disease in poultry, sometimes with loss of egg production [155].

The HA protein of influenza A virus is initially synthesized as a single polypeptide precursor (HA0) which is cleaved into HA1 and HA2 subunits by host cell proteases. The mature HA protein mediates binding of the virus to host cells, followed by endocytosis and HA-mediated fusion with endosomal membranes [2]. Influenza viruses of subtypes H5 and H7 may become highly pathogenic after introduction into poultry and cause outbreaks of HPAI. The switch from an LPAI phenotype to the HPAI phenotype of these H5 and H7 influenza A viruses is achieved by the introduction of basic amino acid residues into the HA0 cleavage site by substitution or insertion, resulting in the so-called multi basic cleavage site (MBCS), which facilitates systemic virus replication [156-159]. The cleavage of the HA0 of LPAI viruses is restricted to trypsin-like proteases which recognize the X-X-X-(R/K)↓G cleavage motif, where ↓ indicates the site of cleavage. Replication of these LPAI viruses is therefore restricted to sites in the host where these enzymes are expressed, i.e. the respiratory and intestinal tract [160,161]. The introduction of an R-X-(R/K)-R↓ G or R-(R/K)-X-R↓G minimal MBCS motif into H5 and H7 subtype viruses facilitates the recognition and cleavage of the HA0 by ubiquitous proprotein convertases, such as furin [161-164]. H5 influenza A viruses with a minimal MBCS motif only have the highly pathogenic phenotype if the masking glycosylation site at position 11 in the HA is substituted into a non-glycosylation site. Otherwise, at least one additional basic amino acid has to be inserted to allow the shift from an LPAI to an HPAI phenotype to occur [97,165-168]. No information is available on the minimal prerequisites for H7 influenza A viruses to become highly pathogenic, but all HPAI H7 viruses have at least 2 basic amino acid insertions in the HA0 cleavage site [166]. HA0 with MBCS is activated in a broad range of different host cells and therefore enables HPAI viruses to replicate systemically in poultry [169]. To date, little is known about the apparent subtype specific nature of the introduction of the MBCS into LPAI viruses and the evolutionary processes involved in the emergence of HPAI viruses. When an MBCS was introduced in a laboratory adapted strain of influenza virus, A/Duck/Ukraine/1/1963 (H3N8), it did not result in a dramatic change in pathogenic phe-

notype [170]. Here, the effect of the introduction of an MBCS into a primary LPAI H6N1 virus, A/Mallard/Sweden/81/2002, is described. The introduction of an MBCS resulted in trypsin-independent replication in vitro and enhanced pathogenesis in a chicken model. Understanding the basis of the HA subtype-specificity of the introduction of an MBCS into avian influenza viruses will lead to a better understanding of potential molecular restrictions involved in emergence of HPAI outbreaks.

MATERIALS AND METHODS

Cells

Madin-Darby Canine kidney (MDCK) cells were cultured in EMEM (Lonza, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes and non-essential amino acids. 293T cells were cultured in DMEM (Lonza) supplemented with 10 % FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodiumpyruvate and non-essential amino acids.

Viruses

Influenza virus A/Mallard/Sweden/81/02 (H6N1) was isolated from a migratory mallard (Anas platyrrhynchos) within the framework of our ongoing avian influenza surveillance program [77], and passaged twice in embryonated chicken eggs. Influenza virus A/Hong Kong/156/97 (H5N1) was isolated from the human index case during the 1997 H5N1 outbreak in Hong Kong [58]. The H6N1WT virus, H5N1WT, H6N1 virus with MBCS in HA (H6N1MBCS) and the H5N1 virus lacking the MBCS (H5N1ΔMBCS) were generated by reverse genetics as described previously [171]. The supernatant of the transfected cells was harvested 48h after transfection and was used to inoculate MDCK cells. The genotypes of all recombinant viruses were confirmed by sequencing. The nucleotide sequences of the 8 gene segments of A/Mallard/Sweden/81/02 (H6N1) are available from GenBank under accession numbers CY060379 - CY060386. The risk potential of the H6N1MBCS virus was assessed prior to start of the experiments and it was determined that the anticipated risk of generating this virus would be equivalent to that of an HPAI virus. Therefore, all in vivo and in vitro experiments were performed under ABSL3+ conditions.

Plasmids

The 8 gene segments of A/Mallard/Sweden/81/2002 (H6N1) and A/Hong Kong/156/97 (H5N1) were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000 [171]. For the construction of the plasmid containing the MBCS in HA of H6N1, a QuickChange Multi Site-directed mutagenesis kit (Qiagen, Venlo, The Netherlands) was used according to instructions of the manufacturer. The following primers were used for the introduction of nucleotides

encoding the RRRKKR↓ MBCS in the HA gene: gaggcttgcaactggactaagaaatgttccacagagaaaaaaaagaggacttttcggagcc and tggactaagaaatgttccacagagaagaagaaaaaaaagaggacttttcg. For the generation of the H5N1ΔMBCS 15 nucleotides encoding 5 amino acids (underlined), PQRERRRKKR↓G, were modulated into PQIETR↓G of the MBCS in HA of the H5N1 virus as previously described [172].

Replication kinetics and virus titrations

Multi step replication kinetics were determined by inoculating MDCK cells in the presence and absence of 1 µg/ml trypsin (Lonza) with a multiplicity of infection (MOI) of 0.01 TCID50 per cell. Supernatants were sampled at 6, 12, 24 and 48 hours after inoculation. Virus titers were determined by end-point titration in MDCK cells in 96-well plates (Greiner Bio-One) as described previously [171]. MDCK cells were inoculated with tenfold serial dilution of culture supernatants or tissue homogenates. One hour after inoculation, cells were washed once with PBS and grown in 200µl of infection media, consisting of EMEM (Lonza) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodiumbicarbonate (Lonza), 10mM Hepes (Lonza), non-essential amino acids (MP Biomedicals) and 20 µg/ml trypsin (Lonza). Three days after inoculation, the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of infection of the cells. Infectious titers were calculated from 5 replicates by the method of Spearman-Karber. For the multi step replication kinetics geometric mean titers were calculated using the infectious titers of two independent experiments.

Western Blotting

293T cells were transfected with plasmids expressing the HA gene of H6N1WT, H6N1MBCS, H5N1WT and H5N1ΔMBCS. Cells were harvested 48h after transfection and were treated either with PBS or 2.5 ug/ml trypsin (Lonza) for 1 hr at 37 ºC. Cells were lysed in hot lysis buffer (1% SDS, 100 mM NaCl, 10 mM EDTA, 10 mM Tris HCl pH 7.5), treated with 3x dissociation loading buffer (2% SDS, 0.01 dithiothreitol, 0.02M Tris-HCL, pH6.8) for 5 min at 96 ºC and proteins were separated in 10% SDS-polyacrylamide gels. A molecular Magic MarkerTM (Invitrogen, Leek, The Netherlands) was run alongside to determine protein sizes. Proteins were transferred onto nitrocellulose membrane by electroblotting for 1 hour in blot buffer (25 mM Tris, 192 mM glycine and 20% methanol). The blots were incubated overnight at 4 ºC in block buffer (PBS with 5% (w/v) non-fat dried milk and 0.05% Tween-10), and incubated with 1:2000 rabbit antisera (1:1 mixture of rabbit α-A/Turkey/Massachutes/65 H6N1 and rabbit α-A/Shearwater/Australia/1/72 H6N1 for H6, rabbit α-A/Hong Kong/156/97 H5N1 for H5) in block buffer for 2 hours at room temperature. Blots were washed with PBS containing 0.05% Tween and incubated for 1 hr with swine anti-rabbit horseradish peroxidase (DAKO, Denmark) 1:3000 in block buffer, washed again, and developed with ECLTM Western Blotting Detection reagents (GE Healthcare, UK).

Intravenous pathogenicity index

The intravenous pathogenicity index (IVPI) of recombinant viruses was determined using 10 six-week-old specific pathogen free (SPF) white leghorn chickens (GDL, Deventer, The Netherlands) according to the OIE standards [173]. In short, chickens were injected intravenously in the ulnar vein with 0.1 ml of the H6N1WT or H6N1MBCS virus at a dose of 10^6 TCID50. The development of clinical signs was monitored for 10 days for each individual chicken. Chickens were classified sick when displaying one clinical sign, such as depression, cyanosis of the comb or wattles, respiratory involvement, diarrhoea, oedema of the face/head and nervous signs and severely sick if they displayed two or more clinical signs. The IVPI index was calculated as the mean score per bird per observation. All animal studies and procedures were reviewed and approved by the Institutional Animal Ethics Committee of Erasmus Medical Center and have been conducted according to the national guidelines of the Netherlands.

Intranasal infection of chickens

Two groups of 10 six-week-old specific pathogen free (SPF) white leghorn chickens (GDL) were inoculated intranasally with $5x10^6$ TCID50 of the H6N1WT or H6N1MBCS virus. Oropharyngeal and cloacal swabs were collected and stored in 1 ml transport media; virus titers in the swabs were determined by end-point titration in MDCK cells. At day 3 and day 6 after inoculation, 5 animals from each group were euthanized and virus titers in the nasal turbinates, trachea, lungs, spleen, liver, heart, intestine and brain were determined for 3 out of 5 animals; remaining chickens were used for immunohistochemistry. Tissues were homogenized in 3ml transport medium, consisting of Hanks balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 mg/ml streptomycin, 100 U/ml polymyxin B sulphate, and 250 mg/ml gentamycin (ICN, The Netherlands), using the Fastprep system (MP biomedicals) with 2 1/4" ceramic sphere balls (MP biomedicals) and centrifuged briefly.

Immunohistochemistry

Immunohistochemistry was performed with tissues of chickens inoculated with the H6N1WT and H6N1MBCS viruses. For each virus, 2 chickens were euthanized 3 and 6 days after inoculation by exsanguination. Necropsies and tissue sampling were performed according to a standard protocol (protocol available on request). After fixation in 10 % neutral-buffered formalin and embedding in paraffin, tissue sections were stained with an immunohistochemical method using a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody for detection of influenza virus antigen (clone Hb65, ATCC, UK). The following tissues were examined: nasal turbinates, trachea, lungs, spleen, liver, heart, intestine, brain and comb.

Nucleotide sequence accession numbers

The nucleotide sequences of the 8 gene segments of A/Mallard/Sweden/81/02 (H6N1) are available from GenBank; accession numbers CY060379 to CY060386

RESULTS

In vitro characteristics of H6N1MBCS virus

In order to investigate the subtype-specificity of the insertion of an MBCS into the HA of LPAI viruses, an MBCS was inserted into the HA0 of A/Mallard/Sweden/81/02 (H6N1WT), resulting in H6N1MBCS. The functionality of the MBCS insertion in the H6 HA was first studied by expression of HA in 293T cells upon transfection and determining the cleavage pattern in the presence and absence of trypsin. The cleavage pattern of H6WT and H6MBCS was compared to the cleavage pattern of HA of HPAI A/ HongKong/156/97 H5N1 (H5WT) and HA of this virus from which the MBCS was removed (H5ΔMBCS). H5WT and H6MBCS displayed similar cleavage patterns upon western blot analysis, with cleavage of the HA0 both with and without supplemental addition of trypsin. H5ΔMBCS displayed a cleavage pattern similar to that of H6WT with efficient HA0 cleavage only upon the addition of trypsin (Fig.1A).

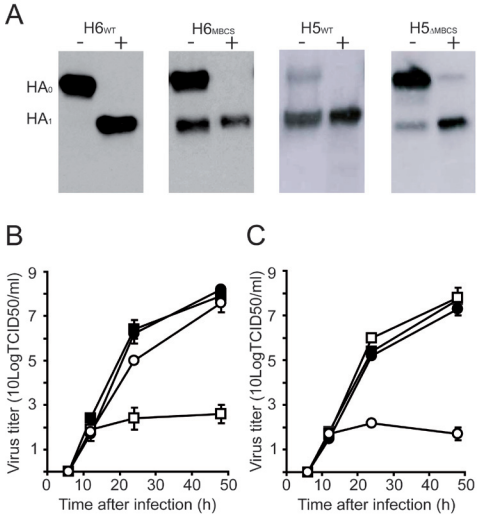

Figure 1. The in vitro phenotype of H6N1MBCS in comparison with H6N1WT, H5N1WT and H5N1ΔMBCS influenza A viruses. (A) Western blots of transfected 293T cells with H6N1WT, H6N1MBCS, H5N1WT and H5N1ΔMBCS HA open reading frame and treated with (+) or without trypsin (-). Replication kinetics in MDCK cells of (B) the H6N1WT (squares) and H6N-1MBCS (circles) viruses in MDCK cells in the presence (filled symbols) or absence of trypsin (open symbols) or (C) H5N1WT (squares) and H5N1ΔMBCS (circles) in MDCK cells in the presence (filled symbols) or absence of trypsin (open symbols). Geometric mean titers were calculated from two independent experiments; error bars indicate standard deviation.

Next, the effect of the introduction of the MBCS on the replication kinetics in MDCK cells was determined. Using reverse genetics 2 wild type (H6N1WT and H5N1WT) and 2 mutant viruses (H6N1MBCS and H5N1ΔMBCS) were produced. The H6N1WT and H6N1MBCS virus contained 7 gene segments of A/Mallard/Sweden/81/02 H6N1 and wild type H6 or H6 HA with MBCS respectively and the H5N1WT and H5N1ΔMBCS virus contained 7 gene segments of A/ HongKong/156/97 H5N1 and wild type H5 HA or H5 HA from which the MBCS was deleted. The H6N1WT virus was able to replicate to high titers in the presence of trypsin; in the absence of trypsin the H6N1WT virus failed to replicate efficiently. The H6N1MBCS virus was able to replicate to comparable titers in the presence and absence of trypsin, with only modest differences in maximum virus titers after 48 hours (Fig 1B). The comparison of the replication kinetics of the H6N1WT and H6N1MBCS virus and the HPAI H5N1WT and H5N1ΔMBCS virus (Fig. 1C) was in agreement with observa-

tions in western blot analysis. The H6N1MBCS and H5N1WT virus displayed similar replication kinetics in the absence of trypsin as H6N1WT and H5N1ΔMBCS virus in the presence of trypsin. The H6N1WT and H5N1ΔMBCS viruses did not replicate efficiently in the absence of trypsin, in agreement with an LPAI phenotype. Thus, the insertion of an MBCS in an LPAI H6N1 virus conferred an in vitro phenotype comparable to an HPAI H5N1 virus.

Intravenous pathogenicity index of the H6N1WT and H6N1MBCS virus

To determine whether the in vitro HPAI phenotype of the H6N1MBCS virus also resulted in an HPAI phenotype in vivo, the intravenous pathogenicity index (IVPI) was determined. The IVPI is the gold standard for the assessment of the pathogenic phenotype of avian influenza viruses in poultry. Avian influenza viruses with an IVPI < 1.2 are considered LPAI viruses and avian influenza viruses with an IVPI ≥ 1.2 are considered HPAI viruses. Ten six-week old leghorn chickens were inoculated intravenously with 0.1 ml $1x10^6$ $TCID_{50}$/ml of the H6N1WT or H6N1MBCS virus and monitored closely for 10 days. The 10 chickens inoculated with the H6N1WT virus did not show clinical signs of disease over the 10 day period, resulting in an IVPI of 0.0, confirming the LPAI phenotype in vivo (Table 1) [173].

The chickens inoculated with the H6N1MBCS virus displayed a rapid progressive disease with 8/10 animals severely sick at 3 days post inoculation (d.p.i.) and 1 deceased animal at 5 and 6 d.p.i. A variety of clinical symptoms was observed in these

Table 1. Overview of the intravenous pathogenicity index for the H6N1WT and H6N1MBCS viruses. Two groups of 10 chickens each were inoculated intravenously with 0.1 ml $1x10^6$ TCID50 of either virus and observed for clinical signs of disease for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick and 3 if dead.

$H6N1_{WT}$	Days post inoculation										Score	Total
	1	2	3	4	5	6	7	8	9	10		
Normal	10	10	10	10	10	10	10	10	10	10	100 x 0	0
Sick[a]	0	0	0	0	0	0	0	0	0	0	0 x 1	0
Severely sick[b]	0	0	0	0	0	0	0	0	0	0	0 x 2	0
Dead	0	0	0	0	0	0	0	0	0	0	0 x 3	0
												0
											IVPI[c]	0
$H6N1_{MBCS}$	Days post inoculation										Score	
	1	2	3	4	5	6	7	8	9	10		
Normal	3	1	1	0	0	0	0	2	3	3	16 x 0	0
Sick[a]	7	5	1	2	1	1	6	5	5	5	38 x 1	38
Severely sick[b]	0	4	8	8	8	7	0	0	0	0	35 x 2	70
Dead	0	0	0	0	1	2	2	2	2	2	11 x 3	33
												141
											IVPI[c]	1,41

[a] Sick birds show one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs.

[b] Severely sick birds show two or more of the signs mentioned undera.

[c] The intravenous pathogenicity index (IVPI) is the mean score per bird per observation over the 10-day period.

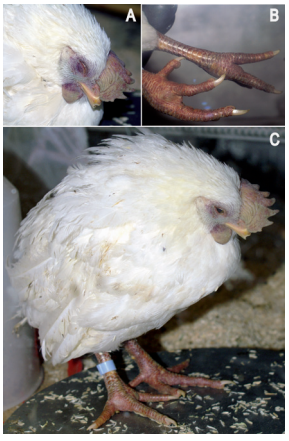

Figure 2. Clinical symptoms of chickens infected with H6N1MBCS virus consistent with HPAI virus infection: cyanosis of the comb and wattles (A), subcutaneous haemorrhage of the shanks (B) and severe depression, ruffled fur with oedema of head and neck and cyanosis of the comb, wattles and paws (C).

birds including respiratory involvement (2/10), depression (10/10) (Fig. 2), diarrhoea (2/10), cyanosis of the exposed skin or wattles (6/10) (Fig. 2), oedema of the face and/or head (6/10) and nervous signs (6/10) (Table 1). The overall IVPI score of the H6N1MBCS virus was 1.4, in concordance with an HPAI phenotype.

Infection of chickens via the intranasal route

Next, clinical signs, virus shedding, and tissue distribution in chickens were studied upon intranasal inoculation, considered more representative of a natural infection than the intravenous inoculation described above. To this end, 10 chickens were inoculated intranasally with $5x10^6$ TCID50/ml of either the H6N1WT or H6N1MBCS virus. The chickens were observed for clinical signs of disease and cloacal and oropharyngeal swabs were obtained daily over a 6-day period. None of the chickens inoculated with the H6N1WT virus displayed clinical signs during the course of the experiment, whereas all H6N1MBCS virus inoculated chickens were (10/10) lethargic and some developed cyanosis of the comb (2/10). Virus shedding from the respiratory tract was observed to start at 1 d.p.i. for both viruses and continued until 4-5 d.p.i. The amount of virus shedding was comparable between the 2 viruses. Virus shedding from the intestinal tract started at 3 d.p.i. for both viruses and continued during the course of the experiment for the H6N1MBCS virus, whereas cloacal shedding of the H6N1WT virus was minimal and below detection limit after 6 days (Fig. 3). On 3 and 6 d.p.i., 5 chickens from each group were euthanized and virus

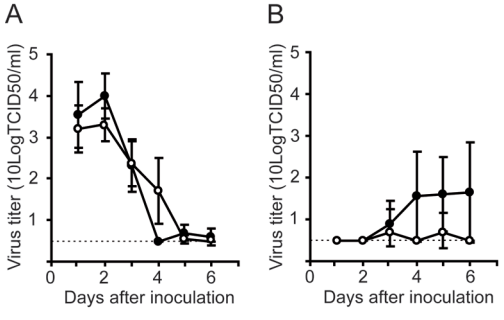

Figure 3. Virus shedding from the respiratory tract (A) and the intestinal tract (B) after intranasal inoculation of chickens the H6N1WT (open circle) and H6N1MBCS (closed circle) viruses. Oropharyngeal and cloacal swabs were taken daily and virus titers were determined by end-point titration in MDCK cells. The geometric mean titers, calculated per day per group, are displayed; error bars indicate 95% confidence interval. The dotted lines indicate the cut off value of the assay.

titers in the nasal turbinates (NT), trachea, lungs, spleen, liver, heart, intestine and brain were determined for 3 of these animals; the remaining chickens were used for immunohistochemistry. The H6N1WT and H6N1MBCS viruses were detected with comparable titers in the upper respiratory tract of inoculated chickens at 3 d.p.i.. In the lungs, the H6N1MBCS virus replicated to higher titers and was detected in all animals, whereas the H6N1WT virus was only detected in 1 animal. The H6N1WT virus was also detected in the spleen (1/3 animals) and intestine (3/3) whereas the H6N1MBCS was detected in the brain (2/3) (Table 2).

Immunohistochemistry was performed in order to confirm influenza A virus replication in tissues obtained at 3 d.p.i. Using an α-NP monoclonal antibody, expression of influenza virus antigen was not detected in any of the tissues of the group inoculated with H6N1WT. For the group inoculated with H6N1MBCS expression of influenza virus antigen was detected in all tissue samples, including tissue of the comb (Figure 4) in agreement with reports on H5 and H7 HPAI infections in chickens [174,175]. At 6 d.p.i. H6N1MBCS virus was still detected in the brain (1/3), heart (1/3) and intestine (2/3) whereas the H6N1WT was cleared from all sampled tissues by this time (Table 2).

Table 2. Virus titers in tissues of chickens inoculated either with the H6N1WT or H6N1MBCS virus. Three chickens of each group were euthanized at 3 d.p.i. and 6 d.p.i. Virus titers in lung, trachea, nasal turbinates (NT), brain, spleen, heart, liver and intestine were determined by end-point titration in MDCK cells. Geometric mean titer ± standard deviation is indicated; the cut-off value is given for negative tissues.

Day 3	$H6N1_{WT}$ virus		$H6N1_{MBCS}$ virus	
Tissue	Virus detection (n/3)	Virus titer (log $10TCID_{50}$/gram tissue) ± standard deviation	Virus detection (n/3)	Virus titer (log $10TCID_{50}$/gram tissue) ± standard deviation
Lung	1/3	1,52 (±0,17)	3/3	2,10 (±0,79)
Trachea	3/3	2,83 (±0,47)	3/3	3,37 (±1,34)
NT	2/3	2,97 (±0,81)	3/3	2,59 (±0,17)
Brain	0/3	<1,41	2/3	1,53 (±0,17)
Spleen	1/3	2,16 (±1,15)	0/3	<1,50
Heart	0/3	<1,51	0/3	<1,51
Liver	0/3	<1,43	0/3	<1,43
Intestine	3/3	2,56 (±0,92)	0/3	<1,65

Day 6	$H6N1_{WT}$ virus		$H6N1_{MBCS}$ virus	
Tissue	Virus detection (n/3)	Virus titer (log $10TCID_{50}$/gram tissue) ± standard deviation	Virus detection (n/3)	Virus titer (log $10TCID_{50}$/gram tissue) ± standard deviation
Lung	0/3	<1,42	0/3	<1,42
Trachea	0/3	<1,91	0/3	<1,91
NT	0/3	<2,01	0/3	<2,01
Brain	0/3	<1,41	1/3	1,57 (±0,27)
Spleen	0/3	<1,50	0/3	<1,50
Heart	0/3	<1,51	1/3	1,56 (±0,08)
Liver	0/3	<1,43	0/3	<1,43
Intestine	0/3	<1,65	2/3	1,92 (±0,31)

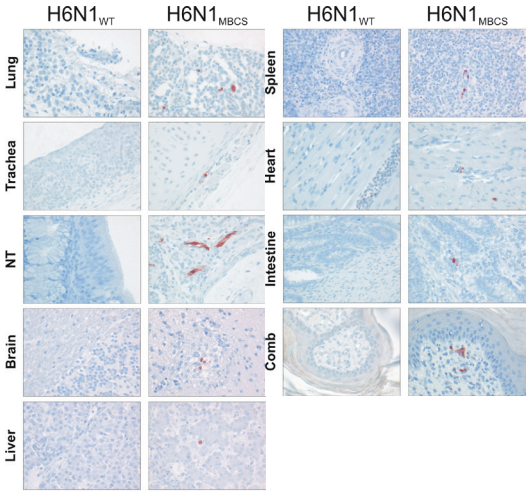

Figure 4. The tissue distribution of the H6N-1WT (left column) and H6N1MBCS (right column) viruses after intranasal inoculation of chickens. Serial sections of these tissues of chickens infected with H6N1WT virus could not detect the expression of viral antigen in lung, tracheal epithelium, nasal turbinates (NT), brain, liver, spleen, heart, intestine and comb. Expression of viral antigen could be detected in all tissues of chickens infected with H6N1MBCS virus. The chickens were inoculated intranasally with $5x10^6$ TCID50 H6N1WT and H6N1MBCS virus and euthanized 3 days post infection. Tissue sections were stained with a monoclonal antibody against influenza A virus nucleoprotein, visible as a red-brown staining.

DISCUSSION

The emergence of HPAI viruses from LPAI ancestral viruses is restricted to influenza A viruses of the H5 and H7 subtypes. All currently known HPAI outbreaks were caused by HPAI H5 and H7 viruses [176]. The emergence of HPAI viruses is primarily thought to occur by introduction of an MBCS in HA of LPAI H5 and H7 viruses during their circulation in poultry. LPAI viruses other than H5 and H7 also frequently cause outbreaks in poultry, in general with mild clinical symptoms [160,177,178]. Despite extensive circulation in poultry, HPAI viruses have never emerged during LPAI outbreaks other than those caused by influenza A viruses of the H5 or H7 subtypes, pointing to a subtype specific nature of the emergence of HPAI viruses. Whereas the structural and molecular mechanism for the emergence of HPAI viruses has been partly elucidated [168,179], the subtype specificity of this emergence remains unknown. The proposed molecular mechanisms underlying the introduction of basic amino acid residues into the HA0 cleavage are nucleotide/amino acid substitution and insertion of additional basic amino acid residues via a strand-slippage [168]. In addition, in the case of two H7 virus strains it has been shown that recombination with host or viral RNA was responsible for the introduction of an MBCS [180,181]. The present study aimed to understand the nature of the subtype specificity of the MBCS introduction by artificial insertion of an MBCS motif identical to that of currently circulating H5 HPAI viruses in an LPAI H6N1 virus. The choice for an LPAI H6 virus was made because multiple introductions of this subtype into poultry have occurred and because of the extensive circulation of H6 viruses in poultry [182,183]. These characteristics make it likely that H6 viruses with an HPAI genotype would have emerged if not for an as yet unidentified restriction. The insertion of an MBCS into the LPAI H6N1 virus resulted in a shift from a trypsin-dependent into a trypsin-independent phenotype, comparable to that of an HPAI H5N1 virus. Moreover, the introduction of the MBCS into the LPAI H6N1 virus also resulted in an in vivo HPAI

phenotype. The clinical signs observed in H6N1MBCS virus infected chickens, such as subcutaneous haemorrhages of the shanks, cyanotic combs and wattles and neurological signs are hallmarks of HPAI infection (Fig. 2). In addition, the IVPI was greater than 1.2 (Table 1), which places the H6N1MBCS virus within the category of HPAI viruses [173]. Our data are in agreement with previous studies which show that the introduction of an MBCS into LPAI H5 viruses increases the tissue tropism to extra-respiratory and extra-intestinal replication [157]. However, the pathogenicity of the generated H6N1MBCS virus is not completely equivalent to the pathogenicity displayed in naturally occurring HPAI H5 and H7 viruses which in general have IVPI scores in the range of 2 to 3 [166,184,185], although lower IVPI scores (1.2) have been reported for naturally occurring HPAI viruses as well [186]. The relatively limited pathogenicity of the H6N1MBCS virus in the chicken model upon intranasal inoculation, as compared to intravenous inoculation, might be caused by the lack of adaptation of the original H6N1WT virus to efficient replication in chickens. The H6N1WT virus was isolated from a wild-caught migratory mallard and as such will be adapted to replication in this wild bird species. It has been shown in a previous report comparing the intranasal infectious dose of LPAI viruses, that infection of white leghorn chickens required very high doses of viruses isolated from wild birds as compared to viruses obtained from chickens [187]. Previous studies have also shown that changes in HA alone are not sufficient for efficient replication of wild bird avian influenza viruses in chickens, and that besides changes in HA, changes in the internal genes such as PB1, PB2 and PA are necessary for adaptation toward efficient replication in chickens [143,188,189]. We suggest that such additional adaptations in the internal genes of the H6N1MBCS virus will likely result into further increased pathogenicity, possibly comparable to the pathogenicity of other HPAI viruses upon intranasal infection and IVPI. It has also been shown for multiple LPAI viruses that adaptation to replication in poultry results in increased virus shedding from both the respiratory tract and the intestinal tract [143,188,190].

For the H6N1WT virus, the low virus shedding from the respiratory tract at 3 d.p.i. (2.3 ± 0.9 Log10 TCID50/ml, 10/10) was accompanied by low or undetectable virus titers in the respiratory tract tissues (Table 2). Likewise, low shedding from the intestinal tract at 3 d.p.i. (0.7 ± 0.6 Log10 TCID50/ml, 3/10) was accompanied by low virus titers in the intestinal tract (colon). Upon immunohistochemistry using multiple cross-sections of tissues of 2 chickens not used for virus isolation, no influenza A virus NP positive cells were detected in tissues of the respiratory or intestinal tract, again indicating the variability and the low level of virus replication within the group of chickens inoculated intranasally with H6N1WT virus. Real-time reverse-transcriptase polymerase chain reaction (RRT-PCR, ([191]) further confirmed the absence of viral genomic RNA in tissues negative in virus isolation (data not shown). In the 10 chickens inoculated with H6N1MBCS virus, low virus shedding was detected in the respiratory tract at 3 d.p.i. (2.3 ± 1.0 Log10 TCID50/ml, 10/10) accompanied by low virus titers in of the respiratory tract tissues. Low shedding from the intestinal tract at 3 d.p.i (0.8 ± 0.6 Log10 TCID50/ml, 3/10) was accompanied by

the absence of virus titers in the intestinal tract. Although viable H6N1MBCS virus could not be detected in all organs at 3 d.p.i., low amounts of viral genomic RNA were detected by RRT-PCR in all tissues of the chickens inoculated with H6N1MBCS virus (data not shown). These data are in agreement with the immunohistochemistry data (Fig.4) and indicate that despite the poor adaptation of the H6N1MBCS virus to chickens, a low level of systemic replication, below the threshold value of the TCID50 assay, had occurred, which is an indication of increased tissue tropism similar to that of natural occurring HPAI viruses [169].

Earlier work indicated that the introduction of an MBCS in the HA of a human seasonal influenza A virus and an LPAI H3 influenza A virus strain ceased the virus' dependence on trypsin for the activation of HA0 upon expression of HA proteins in vitro [170,192-194]. However, in the context of infectious virus H3N8 LPAI viruses with an MBCS did not result in an HPAI phenotype upon intranasal inoculation of white leghorn chickens. Of note, the IVPI of these viruses was not determined [170]. These experimental data also suggested a lack of adaptation in chickens of the wild duck derived H3N8 virus and showed an increased cloacal shedding only of the H3N8 virus with the RRRKKR↓G MBCS out of 3 different MBCSs tested [170]. Collectively, the results from the H3N8 virus and our study suggest that different HA subtypes might require different MBCS motifs.

Now that we have shown that an LPAI H6 virus can become highly pathogenic upon the introduction of an MBCS in HA, the question arises why the HPAI phenotype is restricted to H5 and H7 influenza A viruses in nature. Since it was shown here that there is no functional constraint to H6 with an MBCS with respect to HA0 cleavage and infectivity in vivo - in fact, virus shedding of the H6N1MBCS virus from the intestinal tract was higher as compared to H6N1WT virus - there must different constraints for the emergence of HPAI H6 viruses in nature. Moreover, the insertion of the minimal MBCS motif REKR↓G into H6N1WT virus did not result in a trypsin independent phenotype and replication kinetics were similar to that of H6N1WT virus in the presence and absence of trypsin (data not shown), suggesting that the introduction of a minimal MBCS into H6N1WT virus does not result in increased pathogenicity, comparable to H5 and H7 influenza A viruses [97,166,167]. The prediction of the cleavability of HA by proprotein convertases, is therefore not directly useful to predict the HPAI or LPAI phenotype of influenza viruses [195]. Based on our data it is likely that the subtype restriction of the HPAI genotype and phenotype is not at the molecular, structural or functional level, but potentially at the evolutionary level with sequence restrictions preventing the emergence of HPAI viruses other than those of the H5 and H7 subtypes.

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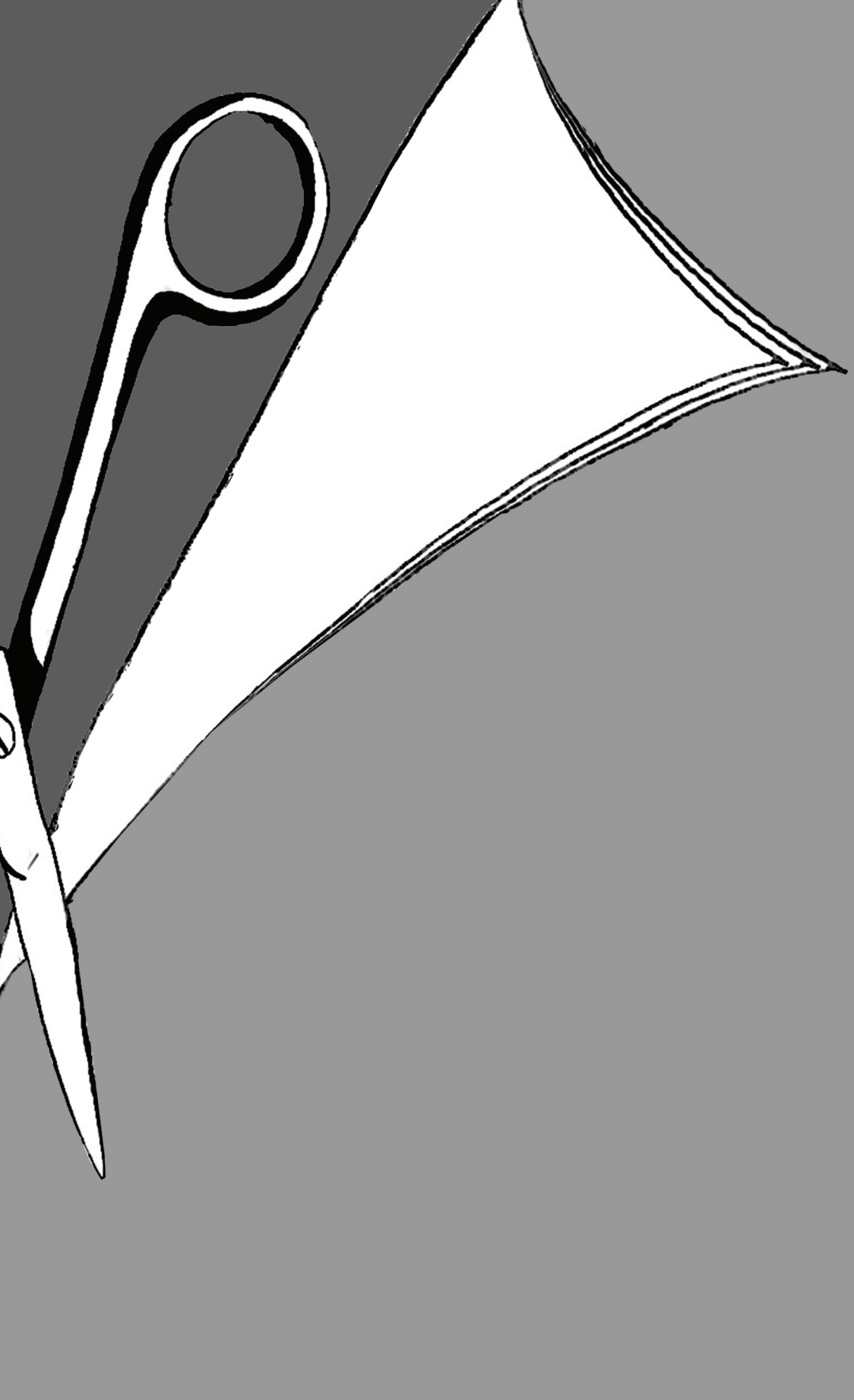

CHAPTER 2
Multi basic cleavage site as a virulence factor of influenza A viruses

2.2
Insertion of a multi basic cleavage site in the hemagglutinin of a human influenza H3N2 virus does not increase pathogenicity in ferrets

Eefje J. A. Schrauwen, Theo M. Bestebroer, Vincent J. Munster, Emmie de Wit, Sander Herfst, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus and Ron A. M. Fouchier

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ABSTRACT

A multibasic cleavage site (MBCS) in the hemagglutinin (HA) protein of influenza A virus is a key determinant of pathogenicity in chickens, and distinguishes highly pathogenic avian influenza (HPAI) viruses from low pathogenic avian influenza (LPAI) viruses. An MBCS has only been detected in viruses of the H5 and H7 subtypes. Here we investigated the phenotype of a human H3N2 virus with an MBCS in HA. Insertion of an MBCS in the H3N2 virus resulted in cleavage of HA and efficient replication in MDCK cells in the absence of exogenous trypsin in vitro, similar to HPAI H5N1 virus. However, studies in ferrets demonstrated that insertion of the MBCS in HA did not result in increased virus shedding, cellular host range, systemic replication, or pathogenicity as compared to wildtype virus. This study indicates that acquisition of an MBCS alone is insufficient to increase pathogenicity of a prototypical seasonal human H3N2 virus.

In wild birds and poultry throughout the world, avian influenza viruses expressing 16 antigenically distinct subtypes of hemagglutinin (HA) and 9 subtypes of neuraminidase (NA) have been described [1,196]. Only subtypes H1, H2 and H3 have circulated extensively in humans [197]. Avian influenza viruses can be classified based on pathogenicity in chickens into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses. Virtually all known HPAI viruses are of the H5 or H7 subtypes. Direct transmission of HPAI H5N1 viruses to humans was first detected in 1997 [58] and has continued to be reported since [59]. In addition, various human cases of HPAI H7 virus infections have been reported [67,198,199].
Pathogenicity of avian influenza viruses in chickens is directly associated with the cleavability of the HA glycoprotein [97,98,156,200]. HA is initially synthesized as a HA0 precursor protein that is subsequently cleaved into the two functional subunits HA1 and HA2. This cleavage step is essential for virus infectivity since uncleaved HA is able to mediate virus attachment but unable to perform the fusion step necessary for the initiation of infection [201]. HA proteins of LPAI viruses have a single arginine at the cleavage site that is recognized by trypsin-like proteases expressed predominantly in the respiratory and intestinal tract. HA of HPAI viruses contain a multibasic cleavage site (MBCS) that can be cleaved by ubiquitously expressed furin and related furin-like proprotein convertases (PC5/6) [10,101]. The cleavage properties of HA and the expression patterns of proteases in the host are considered to be the major determinants of systemic influenza virus replication and pathogenesis in chickens.
The association between HA cleavability and replication outside the respiratory tract is less straightforward for influenza viruses in mammals. Human influenza viruses of subtypes H1, H2, and H3 do not have an MBCS in HA and virus replication is generally restricted to the respiratory tract. For humans infected with HPAI H5 and H7 viruses, virus detection outside the respiratory tract has been reported, but is not a general phenomenon [202]. In Cynomolgus macaques infected with HPAI H5N1 virus, detection of virus antigen was limited to the respiratory tract [102]. Systemic virus replication and associated pathogenesis has been reported for several HPAI H5 and H7 viruses in mice, ferrets, and cats, but certainly not for all viruses tested in these mammalian model systems [203-207]. In mice, it has been shown that removal of the MBCS from some HPAI H5 and lab-adapted H7 virus HAs resulted in reduced virulence [103,115]. Here we followed an opposite – gain of function – approach, by inserting an MBCS in a human H3N2 virus and testing virus replication and pathogenesis in the ferret model. The goal of this work was to increase our basic understanding of the role of the MBCS and trypsin/furin-dependent cleavage of HA on influenza virus pathogenesis in mammals.
Influenza virus A/Netherlands/16190/68 (H3N2) was isolated from an individual in the Netherlands during the 1968 pandemic. Influenza virus A/Hong Kong/156/97 (H5N1) was isolated from the human index case during the 1997 H5N1 outbreak in Hong Kong [58]. The 8 gene segments of these two viruses were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000

[171,208]. For the generation of H5N1 virus without an MBCS (H5N1ΔMBCS), the cleavage site PQRERRRKKR↓G in the H5 HA plasmid was changed to PQIETR↓G by RT-PCR with specific primers as described previously [172]. For the generation of H3N2 virus with an MBCS (H3N2MBCS), the cleavage site PEKQTR↓G in the H3 HA plasmid was changed to PEKQRRRKKR↓G, identical to the MBCS of the control virus A/HongKong/156/97 H5N1. Viruses H3N2wt, H5N1wt, H3N2MBCS and H5N1ΔMBCS were generated by reverse genetics as described previously [209]. The genotypes of all plasmids and viruses were confirmed by sequencing. The risk potential of the H3N2MBCS virus was assessed prior to start of the experiments and it was determined that the anticipated risk of generating this virus would be equivalent to that of a mammal-transmissible HPAI virus. Therefore, all in vivo and in vitro experiments were performed under ABSL3+ containment conditions. Animal studies were approved by an independent Animal Ethics Committee (stichting Dieren Experimenten Commissie consult).

First, the requirement of trypsin for the cleavability of the H3 HA proteins was tested. To this end, 293T cells were harvested 48h after transfection with plasmids expressing HA of H3N2wt and H3N2MBCS, using HA of H5N1wt and H5N1ΔMBCS as controls. Cells were treated either with PBS or 2.5 µg/ml trypsin (Lonza) for 1

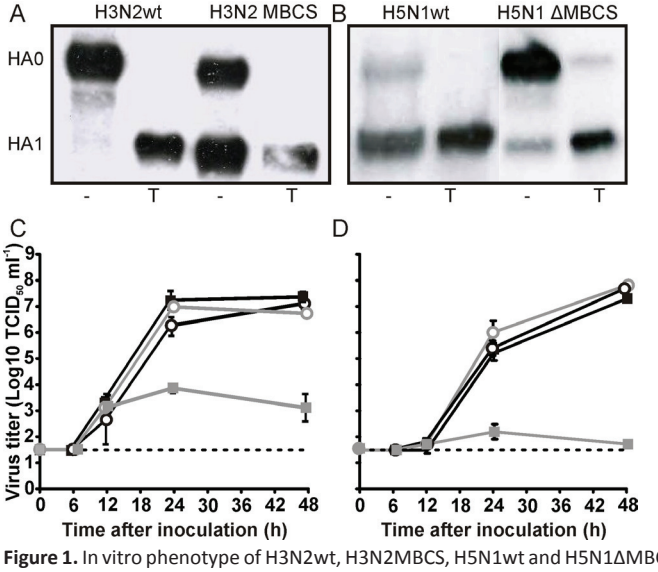

Figure 1. In vitro phenotype of H3N2wt, H3N2MBCS, H5N1wt and H5N1ΔMBCS. Western blots of lysates of 293T cells transfected with HA of H3N2wt, H3N2MBCS (A), H5N1wt and H5N1ΔMBCS (B) upon treatment with (T) or without trypsin (-). Replication of H3N2wt (closed square) and H3N2MBCS (open circle) in the presence (black) or absence of trypsin (grey) (C) or H5N1wt (open circle) and H5N1ΔMBCS (closed square) in the presence (black) or absence of trypsin (grey) (D). MDCK cells were inoculated with 0.01 TCID50/cell and supernatant samples were harvested 6, 12, 24, and 48 hours later. Supernatant samples were titrated in MDCK cells. Geometric mean titers and standard deviation were calculated from two independent experiments. Data for Fig 1B and 1D were taken with permission from the American Society for Microbiology, from [209].

hr at 37 ºC. Cell lysates were subjected to electrophoresis in 10% SDS-polyacrylamide gels, blotted, and western blots were incubated with rabbit serum to A/Hong kong/1/68 (H3) or A/Hong Kong/156/97 (H5) and a peroxidase-labelled swine anti-rabbit antibody as previously described [209]. In the presence of trypsin, the HA of H3N2wt and H3N2MBCS were cleaved to completion (Fig 1A). In contrast, in the absence of trypsin, HA of H3N2wt remained uncleaved while a large fraction of the HA of H3N2MBCS was cleaved. Thus, upon insertion of an MBCS in H3 HA the cleavage of HA was no longer dependent on exogenous trypsin. H3 HA with and without an MBCS thus behaved essentially the same as H5 HA with and without an MBCS (Fig 1A-B).

Next, virus replication of reverse genetics-derived H3N2wt and H3N2MBCS viruses in the presence and absence of 1 µg/ml trypsin (Lonza) was tested in MDCK cells, again using H5N1wt and H5N1ΔMBCS as controls. Cells were inoculated in duplo at a multiplicity of infection (MOI) of 0.01 TCID50 per cell. Supernatants were sampled at 6, 12, 24 and 48 hours after inoculation and virus titers were determined by end-point titration in MDCK cells [209]. The H3N2wt and H5N1ΔMBCS viruses replicated to high titers in the presence of trypsin. In the absence of trypsin both viruses failed to replicate to high titers. The H3N2MBCS and H5N1wt viruses replicated to comparable virus titers in MDCK cells in the presence and absence of trypsin (Fig 1C-D). The trypsin independent phenotype of the human influenza H3N2 virus with an MBCS in vitro was also observed with a second H3N2 virus, A/Netherlands/178/95 (data not shown). Thus, trypsin-independent HA cleavage by insertion of an MBCS can be achieved not only in avian influenza viruses but also in human influenza A/H3N2 virus [192].

Next, we investigated whether the trypsin-independent cleavage of H3N2MBCS resulted in enhanced pathogenicity in ferrets. The ferret model is generally thought to be a good animal model for influenza in humans, because ferrets are naturally susceptible to infection and develop respiratory disease and lung pathology similar to humans when infected with seasonal, avian, or pandemic influenza viruses [210-212]. Groups of six influenza-seronegative female ferrets (Mustella putorius furo) were inoculated intranasally with 10^6 $TCID_{50}$ of H3N2wt or H3N2MBCS virus, divided over both nostrils (2 x 250µl). Animals were observed for the development of clinical symptoms and weighed daily. The percentage mean maximum weight loss was 8.8±1.4% (standard deviation) for animals inoculated with the H3N2wt virus. Animals inoculated with H3N2MBCS had a maximum weight loss of 7.0±1.4% (standard deviation) (Fig. 2A). No differences were observed for clinical parameters such as lethargy, sneezing, ruffled fur, interest in food, and runny nose between the two groups of ferrets. Thus, H3N2MBCS does not appear to cause more severe disease as compared to H3N2WT.

Throat and nasal swabs were collected daily to determine virus excretion from the upper respiratory tract. Infectious virus shedding from the nose peaked at day 2 for both viruses and continued until day 7 and 6 for H3N2wt and H3N2MBCS respectively (Fig. 2B) and shedding from the throat peaked at day 1 and continued until

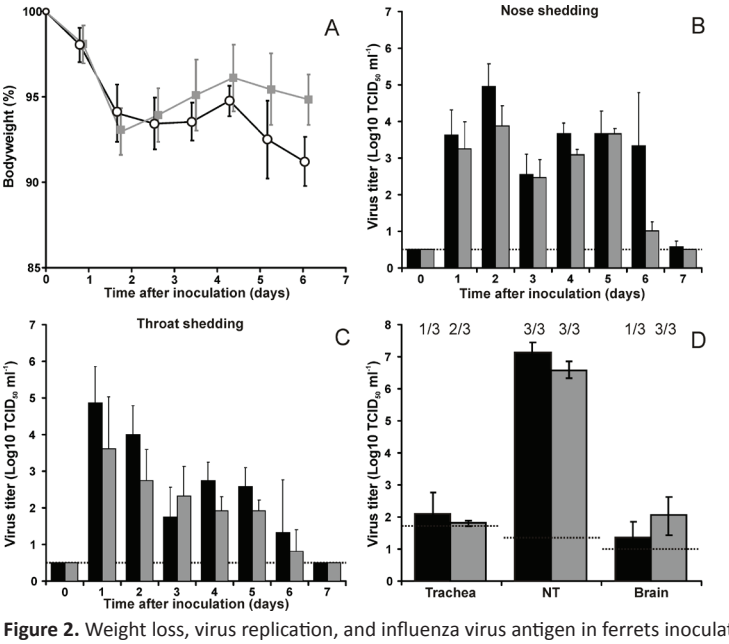

Figure 2. Weight loss, virus replication, and influenza virus antigen in ferrets inoculated with H3N2wt and H3N2MBCS virus. Weight loss of ferrets inoculated with H3N2wt (black, circle) and H3N2MBCS (square, grey) virus (A). Mean bodyweight and standard deviation were calculated as percentages of bodyweight at time of inoculation for each group. After day 3, only 3 animals remained in each group. Virus shedding from the nose (B) and throat (C) of ferrets inoculated with H3N2wt (black) and H3N2MBCS (grey). Virus detection in tissues of ferrets inoculated with H3N2wt (black) and H3N2MBCS (grey) virus at day 3 (D). Geometric mean titers are shown, with error bars to indicate the standard deviation. The lower limit of detection is indicated by the dotted line. The number of positive tissues from the ferrets are shown.

day 6 for both viruses (Fig 2C). We observed a consistent decline in viral shedding from the throat, compared to the nose. Nucleotide sequencing was performed to confirm that the HA cleavage site remained unchanged during the experiment.

Three animals from each group were euthanized at 3 and 7 days post inoculation (dpi), and the nasal turbinates, trachea, lungs, liver, spleen, kidney, colon, and brain were collected to study virus distribution. Necropsies and tissue sampling were performed according to a standard protocol and infectious virus titers were determined by end-point titration in MDCK cells [209]. At 7 dpi, virus was undetectable in samples from the organs in all groups of ferrets. At 3 dpi, virus was not detected in lungs, spleen, liver, kidney and intestine of ferrets inoculated with H3N2wt or H3N2MBCS. Both groups of inoculated ferrets revealed low virus titers in the trachea. Virus titers in the nasal turbinates were high and similar for both groups of ferrets. Virus was detected in the brain of 1/3 animals inoculated with H3N2wt and 3/3 animals inoculated with H3N2MBCS, but only at low titers (Fig 2D). Immunohistochemistry was performed to confirm virus replication in nasal turbinate, trachea and brain at 3 dpi. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained using a monoclonal antibody against the

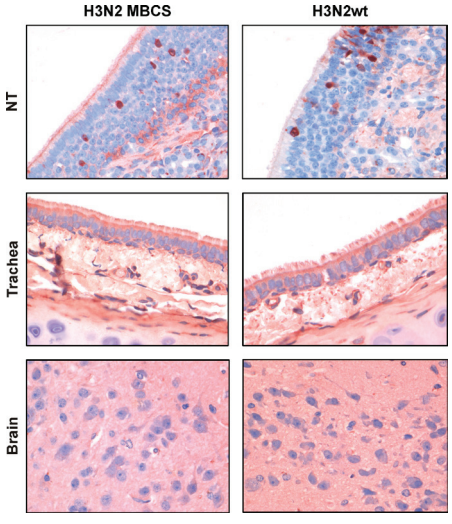

Figure 3. Tissue distribution of the H3N2WT and H3N2MBCS virus in the respiratory tract
Expression of viral antigen in trachea, nasal turbinates (NT) and brain of ferrets inoculated with H3N2WT and H3N2MBCS at 3 dpi. Tissue sections were stained with a monoclonal antibody against influenza A virus nucleoprotein, visible as red-brown staining.

nucleoprotein of influenza A virus [209]. Influenza virus antigen expression was not detected by immunohistochemistry in the brain and trachea of both groups of ferrets. In contrast, virus antigen was readily detected in the nasal turbinates of all three ferrets in both groups at 3 dpi. There was no evidence for different cellular tropism between H3N2MBCS and H3N2WT virus in the nasal turbinates of infected ferrets (Fig 3). These results indicated that replication of H3N2MBCS, like replication of H3N2WT, is mostly restricted to the upper respiratory tract of ferrets.

The current study demonstrates that with an insertion of an MBCS identical to that of currently circulating HPAI H5 viruses, a human H3N2 virus becomes trypsin-independent in vitro. The introduction of an MBCS into other human and LPAI HA subtypes (H3, H6) also resulted in trypsin independent cleavage of HA0 in vitro [170,192,193,209]. Introduction of an MBCS in an LPAI H6N1 virus resulted in an increased intravenous pathogenicity index (IVPI) in chickens [209]. However, the consequences of the introduction of the MBCS on human H3N2 virus replication and pathogenesis in animal models were not investigated previously. Surprisingly, the trypsin independence of a human H3N2 virus did not result in enhanced pathogenicity, tissue tropism, or cellular tropism in the ferret model, as compared with wild type H3N2 virus. If anything, the H3N2MBCS virus was slightly attenuated compared to H3N2WT virus, based on weight loss and virus shedding from the upper respiratory tract.

Both groups of inoculated ferrets revealed no virus replication in the lower respiratory tract. This is not unusual, as strain-to-strain variability among human H3N2 viruses with respect to infection of the lower respiratory tract of ferrets has been demonstrated [154,213-215]. Although the H3N2MBCS virus was detected in the brain of ferrets, virus titers were very low and virus-infected cells were not detected by immunohistochemistry. Given that low virus titers were also detected for a ferret inoculated with H3N2WT virus, and that neither of the groups of infected ferrets

developed neurological symptoms, we conclude that the MBCS in the context of H3 HA did not result in systemic virus replication and enhanced disease. In ferrets infected with more recent wildtype human H3N2 viruses, low virus titers were also detected in the brains [214].

The goal of the present work was to increase the basic understanding of the role of the MBCS and trypsin or furin-dependent cleavage of HA on influenza virus pathogenesis in mammals. Our results indicate that an MBCS is not sufficient to increase the virulence of a human H3N2 virus. For HPAI H5N1 viruses, additional virulence determinants in HA and other viral proteins have been shown to affect virulence independently of the MBCS in ferrets and chickens [204,216-218]. It is possible that an MBCS in H3 HA in combination with other genetic changes, such as changes in the receptor binding site, could result in increased pathogenicity of human H3N2 virus as well, by facilitating replication in extra-respiratory tissues.

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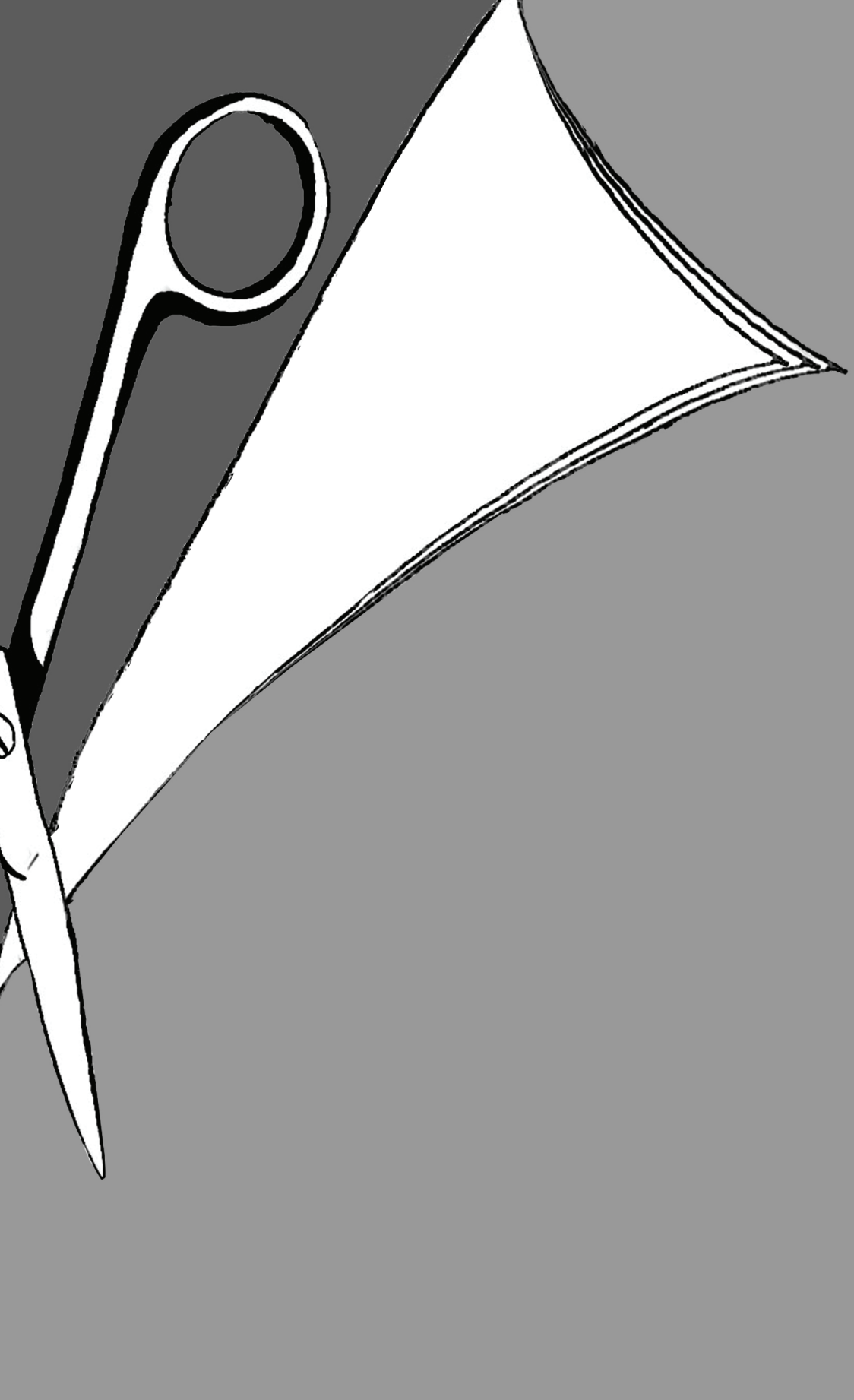

CHAPTER 2
Multi basic cleavage site as a virulence factor of influenza A viruses

2.3
The multi basic cleavage site in H5N1 virus is critical for systemic spread along the olfactory and hematogenous routes in ferrets

Eefje J. A. Schrauwen, Sander Herfst, Lonneke M. Leijten, Peter van Run, Theo M. Bestebroer, Martin Linster, Rogier Bodewes, Joost H. C. M. Kreijtz, Guus. F. Rimmelzwaan, Albert D. M. E. Osterhaus, Ron A. M. Fouchier, Thijs Kuiken and Debby van Riel

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ABSTRACT

The route by which highly pathogenic avian influenza (HPAI) H5N1 virus spreads systemically, including the central nervous system (CNS), is largely unknown in mammals. Especially the olfactory route, which could be a route of entry into the CNS, has not been studied in detail. Although the multi basic cleavage site (MBCS) in the HA of HPAI H5N1 viruses is a major determinant of systemic spread in poultry, the association between the MBCS and systemic spread in mammals is less clear. Here we determined the virus distribution of HPAI H5N1 virus in ferrets in time and space—including along the olfactory route—and the role of the MBCS in systemic replication. Intranasal inoculation with wild type H5N1 virus revealed extensive replication in the olfactory mucosa from where it spread to the olfactory bulb and the rest of the CNS, including the cerebral spinal fluid (CSF). Virus spread to the heart, liver, pancreas and colon was also detected, indicating hematogenous spread. Ferrets inoculated intranasally with H5N1 virus lacking an MBCS demonstrated respiratory tract infection only. In conclusion, HPAI H5N1 virus can spread systemically via two different routes, the olfactory and hematogenous routes in ferrets. This systemic spread was dependent on the presence of the MBCS in HA.

INTRODUCTION

In wild birds and poultry throughout the world, influenza A viruses are represented by 16 hemagglutinin (HA) and 9 neuraminidase (NA) antigenic subtypes [1,2]. Influenza viruses of the H5 and H7 subtype may become highly pathogenic influenza (HPAI) viruses upon transmission from wild birds to poultry. HPAI H5N1 virus, which has been enzootic in poultry for more than a decade, causes severe damage to the poultry industry. Direct transmission of HPAI H5N1 virus to humans was first detected in 1997 [58] and has continued to be reported since, causing severe and often fatal disease with a case-fatality rate of approximately 60% [59]. Unlike most influenza viruses in mammals, which are normally restricted to the respiratory tract, HPAI H5N1 virus is regularly able to spread systemically in humans and other mammals [66,219].

The highly pathogenic phenotype of avian influenza viruses in chickens is primarily determined by the acquisition of a multibasic cleavage site (MBCS) in the HA of a low pathogenic avian influenza (LPAI) virus, and is believed to be a major determinant in tissue tropism and high pathogenicity in poultry [97,98,200]. The HA of low pathogenic avian influenza (LPAI) viruses can be cleaved by trypsin-like proteases. Replication of these LPAI viruses is therefore restricted to sites in the host where these enzymes are expressed, i.e. the respiratory and intestinal tract [10]. In contrast, the HA of HPAI viruses can be cleaved by ubiquitously expressed furin-like proteases, facilitating systemic replication in chickens [101].

In mammals, the association between the presence of an MBCS and systemic spread is less straightforward. None of the human pandemic or seasonal influenza viruses harbor an MBCS, and introduction of an MBCS in a human seasonal H3N2 influenza virus neither increased pathogenicity nor induced systemic spread in ferrets [220]. In contrast, removal of the MBCS from HA of an HPAI H5N1 virus resulted in a virus that caused respiratory tract infection without systemic spread in mice, indicating that the MBCS is a major virulence factor for mice [103]. Whether the MBCS in HPAI H5 is also a major determinant for systemic replication in other mammalian species remains unclear.

Differences in replication sites of human and avian influenza viruses in the mammalian respiratory tract correspond with differences in localization of virus attachment. In humans and ferrets, human influenza viruses attach abundantly to cells in the upper respiratory tract and along the tracheo-bronchial tree. In contrast, avian influenza viruses, including HPAI H5N1 virus, attach rarely to the upper respiratory tract, but attach abundantly to cells of the lower respiratory tract [90,147,206,221]. Interestingly, HPAI H5N1 virus does replicate in the ferret nose, although it does not attach to respiratory epithelial cells of the nose [214].

Extra-respiratory tract replication of HPAI H5N1 virus has been observed—although not consistently— in humans, ferrets, mice, cats, palm civets, tigers, leopard, domestic dog, American mink, fox and stone marten [66,205]. In ferrets, animals commonly used to study influenza virus infections, as they resemble humans in receptor

distribution and disease signs closely, systemic virus replication has been reported for HPAI H5N1 viruses [204,207] although this depends on the route of inoculation and the virus isolate used [222-224].
Several studies have shown that the central nervous system (CNS) is the most common extra-respiratory site of replication after experimental intranasal inoculation. The neurotropism of influenza viruses, including HPAI H5N1 viruses, has been studied in mice [225-230] and more recently in ferrets [222,223,231]. In mice, it was shown that influenza viruses could enter the CNS via the olfactory nerve and trigeminal nerve from the nasal cavity [226-230] but also via the vagal nerves and sympathetic nerves from the lungs [227]. In ferrets, HPAI H5N1 virus entered the CNS most likely via the olfactory nerve [222,231]. However, virus entry via the olfactory nerve, through infection of olfactory receptor neurons (ORN) of which the axons directly extent into the olfactory bulb, has never been proven in ferrets. In mice, virus replication of neurotropic influenza viruses in ORN has been described [228,232].
Here we studied the systemic spread of HPAI H5N1 virus in ferrets, with a special focus on the olfactory route. Additionally, we determined the role of the MBCS of HPAI H5N1 virus in this systemic spread. To this end, ferrets were inoculated with HPAI H5N1 virus either with or without the MBCS, and virus replication and associated lesions were determined in multiple tissues at different time points.

MATERIALS AND METHODS

Cells and viruses

Madin-Darby Canine kidney (MDCK) cells were cultured in EMEM (Lonza, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes and non-essential amino acids. 293T cells were cultured in DMEM (Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodiumpyruvate and non-essential amino acids.
Highly pathogenic avian influenza virus A/Indonesia/5/05 (H5N1) was isolated from a human case and passaged once in embryonated chicken eggs and once in MDCK cells. All eight gene segments were amplified by reverse transcription polymerase chain reaction (RT-PCR), cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 [171,208], and subsequently used to generate recombinant virus (H5N1WT) by reverse genetics as described elsewhere [171]. For the generation of H5N1 virus without an MBCS (H5N1ΔMBCS), the cleavage site PQRERRRKKR↓G in the H5 HA plasmid was changed to PQIETR↓G by RT-PCR with specific primers. Primer sequences are available upon request.

Replication kinetics

Multi step replication kinetics were determined by inoculating MDCK cells in the

presence and absence of 20 µg/ml trypsin (Lonza) with a multiplicity of infection (MOI) of 0.01 TCID50 per cell in two-fold. Supernatants were sampled at 6, 12, 24 and 48 hr after inoculation and virus titers in these supernatants were determined as described below.

Virus titrations

Virus titers in nasal- and throat swabs, homogenized tissue samples from inoculated ferrets, or samples from inoculated MDCK cells, were determined by end-point titration in MDCK cells in 96-well plates (Greiner Bio-One). MDCK cells were inoculated with ten-fold serial dilutions of each sample, washed one hour after inoculation with PBS, and cultured in 200 µl of infection medium, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes, non-essential amino acids and 20 µg/ml trypsin (Cambrex). 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 by the method of Spearman-Karber [233].

Western blotting

293T cells were transfected with the plasmids expressing H5N1WT or H5N1ΔMBCS HA gene segments using CaPO4 method. Cells were harvested 48 hours after transfection and were treated either with PBS or 2.5 µg/ml trypsin (Lonza) for 1 hour at 37 ºC. Cells were lysed in hot lysis buffer and treated with 3x dissociation loading buffer for 5 min at 96 ºC as previously described [209]. Cell lysates were subjected to electrophoresis in 10% SDS-polyacrylamide gels, blotted, and western blots were incubated with 1:2000 rabbit serum to A/Hong Kong/156/97 (H5) and a 1:3000 diluted peroxidase-labelled swine anti-rabbit antibody. Blots were developed with ECLTM Western Blotting Detection reagents.

Ferret experiments

All animal studies were approved by an independent Animal Ethics Committee. All experiments were performed under ABSL3+ conditions. The ferret model used to study the pathogenicity and virus distribution of H5N1WT and H5N1ΔMBCS viruses was described previously [150]. Groups of twelve influenza virus seronegative and Aleutian disease negative female ferrets (Mustela putorius furo) were inoculated intranasally with 10^6 $TCID_{50}$ of H5N1WT or H5N1ΔMBCS virus, divided over both nostrils (2 x 250µl). Throat and nose swabs were collected daily to determine virus excretion from the upper respiratory tract. Nose swabs were collected from only one nostril to keep the respiratory epithelium of the other nostril intact for pathology. Animals were weighed daily and observed for clinical signs as an indicator of disease. Three animals from each group were euthanized at 1, 3, 5 and 7 days post inoculation (dpi) and necropsies were performed according to a standard protocol. The trachea was clamped off so the lungs would not deflate upon open-

ing of the pleural cavity, which allowed visual estimation of the area of affected lung parenchyma. Nasal turbinates (NT) containing both respiratory and olfactory mucosa, trachea, lungs, liver, spleen, kidney, colon, cervical spinal cord, pancreas, heart, cerebellum, cerebrum, serum and cerebral spinal fluid (CSF) were collected to study virus distribution by virus titration. Half the head was fixed in 10% formalin and multiple part of the respiratory and olfactory epithelium from the nasal cavity and the olfactory bulb, cerebrum, cerebellum and trigeminal nerve were collected for pathological investigation. Other tissues collected for pathological investigation were cervical spinal cord, tonsil, trachea, left lung, heart, liver, spleen, pancreas, duodenum, jejunum, colon, kidney and adrenal gland.

Pathology & Immunohistochemistry

Samples for pathological examination were collected in 10% neutral-buffered formalin during necropsy (lungs after inflation with formalin) and fixed for 7 days. Tissues were embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin (HE) for the detection of histological lesions by light microscopy. For the detection of virus antigen by immunohistochemistry, tissues were stained with a monoclonal antibody against influenza A virus nucleoprotein as described previously [206].

Virus histochemistry

The attachment pattern of H5N1WT virus was determined in olfactory epithelium and respiratory epithelium from control ferrets (n=2) as described previously [147]. Briefly, reassortant virus consisting of six gene segments of influenza virus A/PR8/8/34 and the HA and PB2 of A/Indonesia/5/05 was propagated in MDCK cells and concentrated and purified using sucrose gradients. Concentrated virus was inactivated by dialysis against 0.1 % formalin, and labelled with fluorescein isothiocyanate. Paraffin-embedded tissues were incubated with FITC-labeled H5N1WT virus, and detected with a peroxidase-labeled rabbit anti-FITC antibody (DAKO, Heverlee, Belgium) and the signal was amplified with a tyramide signal amplification system (Perkin Elmer, Groningen, The Netherlands). Peroxidase was revealed with 3-amino-9-ethyl-carbozyle resulting in a red precipitate.

RESULTS

H5N1ΔMBCS trypsin dependent in vitro

The functionality of the HA protein after deletion of the MBCS was investigated by expression of the HA of H5N1wt and H5N1ΔMBCS viruses in 293T cells. The cleavage pattern was determined in the presence and absence of trypsin. Upon deletion of the MBCS, efficient cleavage of HA0 into HA1 and HA2 was observed only in the presence of trypsin whereas the H5N1WT HA was cleaved in the absence of trypsin (Fig. 1A). Next, the effect of the deletion of the MBCS on the replication kinetics was

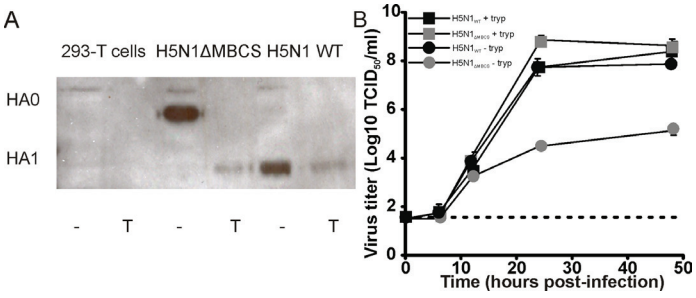

Figure 1. In vitro phenotype of H5N1wt and H5N1ΔMBCS viruses
(A) Western blots of lysates of 293T cells untransfected or transfected with HAs of H5N1ΔMBCS virus and H5N1WT virus upon treatment with (T) or without (–) trypsin. (B) Replication of H5N1WT virus (black) and H5N1ΔMBCS virus (grey) in MDCK cells in the presence (square) or absence (circle) of trypsin. Geometric mean titers were calculated from two independent experiments, error bars indicate standard deviations. The lower limit of detection is indicated by the dotted line.

determined in the absence and presence of trypsin (20 µg/ml). Via reverse genetics, H5N1wt and H5N1ΔMBCS viruses were rescued. H5N1wt replicated to similar virus titers in the presence and absence of trypsin. In contrast, H5N1ΔMBCS virus did not replicate efficiently in the absence of trypsin which is in agreement with an LPAI virus phenotype (Fig. 1B). H5N1ΔMBCS virus is able to replicate one round of infection. Hereafter the HA of progeny released H5N1ΔMBCS cannot be cleaved and therefore the virus is no longer infectious.

Clinical signs and gross lesions in ferrets inoculated with H5N1ΔMBCS virus were less severe compared to H5N1WT virus inoculated ferrets

To determine the pathogenicity of HPAI H5N1 virus in ferrets, including systemic spread, and to elucidate the role of the MBCS, 12 ferrets were inoculated intranasally with 10^6 $TCID_{50}$ of H5N1WT or H5N1ΔMBCS virus. Ferrets inoculated with H5N1WT virus developed anorexia and lethargy. In contrast, ferrets inoculated with H5N1ΔMBCS virus did not reveal any clinical signs. Ferrets were weighed daily as indicator of disease. The maximum weight loss was 20% (20 ± 1.6) for animals inoculated with H5N1WT virus at 7 dpi, the end of the experiment. Ferrets inoculated with H5N1ΔMBCS virus lost a maximum of 6% (6.0 ± 4.0) of their body weight at 3 dpi and there body weight returned to 100% at 7 dpi (Fig. 2A).

Gross pathological examination in ferrets inoculated with H5N1WT virus revealed multifocal dark red areas of consolidation in the lungs in 1 out of 3 (1/3), 2/3, 3/3 and 3/3 ferrets at 1, 3, 5 and 7 dpi respectively, with a maximum of affected lung tissue of 37% (37 ± 39) at 7 dpi. In ferrets inoculated with H5N1ΔMBCS virus, 2/3 at 1, 3 and 7 dpi and 1/3 at 5 dpi showed multifocal dark red areas of consolidation in the lungs with a maximum of affected lung tissue of 7% (7 ± 6) at 7 dpi (Table 1). The relative lung weight had a maximum at 5 dpi for ferrets inoculated with H5N1WT and H5N1ΔMBCS virus of 1.3 (1.3 ± 0.4) and 1.0 (1.0 ± 0.4), respectively (Table 1). Stomachs were empty in all ferrets inoculated with H5N1WT virus, with

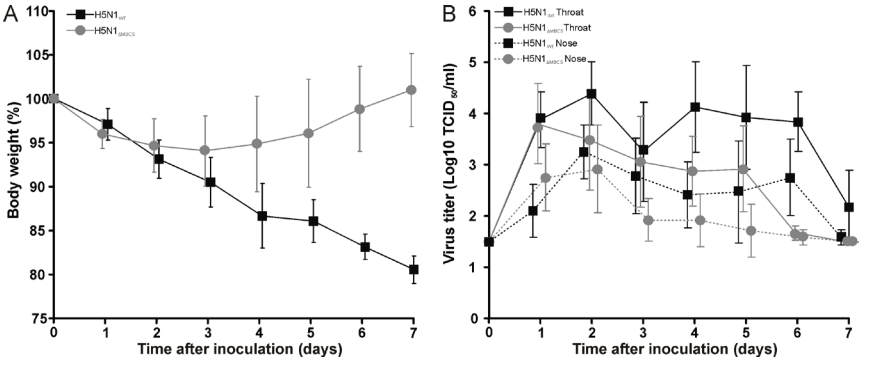

Figure 2. Weight loss and virus shedding in ferrets inoculated with H5N1WT or H5N1ΔMBCS viruses (A) Mean body weights and standard deviations are depicted as percentage of body weight at time of inoculation for each group inoculated with H5N1WT virus (black) and H5N1ΔMBCS virus (grey). (B) Virus shedding from the upper respiratory tract of ferrets inoculated with H5N1WT virus (black) and H5N1ΔMBCS virus (grey) from the nose (dashed line) and throat (solid line) are indicated. Geometric mean titers are shown, with error bars to indicate the standard deviation. The lower limit of detection is 1.5 10log $TCID_{50}$.

the exception of 2 ferrets at 1 dpi. Only two ferrets inoculated with H5N1ΔMBCS virus revealed empty stomachs at 1 and 3 dpi (Table 1). These results indicate that the H5N1ΔMBCS virus induced less severe disease compared to H5N1WT virus considering the body weight, clinical signs and gross lesions.

Virus isolation from swabs and organs differs between H5N1WT and H5N1ΔMBCS virus inoculated ferrets

Nose and throat swabs were collected daily, and virus titers were determined by end-point titration in MDCK cells. Nose swabs remained positive up to 7 dpi in H5N1wt virus inoculated ferrets and up to 6 dpi in H5N1ΔMBCS virus inoculated ferrets. (Fig. 2B). Virus shedding from the throat continued up to 7 or 6 dpi of H5N1WT or H5N1ΔMBCS virus inoculated ferrets, respectively (Fig. 2B).

Table 1. Gross pathology in ferrets inoculated with H5N1WT virus or H5N1ΔMBCS virus

Virus	dpi	Animals with macroscopic lung lesions (n/N)[a]	Area of lung affected (%)[b]	Relative lung weight (%)	Empty stomach (n/N)[c]
$H5N1_{WT}$	1	1/3	2 ± 3	0.7 ± 0.0	1/3
	3	2/3	3 ± 3	0.7 ± 0.0	3/3
	5	3/3	29 ± 23	1.3 ± 0.4	3/3
	7	3/3	37 ± 39	1.3 ± 0.7	3/3
$H5N1_{\Delta MBCS}$	1	2/3	3 ± 3	0.8 ± 0.1	1/3
	3	2/3	5 ± 5	0.8 ± 0.2	1/3
	5	1/3	2 ± 3	1.0 ± 0.4	0/3
	7	2/3	7 ± 6	0.8 ± 0.1	0/3

[a] No. of ferrets in which macroscopic lung lesions were detected/total no. of ferrets is depicted
[b] Area of lung affected and relative lung weight are reported as means ± SD (n=3)
[c] No. of ferrets with empty stomachs/total no. of ferrets is depicted

Shedding from the respiratory tract of H5N1ΔMBCS virus inoculated ferrets appears to be lower than in H5N1wt virus inoculated ferrets, indicative for less efficient virus replication.

At 1, 3, 5 and 7 dpi, three ferrets from each group were euthanized and NT, trachea, lungs, liver, spleen, kidney, colon, spinal cord, pancreas, heart, cerebellum, cerebrum, serum and CSF were collected and virus titers were determined. The virus titers in samples from lung and trachea were similar at 1, 3 and 5 dpi between H5N1WT and H5N1ΔMBCS virus inoculated ferrets. However, in NT the virus titers were 3.0 10log TCID50 lower in H5N1ΔMBCS virus inoculated ferrets than in H5N1WT inoculated ferrets at 1, 3, and 5 dpi, respectively. At 7 dpi, virus was undetectable in all samples from H5N1ΔMBCS virus inoculated ferrets, indicating that the animals cleared the virus by this time point (Table 2). Infectious virus was isolated from extra-respiratory tissues of H5N1WT virus inoculated ferrets from 3 dpi onwards.

Table 2. Virus titers in tissues and CSF of ferrets inoculated with H5N1WT virus or H5N1ΔMBCS virus

	Virus titer(s) (log 10TCID$_{50}$/gram tissue)							
	H5N1$_{WT}$				H5N1$_{\Delta MBCS}$			
Tissue	**day 1**	**day 3**	**day 5**	**day 7**	**day 1**	**day 3**	**day 5**	**day 7**
Respiratory system								
Nasal Turbinates	6.0±0.7 (3/3)[a]	7.2±0.9 (3/3)	6.6±0.8 (3/3)	6.2±0.2 (3/3)	3.0±0.0 (2/3)	4.2±0.6 (2/3)	3.6±0.3 (3/3)	<1.2[b]
Trachea	2.9±0.7 (3/3)	3.2 (1/2)[c]	3.4±0.2 (2/3)	3.9±0.6 (2/3)	3.0±0.3 (3/3)	1.9 (1/3)	3.9±2.8 (3/3)	<1.7
Lung	2.0±0.7 (2/3)	2.9 (1/3)	5.3±1.7 (3/3)	4.2±2.3 (2/3)	2.9±0.2 (3/3)	1.8 (1/3)	3.9±2.2 (2/3)	<1.3
Nervous system								
Cerebrum	<1.5	<1.5	<1.5	2.1±0.2 (3/3)	<1.5	<1.5	<1.5	<1.5
Cerebellum	<1.7	<1.7	<1.7	3.7±0.0 (2/3)	<1.7	<1.7	<1.7	<1.7
Spinal Cord	<1.7	<1.7	<1.7	2.1 (1/3)	<1.7	<1.7	<1.7	<1.7
Cerebrospinal fluid[d]	<1.5	<1.5	<1.5	3.8±0.7 (2/3)	<1.5	<1.5	<1.5	<1.5
Other								
Pancreas	<1.5	<1.5	3.0 (1/3)	2.9±0.5 (2/3)	<1.5	<1.5	<1.5	<1.5
Liver	<1.1	<1.1	2.8±0.9 (3/3)	4.4±1.1 (3/3)	<1.1	<1.1	<1.1	<1.1
Colon	<1.4	2.1 (1/3)	<1.4	1.8±0.1 (2/3)	<1.4	<1.4	<1.4	<1.4
Heart	<1.5	<1.5	2.1±0.3 (2/3)	2.3 (1/3)	<1.5	<1.5	<1.5	<1.5

[a] Virus titers are reported as means ± SD (n=3) or as individual titers if viruses were not isolated from all three animals. No. of ferrets in which virus positive organs were detected/total no. of ferrets is depicted

[b] Cut off values are given for negative tissues

[c] Only two trachea samples were collected

[d] Virus titers of cerebrospinal fluid are in log10TCID50/ml

At 3 dpi, infectious virus was found in one colon sample. At 5 dpi infectious virus was found in the liver (3/3), pancreas (1/3) and heart (2/3). At 7 dpi, extra-respiratory infectious virus was detected in the liver (3/3), pancreas (2/3), colon (2/3), heart (1/3), spinal cord (1/3), cerebellum (2/3) and cerebrum (3/3). In contrast, H5N1ΔMBCS virus inoculated ferrets did not reveal any extra-respiratory infectious virus. Infectious virus was not detected in spleen and kidney at any of the time points in H5N1WT virus inoculated ferrets. Interestingly, at 7 dpi we detected infectious virus in the CSF. No infectious virus could be isolated from the serum of H5N1WT virus inoculated ferrets (Table 2). Nevertheless, low levels of viral genomic RNA were detected in serum by quantitative RT-PCR from 1/3, 2/3, 1/3 and 3/3 ferrets at 1, 3, 5 and 7 dpi respectively (data not shown). No infectious virus was isolated from extra-respiratory tissues, serum and CSF of H5N1ΔMBCS virus inoculated ferrets.

Table 3. Detection of influenza A virus nucleoprotein in tissues from ferrets inoculated either with H5N1WT virus or H5N1ΔMBCS virus

	$H5N1_{WT}$				$H5N1_{\Delta MBCS}$			
Tissue	**day 1**	**day 3**	**day 5**	**day 7**	**day 1**	**day 3**	**day 5**	**day 7**
Respiratory system								
Respiratory epithelium	+/- (3/3) [a]	+/- (3/3)[b]	+/- (3/3)[b]	-[c]	+/- (2/3)	+/- (1/3)[b]	-	-
Olfactory epithelium	+/- (3/3)	+ (3/3)[b,d]	+ (3/3)[b]	+ (3/3)[b]	+/- (2/3)	+ (3/3)[b]	+/- (2/3)[b]	+ (2/3)
Trachea	-	-	-	+/- (1/3)	-	-	+/- (2/3)[b]	-
Bronchus	-	-	-	+/- (1/3)[b]	-	-	-	-
Bronchioles	-	-	+(1/3)[b]	+ (1/3)[b]	-	-	+/- (1/3)[b]	-
Alveoli	-	+ (1/3)[b]	+ (3/3)[b]	-	-	+ (1/3)[b]	+ (1/3)[b]	-
Nervous system								
Olfactory bulb	-	+ (1/3)[b]	+ (2/3)[b]	+ (3/3)[b]	-	-	-	-
Cerebrum	-	-	+ (1/3)[b]	+ (2/3)[b]	-	-	-	-
Cerebellum	-	-	-	+ (1/3)[b]	-	-	-	-
Spinal cord	-	-	-	+ (1/3)[b]	-	-	-	-
Trigeminal nerve	-	-	+ (1/3)[b]	-	-	-	-	-
Other								
Pancreas	-	-	-	+ (1/3)[b]	-	-	-	-
Liver	-	-	-	+ (2/3)[b]	-	-	-	-
Tonsils	-	+ (1/1)[b,e]	+ (1/3)[b]	-	-	-	-	-

[a] +/- < 10 cells contained virus antigen
[b] The detection of virus antigen was associated with histological lesions
[c] - Virus antigen was not detected
[d] + >10 cells contained virus antigen
[e] Only one tonsil was sampled

Distibution of virus antigen is more widespread in H5N1WT virus inoculated ferrets compared to H5N1ΔMBCS virus inoculated ferrets

The distribution of virus antigen was determined in cerebrum, cerebellum, trigeminal nerve, NT, spinal cord, tonsil, trachea, left lung, heart, liver, spleen, pancreas, duodenum, jejunum, colon, kidney and adrenal gland from ferrets inoculated with H5N1WT or H5N1ΔMBCS virus on 1, 3, 5 and 7 dpi (Table 3).

In ferrets inoculated with H5N1WT virus, virus antigen was detected in the respiratory tract from 1 dpi onward. In the NT, virus antigen was observed in only a few respiratory epithelial cells at 1, 3 and 5 dpi. Virus antigen was present in a few olfactory receptor neurons at 1 dpi, but abundantly present at 3, 5 and 7 dpi, with a peak at 3 dpi (Fig. 3). Interestingly, virus antigen was also observed in the Bowman's glands and the olfactory ensheathing cells in the lamina propria of the olfactory mucosa (Fig. 4). In the lower respiratory tract, virus antigen was present in 1/3 ferrets at 3 dpi, 3/3 at 5 dpi and 1/3 at 7 dpi.

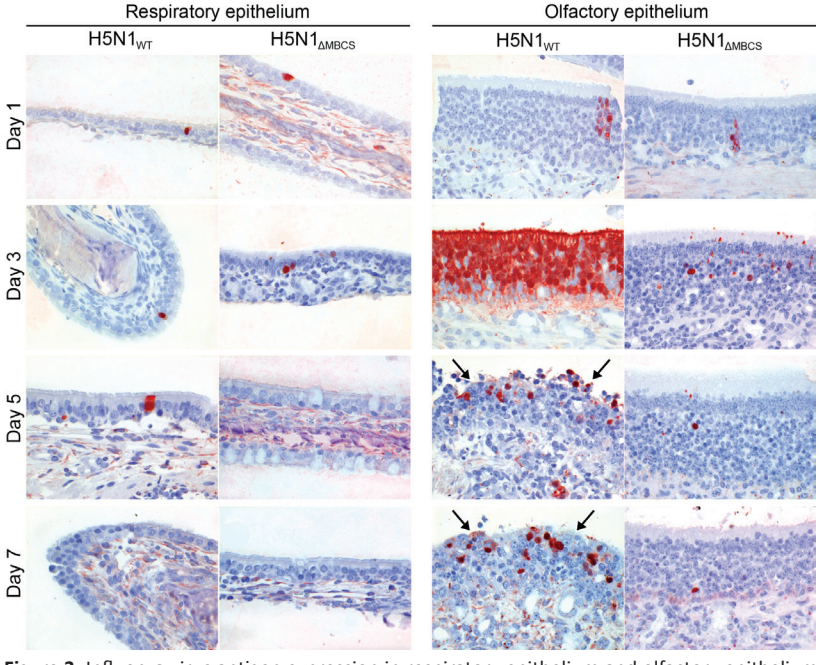

Figure 3. Influenza virus antigen expression in respiratory epithelium and olfactory epithelium of ferrets inoculated either with H5N1WT virus or H5N1ΔMBCS virus

Influenza virus antigen in the respiratory epithelium (left two columns) and olfactory epithelium (right two columns) in ferrets inoculated with H5N1WT or H5N1ΔMBCS virus at 1, 3, 5 and 7 dpi (original magnification 400x). Tissue sections were stained with a monoclonal antibody against influenza A virus nucleoprotein, visible as red staining. In the respiratory epithelium, individual virus antigen positive epithelial cells are observed 1, 3 and 5 dpi in H5N1WT virus inoculated ferrets and at 1 and 3 dpi in H5N1ΔMBCS virus inoculated ferrets. In the olfactory epithelium, virus antigen in H5N1WT inoculated ferrets was present from 1 till 7 dpi, with a peak at 3 dpi. At day 5 and 7 dpi there was focal severe necrosis of olfactory epithelial cells, with infiltration of neutrophils (arrows). In the H5N1ΔMBCS virus inoculated ferrets, virus antigen was present from day 1 till 7.

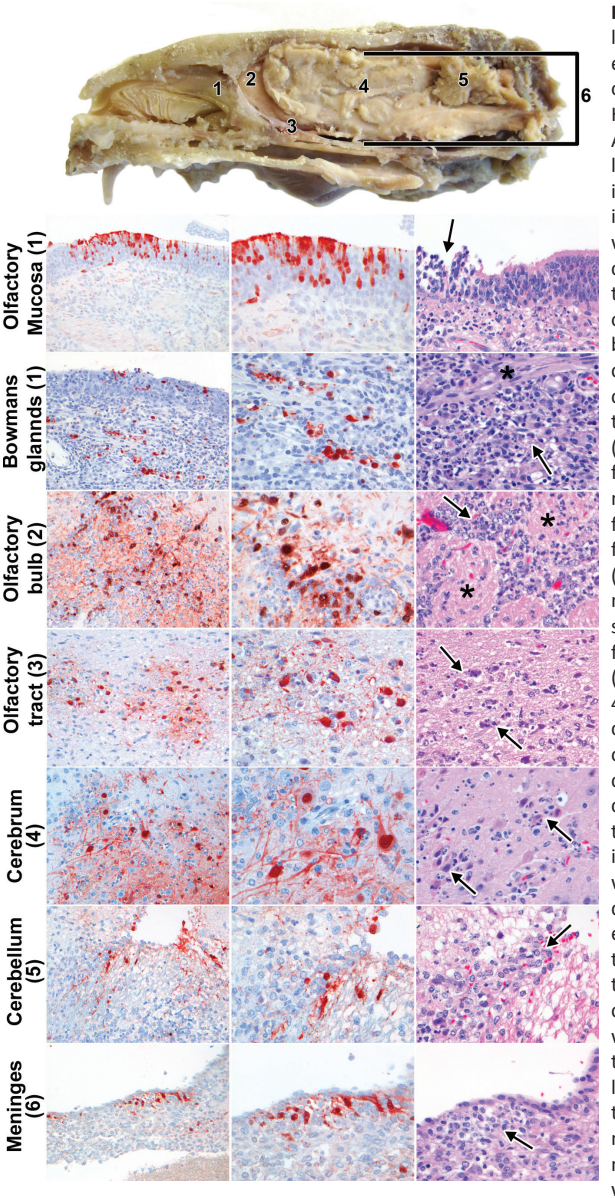

Figure 4. Detection of histological lesions or influenza A virus antigen expression in tissues from the nervous system of ferrets inoculated with H5N1WT virus

A cross-section of a ferret head to illustrate the different anatomical sites is shown at the top. The nasal cavity, containing the nasal turbinates, which are lined by respiratory mucosa, except at the back and top of the cavity where they are lined by olfactory mucosa (1). The olfactory bulb (2) is separated from the nasal cavity by the cribriform plate, and connected via the olfactory tract (3) to the cerebrum (4). The cerebellum (5), cerebrum, olfactory bulb and olfactory tract are all surrounded by the meninges (6). Immunohistochemistry for influenza A virus antigen in different tissues of the olfactory route (left and middle columns, original magnification of 200x and 400x respectively). Histological lesions in different tissues of the olfactory route (right column, original magnification 400x). Olfactory epithelium (1) which contained virus antigen from 1 dpi in olfactory receptor neurons, with focally necrosis of olfactory epithelial cells associated with infiltrating neutrophils (arrow); Olfactory ensheathing cells and Bowman's glands (1), which contained virus antigen from 3 dpi, with necrosis of Bowman's glands epithelial cells associated with infiltrating neutrophils along the nerve twigs (*); Olfactory bulb (2), which contained virus antigen from 3 dpi, with infiltrating neutrophils between the glomeruli (*) in the glomerular layer; Olfactory tract (3), which contained virus antigen from 7 dpi, with neuronal necrosis and infiltrating neutrophils (arrow); Cerebrum (4), which contained virus antigen from 5 dpi, with neuronal necrosis and infiltrating neutrophils (arrow); Cerebellum (5) which contained virus antigen from 7 dpi, with neuronal necrosis and infiltrating neutrophils; and meninges (6), which contained virus antigen from 7dpi, with infiltration of many mononuclear cells and occasional neutrophils (arrow). Tissue sections were stained with a monoclonal antibody against influenza A virus nucleoprotein, visible as red staining.

Virus antigen was most commonly found in alveolar epithelial cells and in 2/3 ferrets (5 and 7 dpi) in epithelial cells of the bronchioles, bronchus and trachea (Table 3).

In the nervous system of H5N1WT virus inoculated animals, virus antigen was found in 0/3, 1/3, 3/3 and 3/3 ferrets at 1, 3, 5 and 7 dpi, respectively (Table 3-4). At 3 dpi, virus antigen was first observed in periglomerular cells in the glomerular layer of the olfactory bulb where the axons of the olfactory receptor neurons have contact with both second order neurons and periglomerular cells in the olfactory bulb. At 5 dpi, virus antigen was observed in the periglomerular cells of the olfactory bulb, in glial cells in the cerebrum and in one neuron in the trigeminal nerve. At 7 dpi, virus antigen was found in mitral cells and granular cells in the olfactory bulb, in glial cells and neuronal cells in the olfactory tract, in glial cell and neuronal cells in the cerebrum and cerebellum, in meningeal cells surrounding the cerebrum, cerebellum and spinal cord, and in ependymal cells in choroid plexus around the cerebellum (Fig. 4).

Beyond the respiratory tract and CNS, virus antigen was observed in lymphoid cells in the tonsils at 3 and 5 dpi, in hepatocytes in the liver at 7 dpi, and in acinar cells in the pancreas at 7 dpi (Table 3, Fig. 5). No virus antigen was detected in tissues of the heart, adrenal gland, kidney, spleen, duodenum, jejunum or colon.

In ferrets inoculated with H5N1ΔMBCS virus, virus antigen expression was restricted to the respiratory tract (Table 3). Virus antigen was only found in a few respiratory epithelial cells at 1 and 3 dpi. In the olfactory epithelium, virus antigen was observed in 2/3, 3/3, 2/3 and 2/3 ferrets at 1, 3, 5 and 7 dpi (Fig. 3). Overall, the number of olfactory receptor neurons that contained virus antigen was lower in the H5N1ΔMBCS inoculated ferrets than in the H5N1WT virus inoculated ferrets. There was no evidence of virus antigen in the Bowman's glands and olfactory ensheating cells in the H5N1ΔMBCS virus inoculated ferrets.

Table 4. Presence of histological lesions and the presence of virus antigen in tissues that belong to the nervous system in ferrets inoculated with HPAI H5N1WT virus

	No. with histological lesions/No. with virus antigen expression at each day			
Nervous tissue	**day 1 (n=3)**	**day 3 (n=3)**	**day 5 (n=3)**	**day 7 (n=3)**
Olfactory epithelium	0/3	3/3	3/3	3/3
Olfactory bulb	0/0	0/1	1/2	3/3
Cerebrum	0/0	0/0	0/1	3/2
Cerebellum	0/0	0/0	0/0	2/1
Spinal cord	0/0	0/0	0/0	1/1
Leptomeninges	0/0	0/0	1/0	3/1
Choroid plexus	0/0	0/0	0/3	0/1

Histological lesions in ferrets inoculated with H5N1WT virus were more severe compared to H5N1ΔMBCS virus inoculated ferrets

Association of histological lesions and influenza virus infection was based on co-localization of lesions and influenza virus antigen. Influenza virus-associated lesions in H5N1WT virus inoculated ferrets predominated in nasal cavity, central nervous system, and lower respiratory tract, and were less frequent in liver, pancreas, and tonsils (Table 3). Influenza virus-associated lesions in H5N1ΔMBCS virus inoculated ferrets were restricted to the nasal cavity and lower respiratory tract.

Lesions in both the olfactory and respiratory epithelium of the nasal cavity occurred commonly in H5N1WT virus inoculated ferrets (Table 3), but the lesions were much more extensive and severe in the olfactory mucosa than in the respiratory mucosa. Olfactory mucosa lesions consisted of a moderate or severe, multifocal necrotizing rhinitis, characterized at 3 dpi by necrosis of olfactory epithelial cells and of epithelial cells of the underlying Bowman's glands, and infiltration by moderate numbers of partly degenerate neutrophils. At 5 dpi, lesions progressed to multiple erosions of the olfactory mucosa adjacent to reactive hypertrophy and hyperplasia of remaining olfactory epithelial cells, and disruption of the histological architecture of the Bowman's glands. The lesions at 7 dpi were similar to those at 5 dpi with the addition of hypertrophy and hyperplasia of remaining epithelial cells in the Bowman's glands. In comparison, respiratory mucosa lesions consisted of a mild, focal rhinitis, characterized by infiltration of a few neutrophils in the respiratory epithelium and underlying connective tissue. Lesions in the olfactory and respiratory epithelium were less common in H5N1ΔMBCS virus inoculated ferrets (Table 3) and milder in character.

Lesions in the central nervous system of H5N1WT virus inoculated ferrets first occurred in the olfactory bulb of single ferrets at 3 and 5 dpi, and subsequently were widespread throughout the central nervous system of all ferrets at 7 dpi (Table 4, Fig. 4). Olfactory bulb lesions consisted of a focal infiltration with rare neutrophils in the glomerular layer at 3 dpi. At 5 dpi, olfactory bulb lesions had extended to multifocal infiltration with moderate numbers of neutrophils in the glomerular and granular layers, as well as perivascular cuffing with mononuclear cells and neutrophils. At 7 dpi, lesions were similar to those at 5 dpi, except that they were restricted to the granular layer and perivascular cuffing consisted predominantly of mononuclear cells. At 7 dpi, lesions also were present in the olfactory tract, cerebrum, cerebellum, and spinal cord. In general, these lesions consisted of multiple foci of neuronal necrosis, infiltration by moderate numbers of partly degenerate neutrophils, and perivascular cuffing with mononuclear cells. The leptomeninges overlying these tissues were infiltrated by many mononuclear cells and occasional neutrophils. In addition to cerebellar lesions, two ferrets also had necrosis and sloughing of adjacent ependymal cells and edema of the underlying neuropil.

Lesions in the lower respiratory tract were present in five H5N1WT virus–inoculated ferrets (Table 3) and were mild and focal except for those in one ferret at 5 dpi, which were moderate and locally extensive. In general, lesions were centred on al-

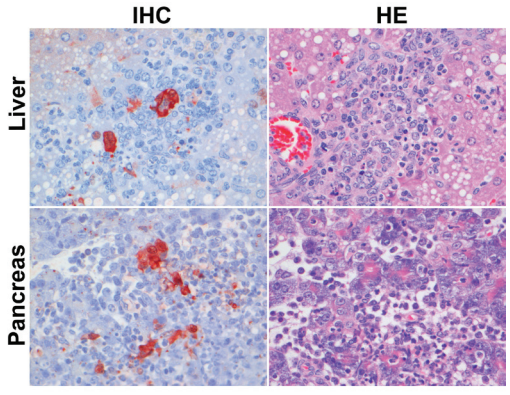

Figure 5. Detection of histological lesions and influenza A virus antigen expression in extra-respiratory tract tissues in ferrets inoculated with H5N1WT virus
In the liver, virus antigen was observed in hepatocytes, with multifocal necrosis of hepatocytes and replacement by variable numbers of neutrophils and mononuclear cells. In the pancreas, virus antigen was observed in acinar cells, with necrosis of acinar cells, widespread edema and infiltration of neutrophils and mononuclear cells.

veoli and bronchioles, with milder and less frequent lesions in bronchi and trachea. At 3 dpi, the lesions were characterized by necrosis and loss of alveolar and bronchiolar epithelial cells, infiltration of neutrophils in alveolar and bronchiolar walls, and presence of neutrophils mixed with fibrin in alveolar and bronchiolar lumina. At 5 and 7 dpi, mononuclear cells progressively predominated over neutrophils in the inflammatory cell infiltrate, and there was hypertrophy and hyperplasia of epithelial cells in both alveoli and bronchioles. Lesions in the lower respiratory tract of ferrets inoculated with H5N1ΔMBCS virus were present in single ferrets at 3, 5, and 7 dpi. These lesions were focal and comparable in appearance to those in ferrets inoculated with H5N1WT virus.

Lesions outside the nervous and respiratory systems were restricted to the liver, pancreas, and tonsils of ferrets inoculated with H5N1WT virus. In the liver, lesions were present in two ferrets at 7 dpi (Table 3, Fig. 5). These lesions consisted of multifocal necrosis of hepatocytes and replacement by variable proportions of neutrophils and mononuclear cells. Additionally, there was bile duct hyperplasia.

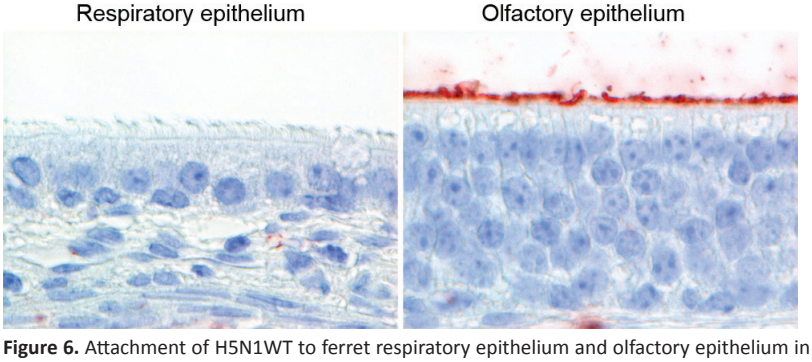

Figure 6. Attachment of H5N1WT to ferret respiratory epithelium and olfactory epithelium in the nose
No attachment of H5N1WT virus to the apical side of respiratory epithelium (left) and abundant attachment of H5N1WT virus to the apical side of the olfactory epithelium (right) (original magnification 1000x). Tissue sections were incubated with H5N1WT virus, attachment is visible as red staining

In the pancreas of one ferret at 7 dpi, there was multifocal necrosis of pancreatic acinar cells, with widespread edema and infiltration of neutrophils and mononuclear cells. In the tonsils of single ferrets at 3 and 5 dpi, there was focal and mild (3 dpi) or multifocal and moderate (5 dpi) lymphocytic necrosis in the lymphoid tissue, with infiltration of neutrophils.

H5N1WT virus attaches abundantly to olfactory epithelium

To explain the differences in number of cells infected between the respiratory epithelium and olfactory epithelium in ferrets inoculated with H5N1WT virus, we determined the attachment pattern of H5N1WT virus on these tissues from uninfected ferrets in the nose. There was no attachment of H5N1WT virus to respiratory epithelium, which is in accordance with previous observations [90]. However, H5N1WT virus was able to attach abundantly to the apical side of the olfactory epithelium (Fig. 6). H5N1ΔMBCS virus reveals the same attachment pattern, since the receptor preference of the virus has not been changed as was shown by Chutinimitkul et al. [90].

DISCUSSION

This study improves our knowledge of systemic spread of HPAI H5N1 virus in a mammalian model in two respects. First, we show that the olfactory epithelium forms an island of susceptible cells in a sea of relatively resistant respiratory epithelium. From this island, HPAI H5N1 virus spreads directly to the CNS via the olfactory route. Second, we show that the MBCS is necessary for systemic spread of HPAI H5N1 virus. In its absence, the virus is restricted to the respiratory tract.

Intranasal inoculation of HPAI H5N1WT virus resulted in respiratory tract infection, characterized by extensive replication and lesions in the olfactory epithelium. The abundant attachment and replication of HPAI H5N1WT virus in cells of the olfactory epithelium explains the origin of the high virus titers found in homogenized NT. Virus antigen in the olfactory epithelium peaks at 3 dpi (Fig. 3) which corresponded with a peak in infectious virus titers in the NT at 3 dpi (Table 2) and nose swabs at 2 dpi (Fig. 2). The lack of abundant virus replication in the respiratory epithelium is consistent with the rare attachment of H5N1 virus (Fig. 6). Furthermore, low replication in the respiratory epithelium explains the relatively low virus titers found in the nasal swabs, compared to titers found after inoculation with a seasonal H3N2 virus [220]. It is therefore tempting to speculate that a switch from replication in the olfactory epithelium to the respiratory epithelium would result in higher virus titers in nasal swabs and possibly efficient respiratory droplet transmission [234]. Interestingly, intranasal inoculation of H5N1 virus did not result in a consistent severe inflammation of the lower respiratory tract which is in accordance with a previous study by Bodewes et al [222].

There was evidence for two routes of extra respiratory spread in the HPAI H5N1wt

virus inoculated ferrets; first via the olfactory route to the CNS and secondly via the hematogenous route. First, HPAI H5N1 virus is able to directly infect and replicate extensively in ORN in the olfactory epithelium, from which it spread to the olfactory bulb and further into the CNS. The ORN, which were influenza virus antigen positive from 1 dpi onwards, have axons that extent through the cribriform plate into the glomerular layer of the olfactory bulb. These axons synaps with periglomerular cells and neurons in the olfactory bulb, which contained virus antigen from 3 dpi onwards. Since it has been shown for HPAI viruses that trans-axonal transport is possible [235,236], infection of ORN provides a direct route for H5N1 virus into the olfactory bulb. From 5 dpi onwards, virus spread along the olfactory tract into the cerebrum and cerebellum was observed. Infectious virus was detected in CSF at 7 dpi together with virus antigen in the meninges, choroid plexus and epedymal cells, providing an additional route of virus spread throughout the CNS from 7 dpi onwards as described before [222]. The observations that HPAI H5N1WT virus spreads to the CNS without severe respiratory disease, fits with the case report of two children with acute encephalitis, without initial respiratory disease. From one of these children, HPAI H5N1 virus was detected in the CSF retrospectively [237]. It remains to be determined if virus entry via the olfactory route is a unique feature of A/Indonesia/5/05 HPAI H5N1 virus, or that other HPAI H5N1 strains and possibly seasonal influenza viruses are also able to enter the CNS via the olfactory route in the ferret model [223].

Besides the olfactory route, there was also evidence for HPAI H5N1WT virus spread via the hematogenous route. The pattern of virus distribution in colon, pancreas, heart and liver is indicative for blood-borne spread which correlates with the detection of viral genomic RNA in serum. Hematogenous spread into the CNS appears to be less likely considering the observed virus distribution along the olfactory route. This is also in agreement with previous studies that also suggest the olfactory tract as a route of entry into the CNS [222,231].

The extra-respiratory spread observed in the HPAI H5N1WT virus inoculated ferrets was dependent on the MBCS, since replication was restricted to the respiratory tract in H5N1ΔMBCS inoculated ferrets. In addition, clinical disease was also dependent on the MBCS since ferrets inoculated with H5N1ΔMBCS revealed less symptoms than H5N1WT inoculated ferrets. The role of the MBCS in H5N1 virus in the extra-respiratory spread in ferrets corresponds with observations in chickens and mice [97,103]. However, the mechanism by which HPAI H5N1 virus spreads beyond the respiratory tract varies largely between chickens and mammals. In chickens, the acquisition of an MBCS results in an endothelial cell tropism, which facilitates systemic replication in endothelial cells and subsequent replication in extra-respiratory organs [238]. In contrast, in mammals—and some bird species—the presence of an MBCS in H5N1 virus results in systemic replication of H5N1 virus, without extensive replication in endothelial cells [239,240]. Interestingly, the presence of an MBCS in a seasonal H3N2 virus did not result in systemic dissemination [220]. This lack of systemic dissemination might be due in part to a difference in cell tropism in

the respiratory tract. Seasonal H3N2 virus and HPAI H5N1 virus recognize different sialic acids and are known to target different cell types in both the upper- and lower respiratory tract [147,221].
Replication of H5N1ΔMBCS virus —which was trypsin dependent in vitro— was strongly reduced in the nose (Table 2) both in virus titers and in numbers of influenza virus positive cells in the olfactory mucosa compared to H5N1WT virus (Fig. 3). However this difference was not observed as clearly in the virus titers from the nose swabs, probably due to the overall low viral shedding from the nose. However, virus replication in the lower respiratory tract was not reduced early after inoculation compared with H5N1WT inoculated ferrets. Since the HA of H5N1ΔMBCS virus can not be cleaved by intracellular furin-like proteases, activation of the HA of this virus is dependent on cell membrane and soluble proteases. This could indicate that expression of these proteases varies between the different anatomical sites and cell types in the respiratory tract of ferrets. In rats, the composition of proteases and inhibitory compounds varies largely between different anatomical sites in the respiratory tract [241]. In humans, mucus on top of the human olfactory epithelium contains protease inhibitors [242]. Further studies are therefore required into the protease and protease inhibitor composition and there role in cell tropism and pathogenicity of influenza viruses in mammals. Ferrets inoculated with H5N1ΔMBCS revealed no systemic replication. Lack of virus spread along the olfactory tract might be the result of low virus titers in the upper respiratory tract and/or the absence of the appropriate proteases outside the respiratory tract in ferrets. The low virus titers in the nose might not be sufficient for a spill over to the olfactory bulb and CNS. However, it is not known if the appropriate proteases are present along the olfactory route in ferrets. Studies in the rat CNS have shown that trypsin I, which was able to cleave the HA of multiple influenza viruses, is present but not equally distributed throughout cells of- and locations in the CNS [241].
In conclusion, HPAI H5N1WT virus is able to directly infect and replicate extensively in olfactory receptor neurons in the olfactory epithelium, from which it spreads to the olfactory bulb and further to the CNS. Interestingly, this entrance via the olfactory route was dependent on the presence of the MBCS in influenza virus A/Indonesia/5/05. Future studies should reveal whether this CNS invasion is a unique feature of HPAI H5N1 virus, or that more influenza viruses are able to enter the CNS via the olfactory route.

ACKNOWLEDGEMENTS

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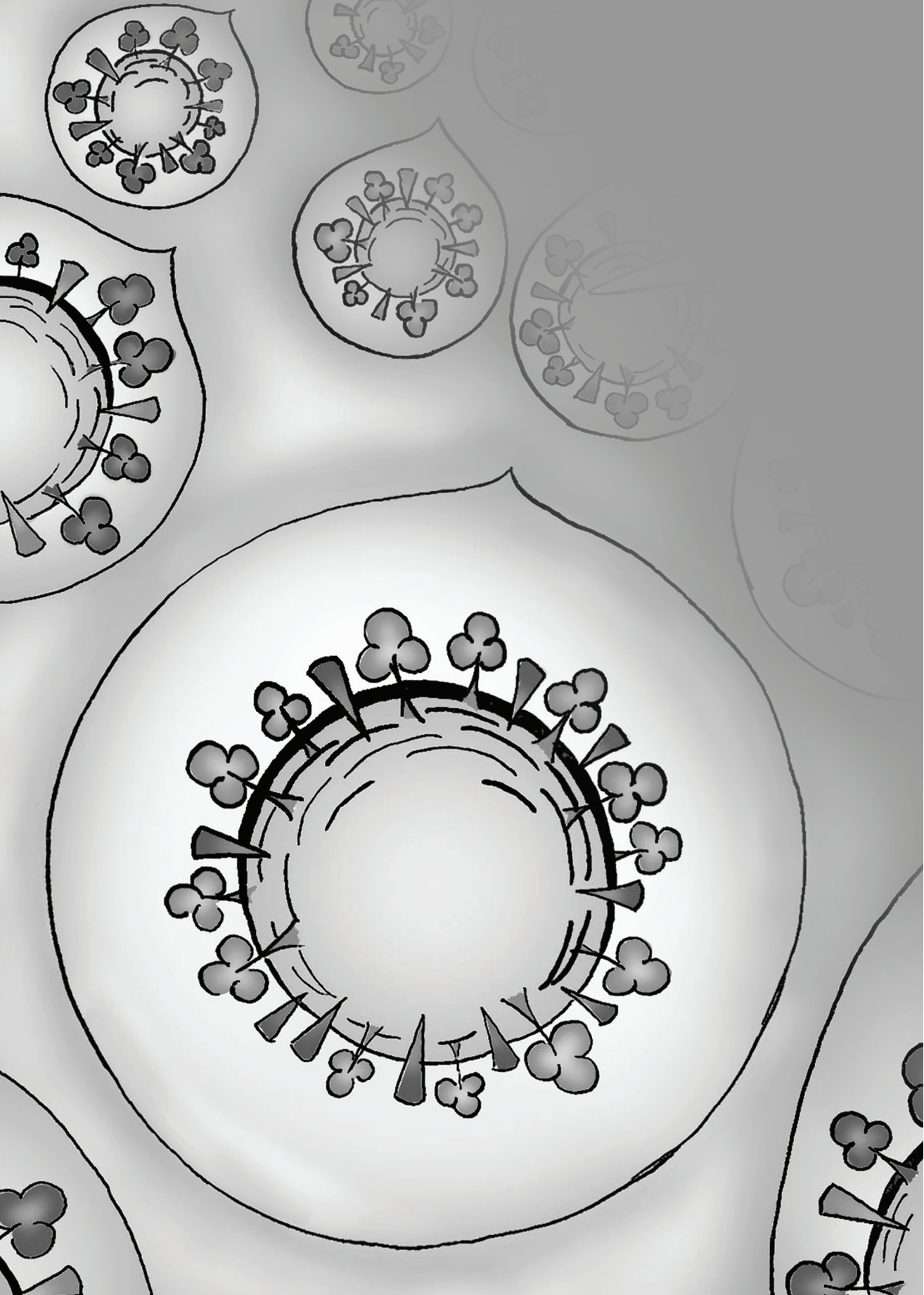

CHAPTER 3
Genome reassortment of influenza A viruses

3.1
Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment

Eefje J.A. Schrauwen, Sander Herfst, Salin Chutinimitkul, Theo M. Bestebroer, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus, Thijs Kuiken and Ron A.M. Fouchier

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ABSTRACT

Since the emergence of the 2009 pandemic H1N1 influenza A virus in April 2009, three influenza A viruses – seasonal H3N2, seasonal H1N1, and pandemic H1N1 - circulate in humans. Genetic reassortment between these viruses could result in enhanced pathogenicity of the virus. Here, four reassortant viruses with favorable in vitro replication property were compared with the wild type pandemic virus with respect to replication kinetics in vitro and pathogenicity and transmission in ferrets. Pandemic H1N1 viruses that contained either PA and PB2 or PB2 alone of seasonal H1N1 virus were attenuated in ferrets. In contrast, pandemic virus with NA of seasonal H3N2 virus resulted in increased virus replication and more severe pulmonary lesions in ferrets. These data demonstrate that the 2009 pandemic H1N1 virus has the potential to reassort with seasonal influenza viruses, possibly resulting in increased pathogenicity while maintaining the capacity of transmission via aerosols or respiratory droplets.

INTRODUCTION

The 2009 H1N1 virus that caused the first influenza pandemic of the 21st century (pH1N1), continues to be detected in a large number of countries around the world [48,243]. The 2009 pandemic has been relatively mild, with disease ranging from sub-clinical infections to sporadic cases of severe pneumonia and acute respiratory distress syndrome [244-249]. The pH1N1 virus is a unique reassortant virus that contains the neuraminidase (NA) and matrix (M) genes from the Eurasian swine influenza virus lineage, while the other six gene segments are derived from the North American triple reassortant swine influenza virus lineage [50]. From the start of the pandemic, there have been concerns that the pH1N1 virus may mutate, or reassort with contemporary influenza viruses to give rise to more pathogenic viruses.

Co-circulation of multiple strains of influenza A virus in humans provides an opportunity for viral genetic reassortment: the mixing of genes from 2 (or more) viruses [250]. Genetic reassortment of the pH1N1 virus with seasonal H3N2 (H3N2) or seasonal H1N1 (sH1N1) viruses might thus represent a route to enhanced pathogenicity of the pH1N1 virus. To date, no reassortment events between pH1N1 and seasonal viruses have been reported in humans. However, a triple-reassortant swine influenza A(H1N1) virus, distinct from the 2009 pandemic virus, containing the HA and NA genes of sH1N1 influenza virus was described recently [251]. Dual infections by sH1N1 and H3N2 viruses have been reported [22] as well as mixed infections of pH1N1 and H3N2 viruses [23,252], thus highlighting the potential for reassortment of currently circulating influenza viruses in humans. H1N2 reassortant influenza viruses with the HA of seasonal H1N1 and the NA of seasonal H3N2 viruses have been isolated from humans during previous influenza seasons, thereby confirming that such HA/NA combination can emerge in humans [25,253], and H1N2 influenza viruses have further been detected frequently in pigs [85].

To investigate the potential of reassortment between seasonal and pandemic influenza viruses further, here an in vitro selection method for reassortants was employed, using reverse genetics and serial passaging under limiting dilution conditions. Four reassortants with different gene constellations were identified. These reassortants were composed of six or seven gene segments of the pH1N1 virus with the PB2 or PB2 and PA gene segments of the sH1N1 virus, or the NA or NA and PB1 gene segments of H3N2 virus. Pathogenicity and transmission of these viruses were tested using a ferret model. Viruses containing the sH1N1 PB2 or PB2 and PA genes were attenuated in ferrets, whereas viruses containing the NA gene of the H3N2 virus displayed increased pathogenicity in vivo. All reassortants were transmitted between ferrets via aerosol or respiratory droplets. These results demonstrated that some reassortants between pH1N1 and H3N2 were viable, remained transmissible and were more pathogenic than the wild type pH1N1 virus and emphasize the importance of monitoring reassortant viruses in surveillance programs, as reassortment events may affect pathogenicity.

MATERIALS AND METHODS

Cells and viruses

Madin-Darby Canine kidney (MDCK) cells were cultured in EMEM as described previously [51]. Influenza virus A/Netherlands/602/09 (pH1N1) was isolated from the first patient with pandemic H1N1 virus infection in the Netherlands [150]. Influenza virus A/Netherlands/213/03 (H3N2) and influenza virus A/Netherlands/26/07 (sH1N1) were isolated from patients during epidemics in the Netherlands. After passaging these viruses in MDCK cells two times, all eight gene segments were amplified by reverse transcription polymerase chain reaction, cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 [171,208], and subsequently used to generate recombinant virus by reverse genetics as described elsewhere [171].

Generation of the reassortant viruses

Mixtures of reassortant viruses were generated in 293T cells using reverse genetics, by co-transfecting eight plasmids that encode the pH1N1 virus genome together with seven plasmids encoding the H3N2 or sH1N1 virus genome, omitting their HA gene. The 293T cell supernatants were passaged in quadruplicate under limiting dilution conditions using ten-fold serial dilutions in MDCK cells three times to enable selective outgrowth of viruses with high in-vitro replication rates. After three passages, the genome composition of these viruses was determined by sequencing using conserved primers targeting the non coding regions of each gene segment.
Reverse genetics was also used to produce specific reassortant viruses – pH1-sH1PB2, pH1-sH1PB2PA, pH1-sH3NA, and pH1-sH3NAPB1 – by transfection of 293T cells and subsequent virus propagation in MDCK cells.

In vitro characterization of viruses

Multicycle replication curves were generated by inoculating MDCK cells at a multiplicity of infection of 0.01 50 percent tissue culture infectious doses (TCID50) per cell in 2-fold [51].
Virus titers from samples of inoculated MDCK cells as well as nasal and throat swabs or homogenized tissue samples from inoculated ferrets, were determined by endpoint titration in MDCK cells in 96-wells plates (Greiner Bio-One)as described elsewhere [254].

Ferret experiments

All animal studies were approved by an independent Animal Ethics Committee. Experiments were performed under ABSL3+ conditions. The ferret model to test pathogenicity and transmission of pH1N1 virus was described previously [51,150]. To study pathogenicity, groups of six influenza virus seronegative female ferrets (Mustella putorius furo) were inoculated intranasally with 10^6 TCID50 of wild type pH1N1 virus, or the reassortant viruses pH1-sH1PB2, pH1-sH1PB2PA, pH1-sH3NA,

and pH1-sH3NAPB1, divided over both nostrils (2 x 250 µl).
In the transmission experiment, four female ferrets for wild type pH1N1 virus and two ferrets for each of the reassortant viruses, were individually housed in transmission cages and inoculated intranasally with 10^6 TCID50 of virus divided over both nostrils (2 x 250 µl).

Immunohistochemistry and histopathology
Immunohistochemistry and pathology were performed using lungs of inoculated ferrets. For each virus, 3 ferrets were euthanized at 3 and 7 dpi by exsanguination. Necropsies and tissue sampling were performed according to a standard protocol. After fixation in 10 % neutral-buffered formalin and embedding in paraffin, samples were sectioned at 4 µm and stained with an immunohistochemical method using a mouse monoclonal antibody against the nucleoprotein of influenza A virus [102]. Influenza virus antigen expression in lung sections was scored for bronchial surface epithelium, bronchial submucosal gland epithelium, bronchiolar epithelium, alveolar type I pneumocytes, and alveolar type II pneumocytes. Scoring was categorized as 0, no positive cells; 1, few positive cells; 2, moderate number of positive cells; and 3, many positive cells. Serial lung sections were stained with hematoxylin and eosin for detection and description of pathologic changes. Samples were scored for influenza-virus-associated inflammation in bronchi (bronchitis), bronchial submucosal glands (bronchoadenitis), bronchioles (bronchiolitis), and alveoli (alveolitis). Scoring of severity of inflammation was categorized as 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, marked inflammation. Sections were examined without knowledge of the identity of the ferrets.

RESULTS

In vitro selection of pH1N1-sH1N1 and pH1N1-H3N2 reassortants
Reassortant viruses were generated in 293T cells using reverse genetics. All eight gene segments of the pandemic virus were co-transfected with all segments of a seasonal virus, except HA. We omitted HA of the seasonal viruses to ensure that only reassortants containing the pH1N1 virus HA could arise, against which a large proportion of the human population is still immunologically naïve [255]. After three passages under limiting dilution conditions in MDCK cells, the virus genome composition within culture supernatants was determined by sequencing (Table 1). The proportion of gene segments 1-8 (except HA) analyzed was approximate 60%, 60%, 65%, 90%, 95%, 100% and 100% respectively. Minor virus variants were not detected. No point mutations were observed in the proportions of the genome analyzed. Upon pH1N1-sH1N1 transfection and passaging, three reassortants contained the PB2 gene of sH1N1 virus, two of which had also incorporated the sH1N1 PA gene. All four pH1N1-H3N2 reassortants had the NA gene of H3N2 and three of four reassortants also incorporated the H3N2 PB1 gene.

Table 1. Predominant virus genome composition upon in vitro selection of mixtures of pH1N1-sH1N1 and pH1N1-H3N2 reassortants

Replicates	PB2	PB1	PA	HA	NP	NA	MA	NS
pH1-sH1 1	**sH1**	pH1	**sH1**	pH1	pH1	pH1	pH1	pH1
pH1-sH1 2	**sH1**	pH1	**sH1**	pH1	pH1	pH1	pH1	pH1
pH1-sH1 3	**sH1**	pH1	pH1	pH1	pH1	pH1	pH1	pH1
pH1-sH1 4	pH1	pH1	pH1	pH1	pH1	pH1	pH1	pH1
pH1-sH3 1	pH1	**sH3**	pH1	pH1	pH1	**sH3**	pH1	pH1
pH1-sH3 2	pH1	pH1	pH1	pH1	pH1	**sH3**	pH1	pH1
pH1-sH3 3	pH1	**sH3**	pH1	pH1	pH1	**sH3**	pH1	pH1
pH1-sH3 4	pH1	**sH3**	pH1	pH1	pH1	**sH3**	pH1	pH1

In vitro characterisation of pH1N1-sH1N1 and pH1N1-H3N2 reassortants

All viruses shown in table 1 were next generated as clonal viruses using reverse genetics: pH1N1, pH1-sH1PB2, pH1-sH1PB2PA, pH1-sH3NA, and pH1-sH3NAPB1. Replication kinetics of wild type and reassortant viruses were studied in vitro. The replication kinetics of pH1-sH1PB2 and pH1-sH1PB2PA were similar to those of wild type pH1N1 virus, while the pH1-sH3NA and pH1-sH3NAPB1 reassortant viruses displayed slightly higher virus titers at 24 and/or 48 hours after inoculation, with a maximum difference in virus titer of 1.0 10log TCID50 (Figure 1). The fact that each reassortant virus replicated at least at the same rate as the wild type pH1N1 virus is in agreement with the results from the in-vitro selection experiment described above.

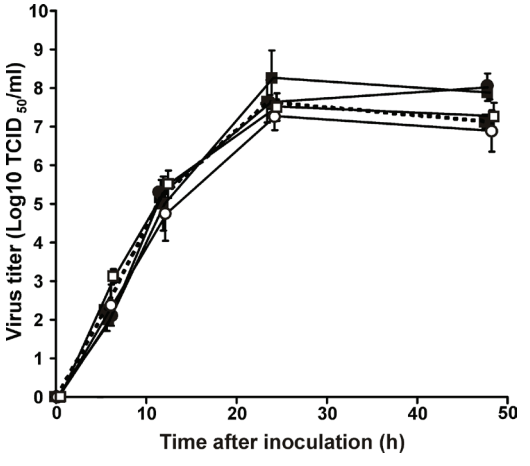

Figure 1. Replication of wild type and reassortant pH1N1 viruses in MDCK cells
MDCK cells were inoculated in duplicate with 0.01 TCID50/cell of the viruses pH1N1 (dotted line, closed square), pH1-sH1PB2PA (open circle), pH1-sH1PB2 (open square), pH1-sH3PB1NA (closed circle), and pH1-sH3NA (closed square), and supernatant samples were harvested 6, 12, 24 and 48 hours later. Supernatant samples were titrated in MDCK cells. Geometric mean titers and standard deviation were calculated from two independent experiments.

Pathogenicity of the reassortant viruses in ferrets

Pathogenicity of the reassortant viruses was investigated in ferrets. Groups of six ferrets were inoculated intranasally with 10^6 TCID50 of pH1N1, pH1-sH1PB2, pH1-sH1PB2PA, pH1-sH3NA, and pH1-sH3NAPB1. The animals were weighed daily as indicator of disease.

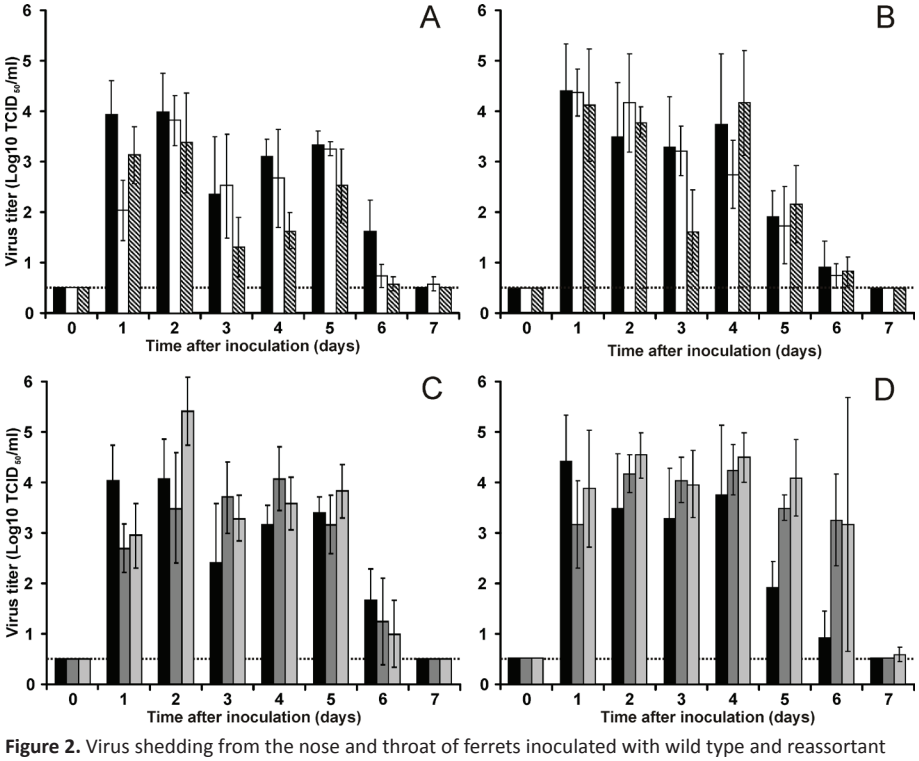

Figure 2. Virus shedding from the nose and throat of ferrets inoculated with wild type and reassortant pH1N1 viruses.
Virus shedding from nose (A, C) and throat (B, D) for pH1-sH1 (A, B) and pH1-sH3 (C, D) reassortant viruses is shown. Colour use is as follows; black: wild type pH1N1, white: pH1-sH1PB2PA, hatched: pH1-sH1PB2, dark grey: pH1-sH3PB1NA, light grey: pH1-sH3NA. Geometric mean titers are shown, with error bars to indicate the standard deviation. The lower limit of detection is indicated by the dotted line. After day 3, only 3 animals remained in each group.

The mean maximum weight loss was 7% for animals inoculated with the pH1N1 virus. Animals inoculated with pH1-sH1PB2PA, pH1-sH1PB2, pH1-sH3NAPB1 and pH1-sH3NA had a maximum weight loss of 4%, 2%, 2% and 6% respectively (data not shown).

Nose and throat swabs were collected from inoculated animals daily, and virus titers were determined. Infectious virus shedding continued until 6 or 7 days post infection (dpi) from the noses (Figure 2A, 2C) and throat (Figure 2B, 2D) of most inoculated animals. The total virus shedding from the nose, as calculated from the "area under the curve" (AUC) for the ferrets that were in the experiment for 7 days (N=3), was significantly lower in animals inoculated with the pH1-sH1PB2 reassortant virus (T-test, p=0.003) and significantly higher in the animals inoculated with the pH1-sH3NA virus (T-test, p=0.023), as compared to the animals inoculated with wild type pH1N1 virus. The total virus shedding from the throat, as calculated from the AUC for the ferrets that were in the experiment for 7 days, was not significantly different between the groups of ferrets.

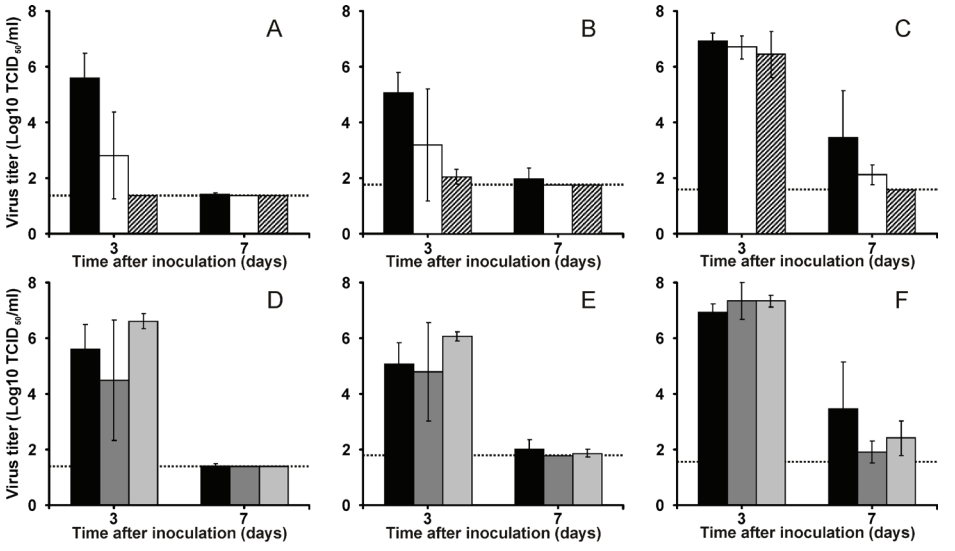

Figure 3. Virus detection in respiratory tissues of ferrets inoculated with wild type and reassortant pH1N1 viruses.
Virus detection in lungs (A, D), trachea (B, E), and nasal turbinates (C, F) for pH1-sH1 (A-C) and pH1-sH3 (D-F) reassortant viruses is shown. Colour use is as follows; black: wild type pH1N1, white: pH1-sH1PB2PA, hatched: pH1-sH1PB2, dark grey: pH1-sH3PB1NA, light grey: pH1-sH3NA. Three ferrets of each group were euthanized at 3 and 7 dpi. Geometric mean titers are shown, with error bars to indicate the standard deviation. The lower limit of detection is indicated by the dotted line.

At 3 and 7 dpi, three ferrets from each group were euthanized, and nasal turbinates, trachea and lungs were collected for virological examination. At 7 dpi, virus was undetectable or detected at only very low levels in samples from lung, trachea, and nasal turbinates in all groups of ferrets. At 3 dpi, no virus or relatively low virus titers were detected in the lungs and trachea respectively, of ferrets inoculated with pH1-sH1 reassortant viruses (Figure 3A, 3B), while titers in the nasal turbinates were similar to those for wild type pH1N1 virus (Figure 3C). These data indicate that both pH1-sH1 viruses were attenuated with respect to replication in the lower respiratory tract of ferrets.
At 3 dpi, virus was detected in the lungs, trachea, and nasal turbinates of ferrets inoculated with viruses pH1-sH3NAPB1 and pH1-sH3NA at approximately the same levels as upon inoculation with wild type pH1N1 virus (Figure 3D, 3E, 3F). Virus titers detected in the lungs and trachea of animals inoculated with the pH1-sH3NA virus at 3 dpi were 1.0 10log TCID50 higher as compared to those in animals inoculated with wild type pH1N1 virus (not statistically significant). These data indicate that both pH1-sH3 viruses tested were not attenuated in ferrets. If anything, shedding of pH1-sH3NA virus from the nose (Figure 2C), lungs (Figure 3D), and trachea (Figure 3E), was higher as compared to wild type pH1N1 virus.

Pathological changes in the respiratory tract of ferrets inoculated with pH1N1 and reassortant viruses

At 3 and 7 dpi, three ferrets from each group were euthanized, and lung tissue was collected for pathologic examination. At 7 dpi, virus antigen expression was undetectable in lung tissue of any of the ferrets, and lesions were absent or resolving. At 3 dpi, neither viral antigen expression nor lesions were detected in lungs of ferrets inoculated with pH1-sH1PB2. Only one of three ferrets inoculated with pH1N1 had scant virus antigen expression and mild associated lesions in bronchial submucosal glands and bronchioles at 3 dpi (Table 2, Figure 4). In contrast, all three ferrets inoculated with pH1-sH3NA had moderate to abundant virus antigen expression in bronchial submucosal glands, bronchioles, or both, associated with moderate to marked inflammation (Table 2, Figure 4). Virus antigen expression and associated lesions in the lungs of ferrets inoculated with pH1-sH3PB1NA were intermediate between those of wild-type pH1N1 and pH1-sH3NA (Table 2).

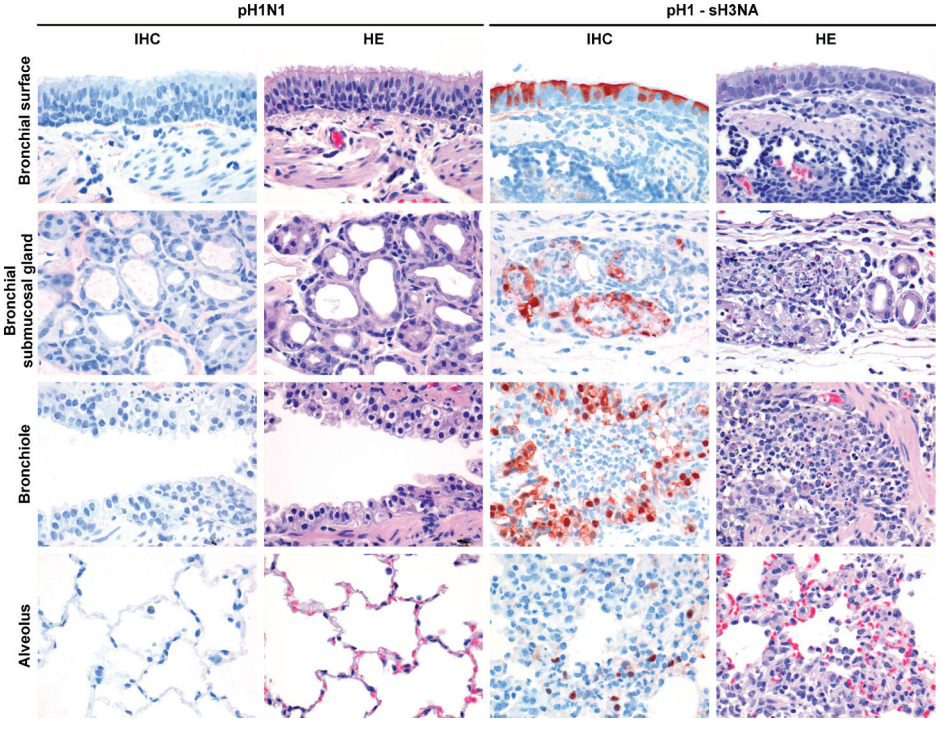

Figure 4. Virus antigen expression and severity of lesions in different tissues of the lungs of ferrets.
Two of three ferrets inoculated with wild type pH1N1 virus had neither virus antigen expression (first column) nor associated lesions (second column) in the lung at day three post inoculation. In contrast, all three ferrets inoculated with reassortant pH1-sH3NA virus had virus antigen expression in bronchi, bronchial submucosal glands, bronchioles, and alveoli (third column), associated with epithelial degeneration and necrosis and infiltration of inflammatory cells, predominantly neutrophils (fourth column).

Table 2. Virus antigen expression and severity of lesion in different tissues of the lung of ferrets inoculated with pH1N1 or reassortant pH1N1 viruses

Virus	N	Cumulative score per tissue							
		Bronchial surface epithelium		Bronchial submucosal epithelium		Bronchiolar epithelium		Alveolar epithelium	
		IHC*	HE†	IHC	HE	IHC	HE	IHC	HE
pH1N1	3	0,0,0‡	0,0,0	0,0,1	0,0,1	0.0.1	0,0,1	0,0,0	0,0,0
pH1-sH1PB2PA	3	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
pH1-sH1PB2	3	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
pH1-sH3PB1NA	3	1,1,0	0,1,0	2,1,0	1,1,0	2,1,0	1,1,0	1,1,0	1,1,1
pH1-sH3NA	3	1,1,1	0,1,1	1,0,3	1,0,3	2,2,3	2,2,3	1,0,1	1,1,1

* IHC = Immunohistochemistry to detected virus antigen expression.
† HE = Hematoxylin and eosin staining to analyze severity of inflammation
‡ Individual scores (as indicated in the methods section) for three ferrets are listed

The cell types in which virus antigen expression was detected were ciliated epithelial cells of bronchi, epithelial cells of bronchial submucosal glands, ciliated and non-ciliated cells of bronchioles, and both squamous and cuboidal epithelial cells – interpreted as type I and type II pneumocytes, respectively – of alveoli (Figure 4). Virus antigen expression also was seen in desquamated epithelial cells and cell debris in lumina of above tissues.
The lesions associated with virus antigen expression can be categorized as acute, focal or multifocal, necrotizing bronchitis, bronchoadenitis, bronchiolitis, and alveolitis. These lesions were characterized by degeneration and necrosis of epithelial cells, infiltration of the affected tissues and their lumina by many neutrophils and few eosinophils, and exudation of edema fluid and fibrin into tissue lumina.

Transmission of the reassortant viruses in ferrets
Transmission of the pH1N1 and reassortant influenza viruses via aerosol or respiratory droplets was tested in the ferret model. Groups of 4 ferrets for pH1N1 virus and 2 ferrets for the reassortant viruses were inoculated intranasally with 10^6 TCID50 of virus. At 1 dpi, a naïve ferret was placed in a cage adjacent to each inoculated ferret. All viruses were transmitted from the inoculated to the naive ferrets in 4 out of 4 for pH1N1 virus and 2 out of 2 for each of the reassortant viruses. The first day of virus detection in the naïve animals was 2 days post exposure (dpe), similar for all viruses tested.

DISCUSSION

Here we used an in vitro selection method to identify reassortant viruses between pandemic H1N1 influenza virus and seasonal H1N1 and H3N2 viruses of interest for testing in a ferret model. Studying the impact of reassortment on changes in influenza virus phenotype is cumbersome, as the number of reassortants that can

be generated between 2 viruses is very high; 28 = 256 different viruses. After three passages a limited number of specific virus populations were selected in-vitro. Of note, minor virus variants representing less than 20 % of the virus population would remain undetected in our approach of PCR amplification and direct determination of the consensus sequence of the amplicons. However, upon repeating the procedure four times for both reassortment combinations, the seasonal influenza virus genes that were selected in the pandemic H1 virus backbone were more or less consistent, with NA of H3N2 virus being selected in 4/4 attempts, PB1 of H3N2 and PB2 of sH1N1 virus in 3/4 attempts, and PA of sH1N1 virus being selected in 2/4 attempts. Replication in MDCK cells may not be the best selection criterion for the identification of reassortants of interest to human health. Nevertheless, we choose this in-vitro selection method, since previous work using the ferret model for selection of reassortants has shown that pH1N1 outcompetes sH1N1 and H3N2 viruses rapidly, reducing the opportunity for reassortment in this model [256]. This growth advantage over the seasonal viruses was in agreement with the fact that the selected viruses mostly contained the pH1N1 genes. The use of reverse genetics allows the production of all gene segments at approximately similar copy numbers upon transfection, while upon double infection with two viruses in vitro or in ovo – the classical ways to generate reassortants – viruses may differ in replication capacity, resulting in a bias of the reassortants that are produced.

Interestingly, the polymerase gene segments of sH1N1 and H3N2 viruses frequently substituted for the polymerase genes of the pH1N1 virus in vitro. In minigenome assays, the polymerase complex activity of the wild type pH1N1 virus was relatively low, and replacement of various polymerase genes of the pH1N1 virus increased this activity. However, polymerase complexes with the highest activity in minigenome assays were not necessarily the ones detected in the reassortant viruses (data not shown). This apparent discrepancy is probably a result of the different parameters under investigation in the two assays, in particular the production of mRNA versus all viral RNAs.

Virus titers for pH1-sH1PB2PA and pH1-sH1PB2 in the lungs and trachea of ferrets were lower as compared to titers in ferrets inoculated with wild type pH1N1 virus, suggesting that both pH1N1-sH1N1 reassortant viruses were attenuated in ferrets, at least for replication in the lower respiratory tract. The reassortants between pH1N1 and H3N2 viruses replicated at slightly higher rates than wild type pH1N1 virus in vitro. Moreover, virus shedding of pH1-sH3NA virus from the nose, lungs, and trachea of inoculated ferrets was slightly higher as compared to wild type pH1N1 virus. Although the differences in replication and shedding were small, and not-statistically significant due to the relatively small numbers of animals in each group, we conclude that the pH1N1-H3N2 viruses were not attenuated in ferrets.

Inoculation of the reassortant pH1-sH3NA – either with or without the PB1 of H3N2 virus – resulted in higher expression of virus antigen and more severe lesions at all levels of the lower respiratory tract compared to inoculation of wild-type pH1N1 virus (Table 2, Figure 4). In a previous study, the wild-type pH1N1 virus was detected

more abundantly in the lower airways of ferrets as compared to the present study [257]. We attribute this difference to the use of a virus isolate rather than a virus generated by reverse genetics, and to a different batch of ferrets in the previous study. In the present study, all viruses were produced with reverse genetics, and can thus be compared directly. Moreover, the reassortant pH1 virus with the NA of the H3N2 virus was more pathogenic than both sources of pH1N1 virus, wild-type isolate or the virus derived by reverse genetics. Increased severity of lesions may be related to higher virus replication in the lung, to stronger host immune responses, or both [257].

We conclude that the pandemic H1N1 virus has the potential to reassort with seasonal H1N1 and H3N2 influenza viruses, and that such reassortment events could result in viruses with increased pathogenicity in ferrets. Although increased pathogenicity in ferrets can not be extrapolated directly to increased pathogenicity in humans, the ferret model is generally thought to be a good animal model for influenza in humans, because ferrets are susceptible to natural infection and develop respiratory disease and lung pathology similar to humans when infected with seasonal, avian, or pandemic influenza viruses [210,211]. Patterns of influenza virus attachment to cells of the respiratory tract are also similar in ferrets and humans [147], and the ferret model has further been used successfully for studies on virus transmission via respiratory droplets or aerosols [150,153].

Although viruses with the NA gene – with or without the PB1 gene – of H3N2 were identified here as potentially "fit" virus reassortants, it is possible that reassortant viruses with other gene constellations may have selective advantages in humans as well. It is of interest to note that the 1968 H3N2 pandemic virus also continued to reassort after the pandemic year, resulting in viruses in 1969-1971 with a different N2 gene as compared to those earlier in the pandemic [258]. H1N2 reassortant influenza viruses with the HA of seasonal H1N1 and the NA of seasonal H3N2 viruses have been isolated from humans during previous influenza seasons, thereby confirming that reassortant influenza viruses with such HA/NA combination can emerge in humans [25,253]. Moreover, H1N2 influenza viruses have frequently been detected in pigs around the world [85].

We recommend that reassortment of pandemic 2009 influenza viruses should be monitored closely in surveillance programs, in particular when changes in pathogenicity and/or transmission in humans become apparent.

ACKNOWLEDGEMENTS

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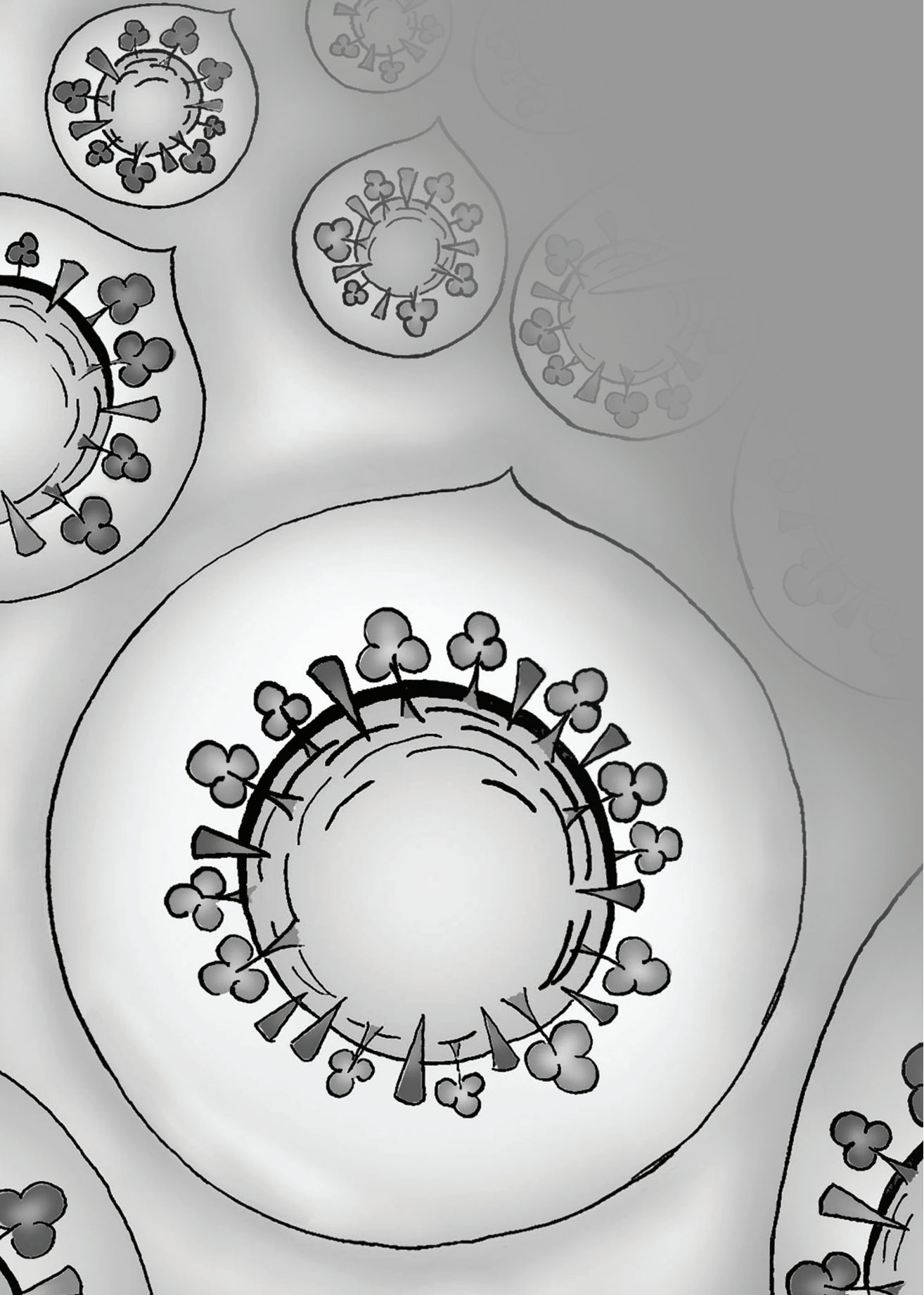

CHAPTER 3
Genome reassortment of influenza A viruses

3.2
Reassortment between avian H5N1 and human influenza viruses is mainly restricted to the matrix and neuraminidase gene segments

Eefje J.A. Schrauwen, Theo M. Bestebroer, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus, Ron A.M. Fouchier and Sander Herfst

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ABSTRACT

Highly pathogenic avian influenza H5N1 viruses have devastated the poultry industry in many countries of the eastern hemisphere. Occasionally H5N1 viruses cross the species barrier and infect humans, sometimes with a severe clinical outcome. When this happens, there is a chance of reassortment between H5N1 and human influenza viruses. To assess the potential of H5N1 viruses to reassort with contemporary human influenza viruses (H1N1, H3N2 and pandemic H1N1), we used an in vitro selection method to generate reassortant viruses, that contained the H5 hemagglutinin gene, and that have a replication advantage in vitro. We found that the neuraminidase and matrix gene segments of human influenza viruses were preferentially selected by H5 viruses. However, these H5 reassortant viruses did not show a marked increase in replication in MDCK cells and human bronchial epithelial cells. In ferrets, inoculation with a mixture of H5N1-pandemic H1N1 reassortant viruses resulted in outgrowth of reassortant H5 viruses that had incorporated the neuraminidase and matrix gene segment of pandemic 2009 H1N1. This virus was not transmitted via aerosols or respiratory droplets to naïve recipient ferrets. Altogether, these data emphasize the potential of avian H5N1 viruses to reassort with contemporary human influenza viruses. The neuraminidase and matrix gene segments of human influenza viruses showed the highest genetic compatibility with HPAI H5N1 virus.

INTRODUCTION

Since the late 1990s, highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype have devastated the poultry industry of numerous countries of the eastern hemisphere. After 2004, H5N1 has spread from Asia to Europe, Africa, and the Middle East, resulting in the killing or culling of hundreds of millions of domestic birds. Occasionally, HPAI H5N1 viruses cross the species barrier and infect humans, sometimes with a severe clinical outcome. This direct transmission of HPAI H5N1 virus to humans was first detected in 1997 [58] and has continued to be reported ever since [59]. Luckily, these viruses do not transmit efficiently between humans. However, they may gain the ability to spread efficiently among humans through either virus adaptation to the new host, genetic reassortment (ie genetic mixing of viruses) with contemporary human influenza viruses, or both [234,259-261]. Reassortment has proven to be an important mechanism for influenza viruses to evolve. The influenza pandemics of 1957, 1968 and 2009 were the result of reassortment events [30,31,50].

To date, reassortment of H5N1 viruses with human influenza viruses has not been detected in nature. However, co-infection of H5N1 and human viruses in humans or pigs may provide a new opportunity for reassortment and the subsequent emergence of viruses with pandemic potential. Therefore, it is important to investigate the genetic compatibility of the genes of potential parental strains.

In previous studies, reassortment of avian H5N1 and human H3N2 has been investigated extensively in vivo [154,262,263]. It was found that H5N1-H3N2 reassortment resulted in a more pathogenic H5 virus in mice [262,263]. Furthermore, reassortment was found to occur readily in vivo, with a high probability in the ferret upper respiratory tract [154]. However, none of the tested H3N2-H5N1 reassortant viruses had gained the ability to be transmitted between ferrets.

Recent studies have also investigated the replication kinetics of many reassortant viruses between the 2009 pandemic H1N1 (pH1N1) and H5N1 influenza virus in vitro and in vivo [264-266]. Co-infection of cultured cells with pH1N1 and H5N1 showed that these two viruses have high genetic compatibility and that some of these viruses displayed better replication kinetics in vitro [264]. In addition, increased pathogenicity was observed for a reassortant pH1N1 containing an H5N1 HA in mice [266].

Avian H5N1 and human influenza viruses display different replication characteristics in primary cell cultures. Avian influenza viruses can infect human airway epithelium cells, although replication may be limited compared to human influenza viruses because of a nonoptimal cellular tropism [267]. This system offers an alternative to study virus and/or host properties required for adaptation or reassortment of influenza viruses. It was studied that co-infection of cells with viruses carrying HA of avian and human influenza viruses take place when the cells provide both receptors [268].

Here, we investigated the ability of HPAI H5N1 and contemporary human H3N2,

H1N1 and pH1N1 influenza viruses to reassort, by means of an in vitro selection method using reverse genetics and serial passaging under limited dilution conditions as described before [269]. In contrast to double infection with 2 viruses, this method allows the production of gene segments at approximately similar copy numbers upon transfection, after which in vitro or in ovo viruses may differ in replication capacity. In addition, avian H5N1 outcompetes human influenza viruses in co-infection experiments [264]. In this way, a bias towards reassortants produced is thus prevented. The reassortants that were selected during this in vitro selection experiment were subsequently evaluated for replication kinetics in MDCK cells and normal human bronchial epithelial (NHBE) cultures. In addition, to study wether reassortants were produced with a replication advantage over the parental viruses in vivo, ferrets were inoculated with a mixture of reassortant viruses between pH1N1 and H5N1. The genetic composition of these reassortant mixtures were followed over time. Simultaneously, the transmissibility of these viruses was evaluated in a ferret model via aerosols or respiratory droplets.

MATERIAL & METHODS

Ethics statement

Animals were housed and 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). All animal experiments were approved by the independent animal experimentation ethical review committee 'stichting DEC consult' (Erasmus MC permit number EUR 1621) and were performed under animal biosafety level 3+ conditions as described elsewhere [261]. Animal welfare was observed on a daily basis, and all animal handling was performed under light anesthesia using ketamine to minimize animal suffering. Influenza virus sero-negative 6 month old female ferrets (Mustella putorius furo), weighing 800-1000 g., were obtained from a commercial breeder. All experiments involving H5N1 transmission were conducted prior to the institution of the current moratorium.

Cells and viruses

Madin-Darby Canine kidney (MDCK) cells were cultured in EMEM (Cambrex, Heerhugowaard, the Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate (Cambrex), 20 mM Hepes (Cambrex), and 0.1 mM non-essential amino acids (MP Biomedicals Europe, Illkirch, France). 293T cells were cultured in DMEM (Cambrex) supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2mM glutamine, 1mM sodium pyruvate, and non-essential amino acids.

All eight gene segments of influenza virus isolates A/Netherlands/602/2009 (pH1N1), A/Netherlands/213/2003 (H3N2), A/Netherlands/26/2007 (sH1N1) and A/Indonesia/5/2005 (H5N1) were amplified by reverse transcription polymerase

chain reaction, cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 [171,208], and subsequently used to generate recombinant virus by reverse genetics as described elsewhere [171]. All in vitro selection experiments, growth curves and virus titrations were performed under ABSL3+ conditions.

Generation of the reassortant viruses

Reverse genetics was used to generate mixtures of reassortant viruses in 293T cells by co-transfecting eight plasmids that encode the H5N1 virus genome together with seven plasmids encoding the pH1N1, H3N2 or sH1N1 virus genome. The HA gene of these human viruses was omitted to make sure that only viruses with the HA of H5N1 virus were generated, as described previously [269]. The 293T cell supernatants were subsequently passaged in quadruplicate under limiting dilution conditions by using ten-fold serial dilutions in MDCK cells three times to enable selective outgrowth of viruses with high in vitro replication rates. Next, the genome composition of these viruses was determined by Sanger sequencing using conserved primers targeting the noncoding regions of each gene segment. Reverse genetics was subsequently used to produce reassortant viruses with the different gene compositions as identified by sequencing.

Virus titrations on MDCK cells

Virus titers in ferret nasal and throat swabs, or samples from replication curves were determined by end-point titration in MDCK cells in 96-well plates (Greiner Bio-One). MDCK cells were inoculated with tenfold serial dilutions of each sample, washed one hour after inoculation with phosphate-buffered saline (PBS), and cultured in 200µl of infection medium, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodiumbicarbonate, 20 mM Hepes, non-essential amino acids, and 20 µg/ml trypsin (Cambrex). Three days after inoculation, the supernatants of inoculated 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 4 replicates by the method of Spearman-Karber [233].

Replication kinetics in MDCK cells

Multicycle replication curves were generated by inoculating MDCK cells at a multiplicity of infection (MOI) of 0.01 50 percent tissue culture infectious doses (TCID50) per cell in duplicate. Supernatants were sampled at 6, 12, 24, and 48 hours after inoculation, and virus titers in these supernatants were determined by means of end-point titration in MDCK cells as described above.

Culture of NHBE cells

Normal human bronchial epithelial (NHBE) cells were obtained from Clonetics (Basel, Switzerland) and used at passage 3-4. Undifferentiated NHBE cells were grown on 30 µg/ml type I collagen-coated 75 cm2 flasks in serum-free bronchial epithelial

cell basal medium (BEBM) supplemented with BEBM SingleQuots (Clonetics). At 60-80% confluency, cells were trypsinized and seeded at a cell density of $1x10^4$ viable cells onto type I collagen-coated 6.5 mm transwell inserts with 0.4 µm pore size (Corning, Amsterdam, The Netherlands). The growth medium consisted of a 1:1 mixture of complete BEBM and DMEM, supplemented with 15 ng/ml retinoic acid (Sigma-Aldrich). The medium was replaced with fresh medium every other day until cells reached confluency. Subsequently, an air-liquid interface (ALI) was created by removing medium from the apical side to promote mucocilliary differentiation. The medium was refreshed basolaterally and the apical side was washed with Dulbecco's PBS (DPBS, Lonza) at 37°C every other day. Well-differentiated (wd-) NHBE cells were inoculated with influenza virus, 21 days after ALI, at which stage beating cilia and mucus production were clearly detectable.

wdNHBE cell characterization

wdNHBE cells on transwell filters were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature (RT) and subsequently washed with PBS 0.1% Tween and permeabilized with 1% Triton X-100 in PBS. After washing with PBS and blocking with PBS 5% BSA-0.1% Tween, ciliated cells were identified by staining with mouse monoclonal β-Tubulin antibody (KLINIPATH, Duiven, The Netherlands). Goblet cells were identified by mouse Mucin 5AC antibody (MUC5AC, ITK DIAGNOSTIC BV, Uithoorn, The Netherlands). The cultures were subsequently incubated with a secondary goat anti-mouse IgG (H+L) texas red labelled antibody (Alexa Fluor ® 594, Invitrogen) to visualize ciliated cells or goblet cells. In addition, cell cultures stained for the presence of ciliated cells were double stained to also visualize the tight junctions: cells were incubated with a ZO-1 N-Term antibody (Invitrogen), followed by incubation with a secondary swine anti-rabbit FITC labelled antibody (DAKO, Enschede, Netherlands). The transwell filters were cut off after staining, mounted on slides with Prolong Gold Mount (Vectashield, Peterborough, UK) and analysed using a fluorescence imaging microscope (Leica Microsystems).

Lectin staining of differentiated NHBE cells

The wdNHBE cultures were washed with DPBS to remove overlaying mucus and incubated for 1 hour at 4ºC with biotin-labeled lectines sambucus nigra agglutinin (SNA; specific for sialic acid α2.6 Gal; 4 µg/ml; SANBIO BV, Uden, Netherlands) or Maackia amurensis agglutinin II (MAAII; specific for sialic acid α2.3 Gal; 20 µg/ml; BIO-CONNECT, Huissen, Netherlands) in Tris-buffered saline (TBS; pH 7.2) containing 1% BSA and 1 mM Mg2+, Ca2+, and Mn2+. Next, the cells were washed with TBS and fixed with 4% paraformaldehyde. After washing, the cultures were incubated for 1 hour at RT with streptavidin-horseradish peroxidise FITC-labeled conjugate (DAKO) in 1% BSA-TBS. Cultures were washed, permeabilized and stained for cilia as described above and analysed by confocal laser scanning microscopy using a LSM700 system fitted on an Axio Observer Z1 inverted microscope (Zeiss). Images were generated using Zen software.

Replication kinetics in differentiated NHBE cells
The wdNHBE cells were washed with DPBS to remove overlaying mucus and duplicates were inoculated via the apical side, with virus of interest at a MOI of 0.02 in 100 µl. After one hour of incubation at 37 ºC the inoculum was removed, cells were washed three times with DPBS and once with growth medium. The last wash step with growth medium was collected for virus titration as time point t=0. At 6, 12, 24, and 48 hours after inoculation, 100 µl of growth medium was added to the apical side of the trans well of each culture to collect virus samples. After 10 min of incubation at 37 ºC, the medium was collected and stored at -80 ºC for virus titration in MDCK cells.

Ferret transmission experiment
The ferret model to test for aerosol or respiratory droplet transmission was described previously [150]. In the transmission experiment, two influenza virus seronegative female ferrets were individually housed in transmission cages and inoculated intranasally, divided over both nostrils (2 x 250µl), with 107.3 TCID50 of MDCK passage 1 (MDCKP1) of the H5N1-pH1N1 reassortant virus mixture (eight H5N1 and seven pH1N1 plasmids, without the pH1N1 HA, were transfected in 293T cells and supernatant was subsequently passaged on MDCK cells). At 1 day post inoculation (dpi), naïve recipient ferrets were individually placed in a transmission cage adjacent to a donor ferret. The animals were separated by two stainless steel grids to allow airflow from the donor to the recipient ferret but to prevent direct contact- and fomite transmission. Nasal and throat swabs were collected at 1, 3, 5 and 7 dpi from donor ferrets and at 1, 3, 5 and 7 days post exposure (dpe) from the recipient animals. Donor ferrets were euthanized at 7 dpi and recipient ferrets at 7 dpe. Virus titers were determined in collected swabs by means of end-point titration in MDCK cells.

Analysis of viral genome composition in ferrets by pyrosequencing
Pyrosequencing, a method to detect single nucleotide polymorphisms (SNP)[270], was used to determine the exact proportion of H5N1 and pH1N1 gene segments in the donor ferrets inoculated with the MDCK P1 H5N1-pH1N1 reassortant mixture. RNA was isolated from the throat swabs collected at 1, 3, 5 and 7 days dpi. After cDNA synthesis, conserved primers were used for each gene segment to amplify a small PCR product of approximately 100 bp (Table 1). These fragments were next sequenced using the Pyromark Q24 pyrosequencing platform (Qiagen, Venlo, The Netherlands) with a specific sequence primer (Table 1).

Table 1. Primers used for pyrosequencing

Primers	Sequence (5'-3')
Forward PB2 H5N1/H1N1	GCAGGTCAAATATATTCAATATGG
Reverse PB2 H5N1/H1N1	GATTATGGCCATATGGTCCAC * biotin
Sequence primer	TCAATATGGAGAGAATAAAA
Forward PB1 H5N1/H1N1	CAGGATACACCATGGACACAGT
Reverse PB1 H5N1/H1N1	CCTCAGGTAGTGGTCCATCAATC* biotin
Sequence primer	ATACACCATGGACACAGT
Forward PA H5N1/H1N1	GTGCGACAATGCTTCAATCCA
Reverse PA H5N1/H1N1	GTGTGCATATTGCAGCAAA * biotin
Sequence primer	CAATGCTTCAATCCAA
Forward NP H5N1/H1N1	GAGCTCTCGGACGAAAAGG
Reverse NP H5N1/H1N1	CTCTGCATTGTCTCCGAAGAA * biotin
Sequence primer	TGCCTTCCTTTGACAT
Forward NA H5N1/H1N1	GGCATAATAACAGACACTATCAAG
Reverse NA H5N1/H1N1	CCATTACTTGGTCCATC * biotin
Sequence primer	GACACTATCAAGAGTTGGAG
Forward MA H5N1/H1N1	GAGTCTTCTAACCGAGGTCGAAAC * biotin
Reverse MA H5N1/H1N1	GTGTTCTTTCCTGCAAAGAC
Sequence primer	GCCTGACGGGATGATA
Forward NS H5N1/H1N1	AGGGTGACAAAAACATAATGGA
Reverse NS H5N1/H1N1	CAAGGAATGGGGCATCACCC * biotin
Sequence primer	GTGACAAAAACATAATGGA

RESULTS

In vitro selection of H5 reassortant viruses

Reverse genetics was used to generate mixtures of reassortant viruses in 293T cells, by co-transfecting eight plasmids that encode the H5N1 virus genome together with seven plasmids encoding the pH1N1, H3N2 or sH1N1 virus genome, omitting their HA gene. The 293T cell supernatants were passaged, in quadruplicate, under limiting dilution conditions to allow selective outgrowth of viruses with high in vitro replication rates. After three passages, the genome composition of these viruses was determined by sequencing (Table 2). The predominant virus population was identified for almost all passaged virus mixtures by sanger sequencing, with minor virus variants representing less than 20% of the virus mixture (estimated detection limit for sanger sequencing). Point mutations were not observed in the proportion of the genome analyzed. Upon H5N1-pH1N1 transfection and passaging of the virus mixtures, wild type (wt) H5N1 was recovered in one attempt and H5N1-pH1N1-reassortants in three attempts (Table 2). These reassortants incorporated the pH1N1 matrix (M) gene (H5-pH1M), or the pH1N1 M, neuraminidase (NA) and non-structural (NS) genes (H5-pH1NA,M,NS), or the pH1N1 M, NA, NS and polymerase complex genes PB2, PB1 and PA (H5-pH1NA,M,NS,PB2,PB1,PA).

Table 2. Predominant virus genome composition upon in vitro selection of mixtures of H5N1-pH1N1, H5N1-H3N2 and H5N1-sH1N1 reassortants

Replicates	PB2	PB1	PA	HA	NP	NA	M	NS
H5-pH1 1	H5	H5	H5	H5	H5	H5	**pH1**	H5
H5-pH1 2	**pH1**	**pH1**	**pH1**	H5	H5	**pH1**	**pH1**	**pH1**
H5-pH1 3	H5	H5	H5	H5	H5	H5	H5	H5
H5-pH1 4	H5	H5	H5	H5	H5	**pH1**	**pH1**	H5**/pH1***
H5-H3 1	H5	H5	H5	H5	H5	**H3**	H5	H5
H5-H3 2	H5	H5	H5	H5	H5	H5	H5	H5
H5-H3 3	H5	H5	H5	H5	H5	**H3**	H5/**H3****	H5
H5-H3 4	H5	H5	H5	H5	H5	**H3**	H5	H5
H5-sH1 1	H5	H5	H5	H5	H5	H5	**sH1**	H5
H5-sH1 2	H5	H5	H5	H5	H5	**sH1**	**sH1**	H5
H5-sH1 3	H5	H5	H5	H5	H5	**sH1**	H5	H5
H5-sH1 4	H5	H5	H5	H5	H5	H5	H5	H5

* A 25/75 population of NS H5 and pH1 was detectable
** A 50/50 population of M H5 and H3 was detectable

Upon H5N1-H3N2 transfection and passaging, wtH5N1 was recovered in one attempt, whereas three reassortant viruses were recovered that had the NA gene of H3N2 (H5-H3NA). In one of the reassortant viruses that contained the NA gene of H3N2, a mixed population was present for the M gene (approximately 50% H5N1 and 50% H3N2; H5-H3NA,M) (Table 2).
Four different genome compositions were identified upon H5N1-sH1N1 transfection and passaging: wtH5N1 was recovered as well as three reassortant viruses containing the sH1N1 M or NA or M and NA genes (H5-H1NA, H5-H1M and H5-H1NA,M) (Table 2).

In vitro characterization of H5 reassortant viruses in MDCK cells

The data obtained from the in vitro selection experiments suggest that the NA and M genes of all three tested human influenza viruses as well as the NS gene of pH1N1 frequently substituted their H5N1 counterparts. Therefore, reverse genetics was used to generate H5 reassortants that contained one (NA, M or NS), two (NA and M), or three (NA, M and NS) genes of pH1N1, H3N2 or sH1N1 (Table 3) and their replication kinetics was subsequently evaluated in MDCK cells. MDCK cells were inoculated with reassortant viruses at an MOI of 0.01, after which the supernatants were harvested at fixed time points and virus titers were determined in MDCK cells. In general, H5 reassortant viruses containing gene segments of pH1N1 showed similar virus titers compared to wtH5N1 (Fig 1A), although H5-pH1M and H5-pH1NA,M,NS reassortant viruses were produced at a slightly higher level at 24 and 48 hours post-inoculation (pi), whereas H5-pH1NA had a lower replication rate compared to wtH5N1. The virus replication of most H5N1-H3N2 reassortant viruses was similar to that of wtH5N1 in MDCK cells (Fig 1B), however H5-H3M and H5-H3NS reassortant viruses had slightly increased virus titers at 24 and 48 hours pi,

Table 3. H5 Reassortant influenza viruses rescued via reverse genetics

Reassortant virus	PB2	PB1	PA	HA	NP	NA	M	NS
H5N1	H5	H5	H5	H5	H5	H5	H5	H5
H5-pH1$_{NA}$	H5	H5	H5	H5	H5	**pH1**	H5	H5
H5-pH1$_{M}$	H5	H5	H5	H5	H5	H5	**pH1**	H5
H5-pH1$_{NS}$	H5	H5	H5	H5	H5	H5	H5	**pH1**
H5-pH1$_{NA,M}$	H5	H5	H5	H5	H5	**pH1**	**pH1**	H5
H5-pH1$_{NA,M,NS}$	H5	H5	H5	H5	H5	**pH1**	**pH1**	**pH1**
H5-H3$_{NA}$	H5	H5	H5	H5	H5	**H3**	H5	H5
H5-H3$_{M}$	H5	H5	H5	H5	H5	H5	**H3**	H5
H5-H3$_{NS}$	H5	H5	H5	H5	H5	H5	H5	**H3**
H5-H3$_{NA,M}$	H5	H5	H5	H5	H5	**H3**	**H3**	H5
H5-H3$_{NA,M,NS}$	H5	H5	H5	H5	H5	**H3**	**H3**	**H3**
H5-sH1$_{NA}$	H5	H5	H5	H5	H5	**sH1**	H5	H5
H5-sH1$_{M}$	H5	H5	H5	H5	H5	H5	**sH1**	H5
H5-sH1$_{NS}$	H5	H5	H5	H5	H5	H5	H5	**sH1**
H5-sH1$_{NA,M}$	H5	H5	H5	H5	H5	**sH1**	**sH1**	H5
H5-sH1$_{NA,M,NS}$	H5	H5	H5	H5	H5	**sH1**	**sH1**	**sH1**

whereas H5-H3NA had a lower virus titer at 48 hours pi. Almost all H5 reassortant viruses with sH1N1 gene segments replicated to similar virus titers compared to wtH5N1, although H5-sH1NA had a lower virus titer at 48 hours pi, compared to wtH5N1. Although the differences in replication kinetics between the wtH5N1 and the H5 reassortant viruses were rather small, it seemed that incorporation of any of the three NA's attenuated virus replication at 48 hours pi.

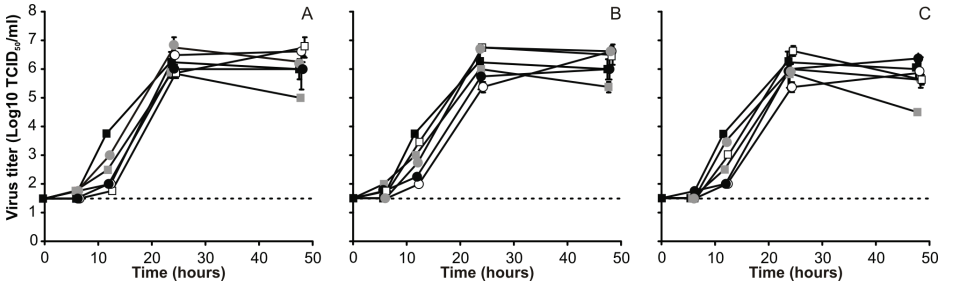

Figure 1. Replication kinetics of H5N1-pH1N1, H5N1-H3N2 and H5N1-sH1N1 reassortant viruses in MDCK cells

MDCK cells were inoculated with 0.01 TCID50/cell of H5N1 (black square), H5 reassortant viruses harboring the NA (grey square), M (grey circle), NS (white square), NA and M (black circle) and NA, M and NS (white circle) of pH1N1 (A), H3N2 (B) or sH1N1 (C) and supernatant samples were harvested 6, 12, 24, and 48 h later. Geometric mean titers were calculated from two independent experiments, error bars indicate standard deviations. The lower limit of detection is indicated by the dotted line.

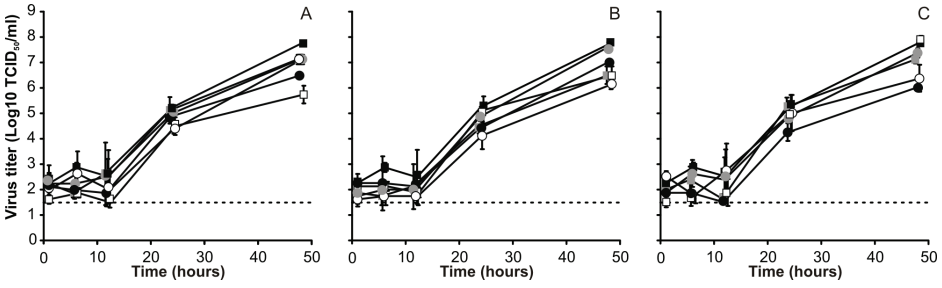

Figure 2. Replication kinetics of H5N1-pH1N1, H5N1-H3N2 and H5N1-sH1N1 reassortant viruses in wdNHBE cells

wdNHBE cells were inoculated with 0.02 TCID50/cell of H5N1 (black square), H5N1 reassortant viruses consisting of the NA (grey square), M (grey circle), NS (white square), NA and M (black circle) and NA, M and NS (white circle) of pH1N1 (A), H3N2 (B) or sH1N1 (C) and samples were harvested 1, 6, 12, 24, and 48 h later. Geometric mean titers were calculated from two independent experiments, error bars indicate standard deviations. The lower limit of detection is indicated by the dotted line.

In vitro characterization of H5 reassortant viruses in wdHBE cells

Influenza viruses infect cells of the respiratory tract of humans. Therefore, we evaluated the replication kinetics of the reassortant viruses in wdNHBE cells.

To characterize the wdNHBE cells, immunohistochemistry was used to identify ciliated cells, mucus-producing goblet cells and tight junctions. Cultures were also double stained for ciliated cells and tight junctions. For further characterization, the sialic acid (SA) receptor distribution on wdNHBE cells was determined by lectin histochemistry using MAL-II which recognizes the α2.3-linked SA and SNA which recognizes α2.6-linked SA, the receptors for avian and human influenza viruses respectively. This data is in agreement with the general pattern of SA receptor distribution on wdNHBE cells cultured in vitro: α2.3-linked SA receptors are expressed predominantly on ciliated cells and to a lesser extent on nonciliated cells and α2.6-linked SA receptors are expressed mainly on nonciliated cells and to a lesser extent on ciliated cells [267].

To study the replication kinetics of the H5 reassortant viruses, wdNHBE cells were inoculated with an MOI of 0.02. Growth medium was added for ten minutes to harvest virus at fixed time points. None of the H5N1-pH1N1 (Fig 2A), H5N1-H3N2 (Fig 2B) and H5N1-sH1N1 (Fig 2C) reassortant viruses replicated to higher virus titers then wtH5N1 in wdNHBE cells. Some H5 reassortant viruses (H5-pH1NS, H5-H3NA,M,NS and H5-sH1NA,M) even displayed virus titers at 48 hours p.i. that were >1.5 log10 TCID50/ml lower compared to the wtH5N1 virus titer.

H5N1-pH1N1 reassortant viruses in ferrets

The ferret model was used to select for H5N1-pH1N1 reassortant viruses with highest replication in vivo, and to evaluate the ability of this virus to be transmitted via aerosols or respiratory droplets. Two ferrets were inoculated intranasally with $10^{7.3}$ TCID50 of the MDCKP1 H5N1-pH1N1 reassortant virus mixture.

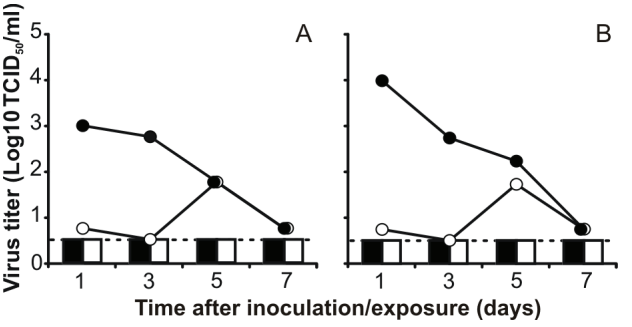

Figure 3. Replication and transmission of H5N1-pH1N1 reassortant virus in ferrets

Two ferrets were inoculated intranasally with 107.3 TCID50 of the MDCKP1 H5N1-pH1N1 reassortant virus mixture and subsequently housed individually in transmission cages (A, B). A naïve recipient ferret was added to a cage adjacent to each transmission cage at 1dpi (A, B). Virus titers in throat (black) and nose swabs (white) of the donor ferrets (lines) and recipient ferrets (bars) were determined by endpoint titration in MDCK cells. The lower limit of detection is indicated by the dotted line.

A recipient ferret was placed in a transmission cage adjacent to each donor ferret one day later. Throat and nose swabs were collected at 1, 3, 5 and 7 dpi and dpe. Virus shedding peaked in the inoculated animals at 1 dpi, with virus titers up to 10^4 TCID50/ml in throat swabs (Fig 3), and this shedding continued until 7 dpi. Overall, the amount and duration of virus shedding of ferrets inoculated with the reassortant H5N1-pH1N1 mixture was lower compared to those of wild type H5N1-inoculated ferrets in our previous experiment [150].

None of the viruses in the H5N1-pH1N1 reassortant mixture was transmitted to the recipient ferrets via the aerosol or respiratory droplet route, since no virus could be detected in the throat and nose swabs collected from the recipient ferrets (Fig 3).

The genetic composition of the H5N1-pH1N1 reassortant viruses in throat swabs collected from the donor ferrets as well as in the MDCKP1 virus stock that was used as inoculum, was analyzed using pyrosequencing. In the MDCKP1 virus stock, the vast majority of the PB2, PB1, PA, NP and NS genes was derived from H5N1, and these H5 genes remained dominant in both ferrets until 7 dpi: 95±5%, 100±0%, 98±2%, 90±4% and 96±3% respectively (Fig 4). In contrast, the NA and M gene segments of pH1N1 virus origin were dominant in the inoculum. In addition, these pH1N1 genes were still present in the reassortant mixture collected from ferrets at 7 dpi, although the proportion of these genes had decreased during the course of infection to 46±4% and 28±4% for NA and M respectively (Fig 4).

DISCUSSION

Genetic reassortment is an important mechanism in the evolution of influenza viruses yielding strains with novel genetic and phenotypic traits. At least two human

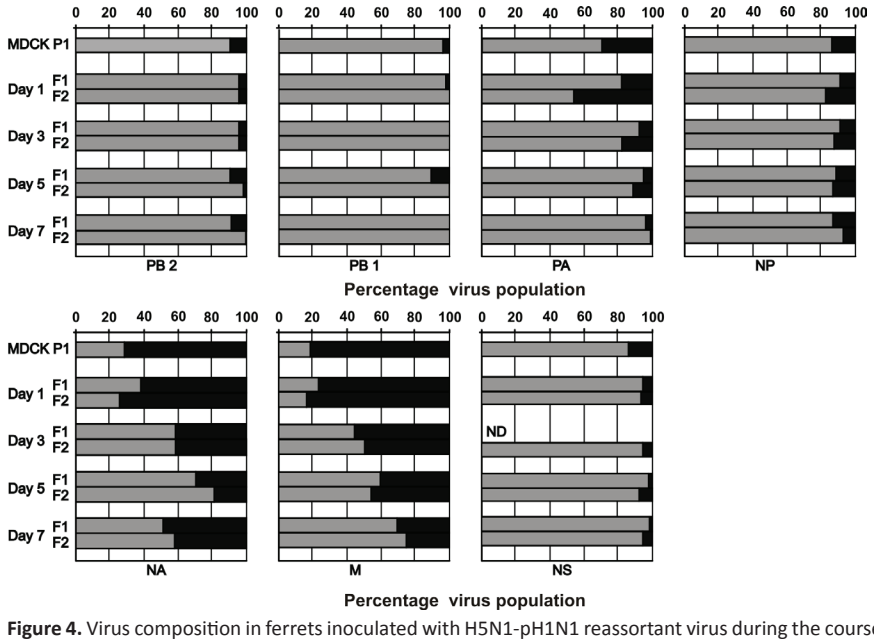

Figure 4. Virus composition in ferrets inoculated with H5N1-pH1N1 reassortant virus during the course of infection

Two ferrets were inoculated intranasally with 107.3 TCID50 of the MDCKP1 H5N1-pH1N1 reassortant virus mixture. At day 1, 3, 5 and 7 dpi throat swabs were collected from both ferrets (F1 and F2). In these throat samples, as well as the MDCKP1 inoculum, the ratio of the H5N1 (grey) and pH1N1 (black) gene segments (PB2, PB1, PA, NP, NA, M and NS) was determined by pyrosequencing. ND: no detection of viral gene segments by PCR.

influenza pandemics in the last century were linked to lineages where circulating human influenza viruses reassorted with influenza genes of non-human (probably avian) origin [30]. To study the potential of reassortment between the HPAI H5N1 virus and contemporary human influenza viruses (pH1N1, H3N2 and sH1N1), we used an in vitro selection method to identify the reassortant viruses that are most likely to emerge. The HA of the contemporary human influenza viruses was omitted in these experiments to make sure that only viruses containing an H5 HA were generated. In this way, a mixture of up to 128 (27) possible different reassortant viruses was generated that was subsequently passaged in MDCK cells under limiting dilution conditions to allow selective outgrowth of viruses with high in vitro replication rates. It should be noted that virus replication in MDCK cells may not reflect natural selection of reassortant viruses in humans. However, we have previously shown that with this method, reassortant viruses with enhanced pathogenicity in ferrets could be identified, thereby emphasizing the usefulness of this method [269].

Analysis of the genetic composition of the viruses that were obtained using the in vitro selection method in the present study, showed that wtH5N1 virus was recovered in 1 out of 4 attempts in all three H5N1-human influenza genome mixing experiments. In all other attempts, the NA and M genes of pH1N1, H3N2 and sH1N1 as well as the NS gene of pH1N1 were selected by HPAI H5 virus, after replication in

mammalian cells (Table 2).
In addition, the ability of H5N1 and pH1N1 influenza viruses to reassort has recently been investigated by others. In this study, MDCK cells were coinfected with these two viruses, resulting in the selection of similar reassortant viruses, harboring the NA, M, and NS gene segments of pH1N1 [264]. In another study, ferrets were co-infected with avian H5N1 and human H3N2 resulting in reassortant viruses that had also incorporated the NA, M and NS gene segments of a human H3N2 virus [154]. Thus, our transfection based approach yielded similar data as with other methods.
When we studied the replication kinetics of the H5 reassortant viruses that were detected after in vitro selection in MDCK cells, we found that only a few H5 reassortant viruses had a slightly increased replication capacity compared to the wt H5N1 virus (Fig 1). This effect could for the most part be attributed to the M gene segment of pH1N1 and H3N2. In contrast, when the NA of H5N1 was exchanged by the NA of one of the three human influenza viruses, the replication kinetics were slower compared to the wtH5N1 virus, which is in agreement with previous findings when H5N1-H3N2 reassortment was studied [154].
In wdNHBE cells, HPAI H5N1 replicates to lower titers compared to pH1N1, H3N2 and sH1N1 (data not shown and [271]). However, when the replication capacity of H5 reassortant viruses was investigated, no increased replication was demonstrated for any of the H5 reassortants, when compared to wtH5N1. It is possible that this poor replication of H5N1 virus compared to the human influenza viruses is the result of the avian receptor specificity of H5N1. Modification of the receptor binding preference of H5N1 to the human type receptors may result in increased replication in wdNHBE cells, and may to some extent compensate for the need of reassortment with human influenza viruses.
The data obtained with the in vitro selection experiments showed that especially the M and NA gene segments from human influenza viruses are preferentially selected by avian H5N1. However when these reassortant viruses were investigated in vitro none of the reassortant H5 viruses had an apparent increase in replication capacity compared to wtH5N1. This observation may explain why wtH5N1 was also recovered in all three in vitro selection experiments.
To study if reassortment between H5N1 and pH1N1 can be beneficial for virus replication in mammals, ferrets were inoculated with a mixture of reassortant viruses (MDCKP1 virus stock) and the virus composition was determined at different time points during the course of infection using pyrosequencing. In the inoculum, the H5 polymerase complex genes PB2, PB1 and PA, as well as the H5N1 NP and NS genes were predominant, with only a small percentage of these genes being derived from pH1N1. Although the H5N1 polymerase genes did not contain the well-known mutations in PB2 (E627K or D701N) that have been shown to be required for optimal replication of avian influenza viruses in mammals [104,105], the proportion of the pH1N1 polymerase complex genes in the virus mixture remained low during the course of infection. In contrast, the proportion of the pH1N1 NA and M genes in the MDCKP1 virus mixture were higher than those of the H5N1 counterparts, but this

proportion decreased slowly over time. It should be noted that the assessment of the preference of reassortant H5 viruses for H5N1 or pH1N1 gene segments would be more reliable if all gene segments would be present in equal copy numbers in the inoculum. Unfortunately, the generation of reassortants by transfecting 293T cells with plasmids harboring the different gene segments apparently resulted in a biased virus population with already a strong preferred use of some of the H5 genes. However, gene segments that represented only a small proportion of the population (like the pH1N1 polymerase genes) should have increased in number if there would have been an apparent selective advantage for the viruses.

In the same ferret experiment, we investigated whether an airborne-transmissible H5N1 virus was present in the reassortant virus mixture. However, no aerosol or respiratory droplet transmission was detected, since no virus could be detected in respiratory samples collected from the naïve recipient ferrets. This may be the result of the low amount of virus that was shed by the donor ferrets, combined with the receptor binding preference of the H5N1 HA for α2,3-linked SA that are absent in the upper respiratory tract of ferrets. This switch in receptor preference was recently shown to be crucial for airborne transmission of H5N1 viruses [259,261]. However, H5N1 viruses that were only mutated to acquire a preference for human α2,6-linked SA receptors were not transmitted between ferrets, suggesting that additional genetic changes are needed [92,261]. Recently Imai et al. showed that in addition to receptor binding preference for α2,6-linked SAs, two additional mutations in HA are required to confer a H5N1-pH1N1 reassortant, carrying the H5 HA and the other genes from pH1N1, airborne-transmissible between ferrets [259]. Moreover, recently we discovered that a fully avian H5N1 virus, with a preference for for human α2,6-linked SA receptors, can acquire the ability to be transmitted between ferrets without the need for reassortment [261].

Although none of the reassortant viruses identified and evaluated in our study have an evident replication advantage over their parental viruses, the generated reassortant viruses were also not found to be severely attenuated. Given that only a few mutations are necessary to confer airborne transmission of a H5N1-pH1N1 reassortant between ferrets, the emergence of reassortant viruses between human and avian influenza viruses but also between human and porcine influenza viruses should be monitored carefully.

ACKNOWLEDGEMENTS

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CHAPTER 4
Transmission of influenza A viruses

4.1
Predicting Airborne Influenza Viruses: (Trans-) mission Impossible?

Erin M. Sorrell, Eefje J.A. Schrauwen, Martin Linster, Miranda De Graaf, Sander Herfst and Ron A.M. Fouchier

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ABSTRACT

Repeated transmission of animal influenza viruses to humans has prompted investigation of the viral, host, and environmental factors responsible for transmission via aerosols or respiratory droplets. How do we determine – out of thousands of influenza virus isolates collected in animal surveillance studies each year – which viruses have the potential to become airborne, and hence pose a pandemic threat? Here, using knowledge from pandemic, zoonotic and epidemic viruses, we postulate that the minimal requirements for efficient transmission of an animal influenza virus between humans are: efficient virus attachment to (upper) respiratory tissues, replication to high titers in these tissues, and release and aerosolization of single virus particles. Investigating airborne transmission of influenza viruses is key to understand – and predict – influenza pandemics.

INTRODUCTION

The virus or virus subtype that will cause the next influenza pandemic is a highly debated topic in the field. Some believe that only influenza virus subtypes H1, H2, and H3 can cause pandemics in humans, and therefore – beyond isolated cases of zoonotic infections – we should not worry about e.g. H5N1, H7N7 or H9N2 viruses for human health. Many believe that swine viruses, rather than avian viruses, are more likely to cause the next pandemic. However, beyond the fact that there will be future pandemics, there is little known in terms of the viral origin, subtype, and virulence of the next pandemic. One other assumption can be made: the virus will be transmissible via aerosols or respiratory droplets (shortened hereafter as airborne transmissible). Influenza A viruses are constantly undergoing genetic and phenotypic changes during their circulation in avian and mammalian species. Our knowledge of viral traits necessary for host switching and virulence has increased significantly over the last decade. However, what exactly determines airborne transmission of influenza viruses in humans has remained largely unknown. Only when we fully understand the viral (genetic and phenotypic), host, and environmental factors that drive airborne transmission can we start to make predictions about which influenza viruses may cause future influenza pandemics.

Past pandemics

Four major pandemics have been recognized for which viral genome sequence data is available. While it was initially proposed that the 1918 H1N1 Spanish influenza pandemic was caused by a wholly avian virus that adapted to humans [29], recent evidence suggests that some of its genes were derived from mammalian viruses circulating as early as 1911 [32]. The 1957 H2N2 Asian influenza pandemic resulted from the reassortment of avian HA, NA, and PB1 virus genes with the then circulating seasonal human H1N1 influenza virus [30]. The H3N2 Hong Kong influenza pandemic of 1968 was also a product of reassortment between avian and human virus genes; HA and PB1 genes of the H2N2 virus were replaced by those of an avian H3 virus [30]. In 2009, an H1N1 influenza virus of swine origin caused the first pandemic of the 21st century [50]. The gene constellation of this virus showed clear evidence of multiple reassortment events that had presumably occurred in pigs over a period of years [272] (Figure 1). The role of swine as a mixing vessel for the generation of reassortant influenza A viruses with pandemic potential is generally accepted, yet still underestimated (reviewed in [20]). However, it should be noted that reassortment can conceivably take place in avian or human hosts; for pandemics prior to 2009, there is no evidence that reassortment events occurred in pigs. Regardless of the identity of the mixing vessel it is important to emphasize that most, if not all, recent influenza pandemics were caused by reassortant viruses. The expansion in surveillance efforts in pigs in response to the 2009 pandemic will most likely reveal many more reassortant viruses that may or may not have the potential to infect and spread in humans.

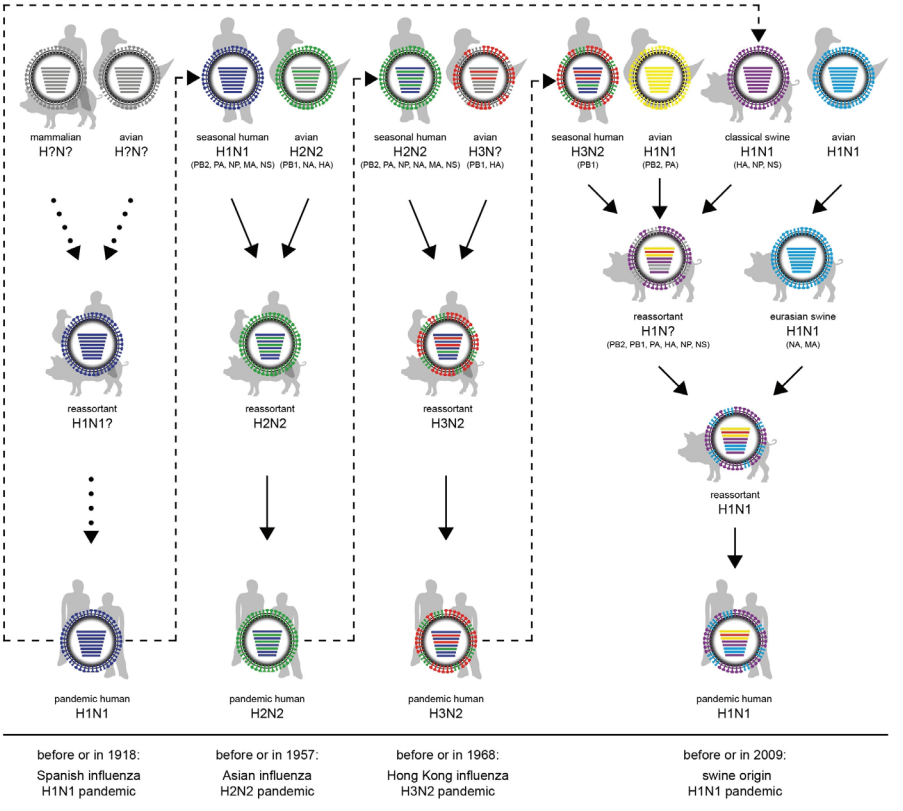

Figure 2. Reassortment and adaptation events of pandemic influenza A viruses
For the 1918 H1N1 "Spanish influenza" pandemic, evidence for two mutually exclusive scenarios has been presented: the gradual adaptation of avian genes to the human host and a reassortment event between avian and mammalian viruses. After 1918, the same H1N1 virus caused seasonal epidemics until 1957, when the H2N2 virus emerged upon reassortment between the seasonal H1N1 and an avian H2N2 virus, introducing the avian HA, NA, and PB1 genes. This H2N2 virus circulated in humans until 1968, when reassortment of the H2N2 with an avian H3 virus resulted in exchange of the H3 HA and PB1 genes to yield a new pandemic virus of subtype H3N2. The 2009 H1N1 pandemic virus included the NA and M genes of the Eurasian swine lineage, and the other genes of a "triple reassortant" swine influenza virus that had previously acquired its genes upon reassortment between human, avian, and (classical) swine viruses. Grey colour in virus particles indicates uncertainty of viral gene segment origin or lack of data. Dotted arrows indicate uncertain scenarios and solid arrows indicate events that are supported by scientific evidence. Dashed arrows represent pandemic viruses circulating in subsequent influenza seasons.

But how, out of thousands of animal influenza viruses from surveillance studies, can we select the ones we should prepare for as potential causes of new pandemics?

Viral determinants of transmission

Retrospective analysis of pandemic H1 (1918), H2 and H3 viruses has revealed that only one to two mutations in the HA receptor binding site are required to confer binding preference for virus receptors on cells of the upper respiratory tract (URT)

of humans, α2,6-linked sialic acids (SAs) [89]. Partially borrowing from this knowledge, several mutations in the HA protein, including Q226L, G228S, E190D, K193R, S227N and N182K, have been shown to change and/or increase receptor binding of avian H9N2 and H5N1 viruses to human URT tissues [74,90-93] with changes like Q226L improving replication and transmission of H9 viruses [273]. However, to date, none of the "designer" H5N1 viruses carrying these mutations have resulted in airborne transmission [92,274]. Efficient H5 transmission may thus require more subtle differences in receptor preference than simple α2,3 versus α2,6 SA linkage specificity.

Previous research has also pointed to key changes in the polymerase proteins that increase virus replication efficiency at 33 ºC, the accepted temperature for efficient replication in the mammalian URT [111,275]. These changes at positions 627, 701 and 591 of PB2 have also been shown to support transmission of multiple subtypes in mammalian models [108,110,151]. A decrease in association of the PB2 and NP proteins in mammalian cells is thought to be the mechanism behind the increase in replication efficiency [276].

PA and NP genes have also been associated with viral host restriction but the key amino acids have yet to be identified. Even fewer experiments have looked at the roles of NA, M and NS proteins in determining host range and transmission. It is likely that virus tropism, efficiency in replication, amount of virus shed, and the duration of shedding are important factors for transmission efficiency. A longer duration of peak virus shedding will increase the chance for the virus to reach susceptible host(s) and therefore increase transmission events [277].

Airborne transmission; size does matter

Human-to-human transmission of influenza viruses can occur through contact, direct or indirect, and/or respiratory droplets (large droplets and aerosols). The role of each has been well studied in mammalian models, focusing on the ferret and guinea pig (reviewed in [146] and Table 1). Opinions differ on the importance of each mode and data has been published to support each (reviewed in [278,279]). Efficient aerosolization of viral particles is however crucial for a virus with transmission efficiency and pandemic potential. There is no exact particle size cut-off at which transmission changes from exclusively large droplet to aerosol. However it is generally accepted that for infectious particles with a diameter of 5μm or less, transmission occurs through aerosols. Large aerosol droplets do not remain suspended in air and typically travel < 1m before settling on the mucosa of close contacts or environmental surfaces. In contrast, smaller particles, < 5μm, have a slow settling velocity and can thus travel further than large droplets [280]. Humans exhale droplets of widely varying size and quantity [281] and the generation of aerosol particles by coughing or sneezing has been well documented, with the majority of particles expelled during breathing and sneezing measured at < 1μm [282,283]. Evidence to support the role of aerosols in influenza transmission include the prolonged persistence of infectivity in aerosolized influenza at low humidity [284,285], transmission

Table 1. Mammalian Models for Influenza Transmission

Model/Species	Virus	Subtype	Transmission[a]	Reference
Mouse (MF-1/CFW)[1]	H1N1	Seasonal	A[1]	[295-298]
	H2N2	Seasonal	A[1]	[297]
		Pandemic	A[1]	[297]
Mouse (Balb/C)	H1N1	Seasonal	None	[289]
		Pandemic	None	[289]
	H3N2	Pandemic	None	[289]
	H5N1	HPAI	None	[289]
Ferret	H1N1	Seasonal	A	[299,300]
		Pandemic	A	[88,150,256,301]
	H3N2	Seasonal	A	[149,273,274]
		Pandemic		
	H1/H3	Oseltamivir resistant	D/None	[302-304]
	H2N2	Pandemic	D/A	[152]
	H7N7	HPAI	D	[305]
	H7N2		D/None	[305]
	H9N2		D/A	[153,273]
Guinea Pigs	H1N1	Seasonal	D[2]/A[2]	[289,307,308]
		Pandemic	D/A	[309]
	H3N2	Seasonal	D/A	[289,307,308]
	H1/H3	Oseltamivir resistant	D	[310]
	H5N1	HPAI	D[2]/None	[275,309]
	H1	Swine	A[2]/None	[108,308]
	H3N2	Swine	None	[308]
	H9N2		None	[308]
Hamsters	H1N1	Seasonal	D[2]	[311]
	H3N2	Seasonal	D	[311]

a A = aerosol, D = direct contact transmission, None = no transmission direct or aerosol
1 Transmission may have been due to differences in mouse strain and/or husbandry techniques (bacterial co-infections were likely to play a role in the 1960s studies)
2 Partial transmission found

to volunteers by aerosols reproducing the disease at doses much less than required by intranasal infections [286] and the abolishment of transmission when virus aerosolization is blocked with UV treatment of upper room air [278].

H?N?, the next pandemic

The continuing spread of highly pathogenic avian influenza (HPAI) H5N1 viruses in poultry and the consistent, albeit infrequent, transmission to humans with high mortality rates [59,287] has kept HPAI H5N1 on high alert as the next potential pandemic. It has been suggested that human-to-human transmission between family members in close contact has occurred [60-62] however, sustained human-to-human transmission has not been confirmed. It is this lack of human-to-human transmission that has prevented extensive infection, and therefore prevented an H5N1 pandemic.

The inefficient airborne transmission of HPAI H5N1 virus has been confirmed in

several mammalian models including ferrets, mice and guinea pigs (Table 1). Numerous studies with wild type H5N1 viruses, reassortants between H5N1 and human viruses, H5N1 viruses adapted by repeated passage, and "designer" H5N1 viruses with mutations known to increase virus binding and/or replication have failed to yield airborne H5 viruses, showing direct contact transmission at best [108,154,274,288,289]. This highlights the complexity of the mechanism(s) of influenza virus transmissibility and confirms that H5N1 viruses require further adaptation to become a pandemic threat.

Along with H5N1, H9N2 viruses have become enzootic in poultry in large parts of Eurasia [290]. These H9N2 viruses increasingly display human-like receptor specificity [74,273] and have occasionally transmitted to humans and pigs [71,291] with most human cases likely going unreported due to the relatively mild symptoms associated with infection. In the laboratory, H9N2 viruses have been shown to transmit to direct contact ferrets with no prior adaptation or mutations [273] and compared to H5N1 virus, were easily adapted after reassortment in a human H3N2 backbone to become airborne in the ferret model [153]. Recent work indicates that reassortment of an H9N2 virus, within the backbone of the pandemic 2009 H1N1 virus, supports aerosol transmission in the ferret without any further adaptation [292]. Therefore, currently circulating avian H9N2 viruses are able to create a potentially pandemic virus when provided the opportunity for reassortment with a human-adapted virus.

Virus design; why and how do influenza viruses become airborne?

The major challenges for influenza virus transmission research going forward are the types of studies needed to elucidate mechanisms for transmission. In our opinion, the focus should be on "gain of function" approaches rather than "loss of function". For the purpose of virus transmission studies, loss of function experiments are like destroying a car engine; remove any crucial part and the engine will stop running. In analogy, mutating a transmissible virus so it no longer transmits is a pointless exercise, giving us none to little mechanistic information; there are a thousand ways to accomplish that. Gain of function experiments mimic tuning the car's engine; only one or a few parts need tuning but the key is determining which part(s) out of the possible thousand they are. To investigate which viral parts need "a tune up" before it becomes transmissible, at least two options are available, and both should be followed. First, we could "replay" the evolutionary events leading to the pandemic viruses of e.g. 1957 and 1968. Which genetic changes in the avian-origin viral genes made these reassortant viruses airborne? Such experiments are now ongoing in several laboratories. Secondly, we can hypothesize, based on accumulated data, which viral characteristics would facilitate airborne transmission. Below, we discuss several of these features that we believe are important for influenza to become airborne. We postulate that the minimal requirements for airborne influenza viruses are 1) Attachment to and replication in appropriate cells of the URT; 2) High virus yields in the URT; 3) Virus shedding as single particles.

Attachment and Replication

Viruses with binding preference for α2,3 SAs can infect and replicate in human lungs and can lead to severe clinical symptoms and even death, yet these viruses are limited in their ability to infect the URT and subsequently transmit via aerosols (Figure 2A). Thus far, all transmissible viruses bind to α2,6 SAs and are capable of attaching to, replicating in, and transmitting from the URT (Figure 2B). However, the gross receptor binding profile alone does not guarantee transmission. Therefore, efficient transmission may require more subtle differences in receptor preference than α2,3 or α 2,6 SA linkage only. Glycan arrays and other assays for virus attachment may facilitate investigation of these subtle differences in binding specificity, but identification of the critical influenza virus receptors on the cells of the URT and LRT is needed to facilitate future research on host and tissue specificity as well as transmission.

High virus yields in the URT

When the extent of virus replication of airborne viruses is low – e.g. investigated through vaccination -, transmission generally does not occur [293,294] (Fig 2C). To date, all pandemic viruses have established efficient and productive infections in the URT (Fig 2D). The mechanism necessary for URT tropism has been linked to affinity for α2,6-SAs and adaptive mutations supporting replication at ~33oC, which optimizes molecular interactions between viral proteins and cellular host factors [108,110,111,151]. High levels of virus replication in the URT may ensure that large amounts of progeny virus are released into aerosols from the nose and mouth upon sneezing, coughing, or breathing.

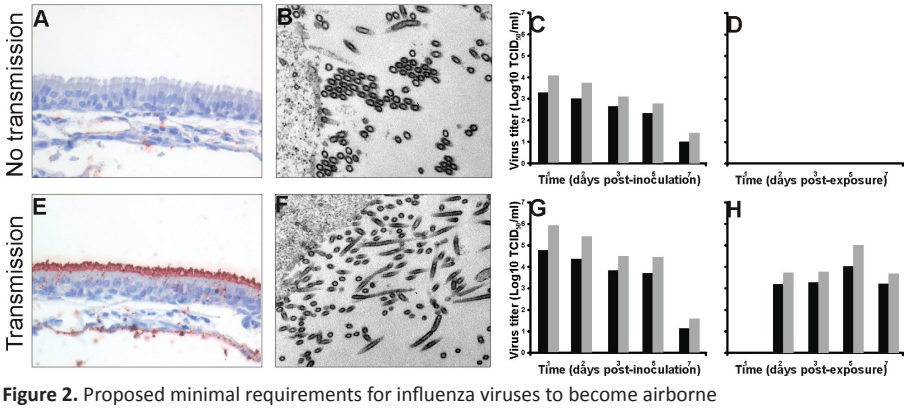

Figure 2. Proposed minimal requirements for influenza viruses to become airborne
Virus attachment to ferret URT is visualized with FITC-labeled viruses. (A) Shows no attachment of virus 1 (non-transmissible virus) to URT whereas (B) shows red staining indicative for attachment of virus 2 (transmissible virus) to the ferret URT. Virus yield in the URT of ferrets is shown for virus 1 (C) and virus 2 (D), with grey bars representing virus shedding from nose swabs and black bars from throat swabs. Electron microscopy of viruses budding from human 293T cells. (E) Shows viral aggregates released in clusters of spherical particles of virus 1 while (F) shows single virus 2 particles being released from the infected cell as both spherical and filamentous virions.

Virus shedding as single particles

Low NA activity can result in inefficient cleavage of SAs and as a consequence, inefficient release and aggregation of virus particles resulting in little to no successive rounds of replication (Figure 2E). The substrate specificity and activity of NA and HA must be in some balance with respect to receptor binding and cleavage in order to maximize the yield of progeny shedding as single virus particles that are efficiently transmitted (Figure 2F). In addition to HA, the NA's role in virus transmission warrants further investigation.

Unknowns

There are many unknowns when it comes to airborne transmission. The above factors are only a starting point for research. More work is needed to elucidate the relative contribution of human influenza virus transmission via contact, (large) respiratory droplets, and aerosols. With respect to airborne routes, how much does sneezing and coughing add to transmission in comparison to breathing alone? How representative are the available animal models (guinea pigs, ferrets) for transmission in humans? Guinea pigs generally do not sneeze upon influenza virus infection yet transmit viruses via the airborne route and some viruses are not transmitted between ferrets despite frequent sneezing of the animals [146]. Neither of these results provide definitive proof for a role of coughing and sneezing in transmission. Other important questions are whether airborne viruses come from one or many cell types, whether virus shape is important for transmission, or specific virus stability and whether key changes that drive transmission of one viral subtype applicable to every other virus (subtype). Research and attention are focused on answering these questions regarding the mechanisms involved in airborne transmission, and it is crucial to sustain the current funding, energy and collaboration in order to answer these key questions.

ACKNOWLEDGEMENTS

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CHAPTER 4
Transmission of influenza A viruses

4.2
Airborne transmission of influenza A/H5N1 virus between ferrets

Sander Herfst, Eefje J.A. Schrauwen, Martin Linster, Salin Chutinimitkul, Emmie de Wit, Vincent J. Munster, Erin M. Sorrell, Theo M. Bestebroer, David F. Burke, Derek J. Smith, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus and Ron A.M. Fouchier

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ABSTRACT

Highly pathogenic avian influenza A/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 A/H5N1 virus by site-directed mutagenesis and subsequent serial passage in ferrets. The genetically modified A/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 A/H5N1 viruses. Four amino acid substitutions in the host receptor-binding protein hemagglutinin, and one in the polymerase complex protein PB2, 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 A/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.

Influenza A viruses have been isolated from many host species including humans, pigs, horses, dogs, marine mammals, and a wide range of domestic birds, but 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 [2]. Influenza A viruses belong to the family Orthomyxoviridae, which have a segmented RNA genome consisting of eight gene segments [5,312]. Segments 1-3 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 [15,313]. Segments 4 and 6 encode the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) respectively. HA is responsible for binding to sialic acids, 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 sialic acids 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 non-structural 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, while NEP is involved in the nuclear export of RNPs into the cytoplasm prior to virus assembly [5,312].

Influenza A viruses show pronounced genetic variation of the surface glycoproteins HA and NA [2]. 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 nine antigenic variants of NA have been recognized in wild birds, and are found in numerous combinations that are designated as virus subtypes (e.g., H1N1, H5N1, H7N7, H16N3), which are used in influenza A virus classification and nomenclature [1,2]. This classification system is biologically relevant, as natural host antibodies that recognize one HA or NA subtype will generally not cross-react with other HA and NA subtypes.

Influenza A viruses of the H5 and H7 subtypes can be further classified based on their virulence in chickens into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses. Viruses of subtypes H1-H4, H6, and H8-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 [176,314]. 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 the LPAI to the 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, and this cleavage facilitates systemic virus replication and results in mortality of up to 100% in poultry [98,156].

Since the late 1990s, HPAI A/H5N1 viruses have devastated the poultry industry of numerous countries of the eastern hemisphere. To date, A/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 cases of human deaths directly attributable to avian A/H5N1 virus were recorded [58]. Since 2003, more than 600 laboratory-confirmed cases of HPAI A/H5N1 virus infections in humans have been reported from 15 countries [59]. Although limited A/H5N1 virus transmission between persons in close contact has been reported, sustained human-to-human transmission of HPAI A/H5N1 virus has not been detected [60-62]. Whether this virus may acquire the ability to be transmitted via aerosols or respiratory droplets among mammals, including human beings, 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 [315,316], what determines airborne transmission of influenza viruses among mammals, a trait necessary for a virus to become pandemic, has remained largely unknown [92,153,154,274]. Therefore, investigations of routes 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 (i.e., genetic mixing) of animal and human influenza viruses [234]. 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 A/H5N1 and seasonal human influenza viruses do not yield viruses that are readily transmitted between ferrets [92,154,274,288]. Here, we investigated if A/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 A/H5N1 virus infections and fatalities in Indonesia remains fairly high [59] 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. Since no reassortants between A/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 A/H5N1 infection.

The ferret (Mustela putorius furo) was used as the animal model in our studies. Ferrets have been used in influenza research since 1933, because they are susceptible to infection with human and avian influenza viruses [317]. Following infection with human influenza A virus, ferrets develop respiratory disease and lung pathol-

ogy 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 [318] (Fig. S1) [150,210]. Host restriction of replication and transmission of influenza A viruses is in part determined by specific sialic acid (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 SA and α-2,6-linked SA are present throughout the respiratory and enteric tracts (Fig. S2) [319]. 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 the avian α-2,3- to the 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 [234]. 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 [108,110,151]. Whereas avian viruses in principle replicate at temperatures around 41ºC, the temperature in the intestinal tract of birds, for replication in humans the viruses need to adapt to 33 ºC, the temperature of the human URT. Amino acid substitution E627K in the polymerase complex protein PB2 has been associated with increased virus replication in mammalian cells at such lower temperatures [104,315,316]. In addition, when newly formed virus particles bud from the host cell membrane after virus replication, the NA that is present on the virus membrane facilitates the release of particles. For A/H5N1, this process is rather inefficient, and released particles tend to form virus aggregates [234]. Therefore a balance between the properties endowed by HA and NA may be required to generate single particles. These established effects were therefore 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 in 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 droplet to aerosol. However, it is generally accepted that for infectious particles with a diameter of 5 µm or less, transmission occurs via aerosols. Since we did not measure particle size during our experiments we will use the term 'airborne transmission' throughout this manuscript.

Biosafety and biosecurity concerns have remained foremost in our planning for this research program. The details are explained in the supporting online material and are summarized here. The enhanced Animal Biosafety Laboratory level 3 (ABSL3+) facility at Erasmus Medical Center (MC) Rotterdam 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 and Dutch ad United States government inspectors.

Before and during the research, biosafety officers of Erasmus MC and inspectors from the Dutch government, as well as from the United States Centers for Disease Control and Prevention, approved the facilities and procedures. Explicit permits for research on genetically modified airborne-transmissible A/H5N1 virus were obtained from the Dutch government. The research was performed strictly in accordance with the Dutch Code of Conduct for Biosecurity [320]. All personnel were instructed and trained extensively for working in the ABSL3+ facility, for handling (highly pathogenic) influenza virus, and in incident control (e.g., spills). To further prevent occupational risks, personnel use protective equipment and are offered seasonal and A/H5N1 influenza vaccines [318]. 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 A/H5N1 virus can acquire mutations that would increase the risk of mammalian transmission [321]. 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 α2,3-linked SA receptors to the human α2,6-linked SA receptors [90]. 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) in HA display attachment patterns similar to those of human viruses to cells of the respiratory tract of ferrets and humans [90]. Of these changes, we know Q222L and G224S together 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 [13]. N182K has been found in a human case of A/H5N1 virus infection [322].

Our experimental ratonale to obtain transmissible A/H5N1 viruses was to select a mutant A/H5N1 virus with receptor specificity for α2,6-linked SA shed at high titers from the URT of ferrets. Therefore, we used the QuickChange multi site-di-

rected mutagenesis kit to introduce amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S in the HA of wildtype A/Indonesia/5/2005, resulting in A/H5N1HA N182K, A/H5N1HA Q222L,G224S and A/H5N1HA N182K,Q222L,G224S. Experimental details for experiments 1 to 9 are provided in the Supplemental Online Material (SOM, [318]). For experiment 1, we inoculated these mutant viruses and the A/H5N1wildtype virus intranasally into groups of six ferrets for each virus (Fig. 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 post-mortem from the euthanized ferrets. Throughout the duration of experiment 1, ferrets inoculated intranasally with A/H5N1wildtype virus produced high titers in nose and throat swabs, by up to ten times more than A/H5N1HA Q222L,G224S, which was the mutant that yielded the highest virus titers of all three mutants during the 7-day period (Fig. 1). However, no significant difference was observed between the virus shedding of ferrets inoculated with A/H5N1HA Q222L,G224S or A/H5N1HA N182K, during the first three days when six animals per group were present. Of the viruses with specificity for α2,6-linked SA, A/H5N1HA Q222L,G224S thus yielded the highest virus titers in the ferret URT (Fig. 1).

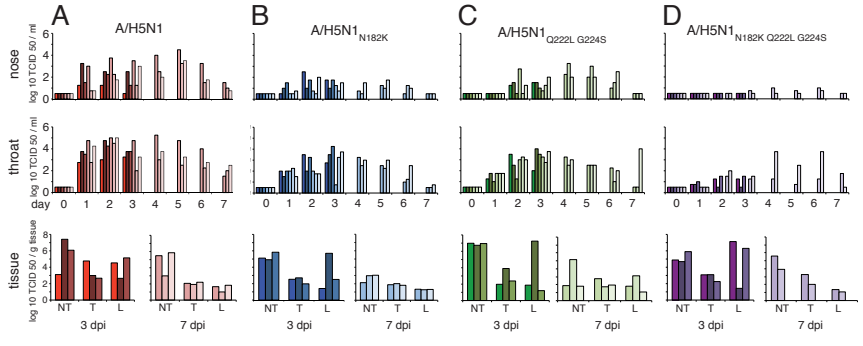

Figure 1. In experiment one, we inoculated groups of six ferrets intranasally with 1 x 106 TCID50 of (A) influenza A/H5N1wildtype virus and the three mutants (B) A/H5N1HA N182K, (C) A/H5N1HA Q222L,G224S, and (D) A/H5N1HA N182K,Q222L,G224S. Three animals were euthanized at day 3 for tissue sampling and at day 7, when this experiment was stopped. Virus titers were measured daily in nose swabs (top) and throat swabs (middle), and on 3 and 7 dpi in respiratory tract tissues (bottom) of individual ferrets. Virus titers in swabs and nasal turbinates (NT), trachea (T), and lung (L) were determined by end-point titration in MDCK cells. (One animal inoculated with A/H5N1HA 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 A/H5N1wildtype-inoculated animals. The mutant that yielded the highest virus titers during the 7-day period was A/H5N1HA Q222L,G224S, but titers were ~1 log lower than for the A/H5N1wildtype–inoculated animals. In the first 3 days, when 6 animals per group were present, no significant differences were observed between A/H5N1HA N182K- and A/H5N1HA Q222L,G224S-inoculated animals as calculated by comparing the viral titer (Mann-Whitney test, P=0.589 and P=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 marks a single animal.

As described above, amino acid substitution E627K in PB2 is one of the most consistent host-range determinants of influenza viruses [108,110,151]. For experiment 2 (Fig. S4), we introduced E627K into the PB2 gene of A/Indonesia/5/2005 by site-directed mutagenesis, and produced the recombinant virus A/H5N1HA Q222L,G224S PB2 E627K. The introduction of E627K in PB2 did not significantly affect virus shedding in ferrets, since virus titers in the URT were similar to those seen in A/H5N1HA Q222L,G224S-inoculated animals (up to 1 x 10^4 fifty-percent tissue culture infectious doses (TCID50)) (Mann-Whitney, p=0.476) (Fig. 1, Fig. S5). When four naïve ferrets were housed in cages adjacent to those with four inoculated animals to test for airborne transmission as described previously [150], A/H5N1HA Q222L,G224S PB2 E627K was not transmitted (Fig. S5).

Since 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 A/H5N1HA Q222L,G224S PB2 E627K virus and A/H5N1wildtype virus in the ferret URT (Fig. 2, Fig. S6). In experiment 3, one ferret was inoculated intranasally with A/H5N1wildtype and one ferret with A/H5N1HA Q222L,G224S PB2 E627K. Throat and nose swabs were collected daily from live animals until 4 days post inoculation (dpi), at which time the animals were euthanized to collect samples from nasal turbinates and lung. The nasal turbinates were homogenized in 3ml 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).

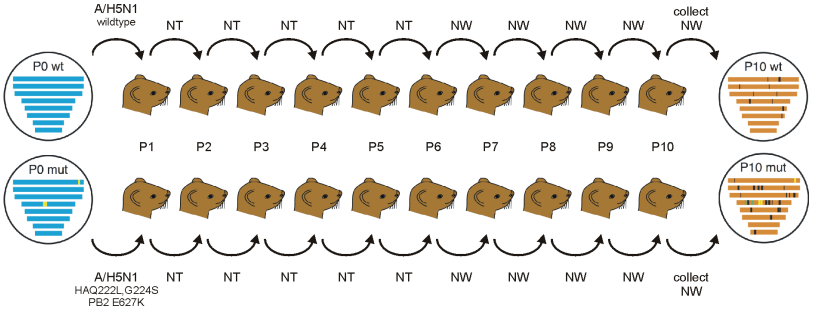

Figure 2. Experiment 3, virus passaging in ferrets. Since no airborne transmission was observed in experiment 2, A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K were serially passaged in ferrets to allow adaptation for efficient replication in mammals. Each virus was inoculated intranasally with 1 x 106 TCID50 in one ferret (2 x 250 µl, 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 (Fig. 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, 1 ml of PBS was delivered drop wise into 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 passages in ferrets. The passage-10 nasal washes were subsequently used for sequence analyses and the transmission experiments to be described in experiment 4. For details, see SOM.

This procedure was repeated until passage 6. From passage 6 onwards, 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 drop wise to the nostrils of the ferrets to induce sneezing. Approximately 200 µl 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 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. Since influenza viruses mutate rapidly, we anticipated that ten passages would be sufficient for the virus to adapt to efficient replication in mammals.

Virus titers in the nasal turbinates of ferrets inoculated with A/H5N1wildtype ranged from ~1 x 10^5 – 1 x 10^7 TCID50/gram tissue throughout the ten serial passages (Fig. 3A, Fig. S7). In ferrets inoculated with A/H5N1HA Q222L,G224S PB2 E627K virus, a moderate increase in virus titers in the nasal turbinates was observed as passage

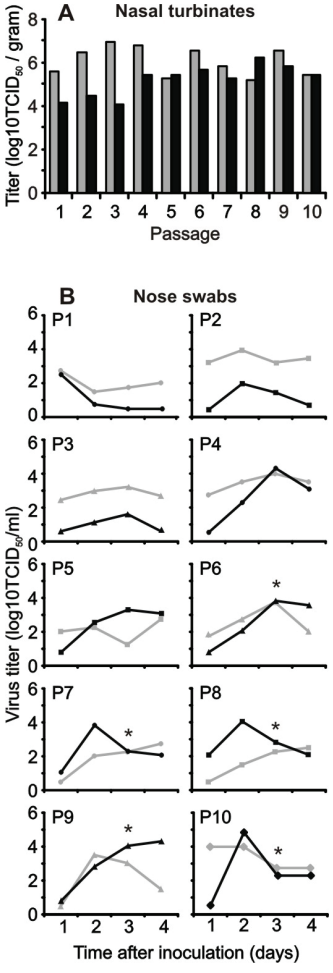

Figure 3. Virus titers in (A) the nasal turbinates collected at day 4 and (B) nose swabs collected daily until day 4, from ferrets inoculated with A/H5N1wildtype (in grey) and A/H5N1HA Q222L,G224S PB2 E627K (in black) throughout the ten serial passages described in Figure 2. Virus titers were determined by end-point titration in MDCK cells. After inoculation with A/H5N1wildtype, virus titers in the nasal turbinates were variable but high, ranging from 1.6 x 105 to 7.9 x 106 TCID50/gram tissue (panel A), with no further increase observed with repeated passage. After inoculation with A/H5N1HA Q222L,G224S PB2 E627K, virus titers in nasal turbinates averaged 1.6 x 104 in the first three passages, 2.5 x 105 in passage four to seven and 6.3 x 105 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 A/H5N1HA Q222L,G224S PB2 E627K (panel B). These titers also increased during the successive passages, with peak virus shedding of 1 x 105 TCID50 at 2 dpi after ten passages. Altogether, these data indicate that A/H5N1HA 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 1-4 dpi throughout the ten serial passages with A/H5N1wildtype revealed no changes in patterns of virus shedding. The asterisk indicated that a nose wash was collected before the nose swab was taken. This may influence the virus titer that was detected.

number increased. These titers ranged from 1 x 10^4 TCID50/gram tissue at the start of the experiment to 3.2 x $10^{5,1}$ x 10^6 TCID50/gram tissue in the final passages (Fig. 3A, Fig. S7). Strikingly, virus titers in the nose swabs of animals inoculated with A/H5N1HA Q222L,G224S PB2 E627K also increased during the successive passages, with peak virus shedding of 1 x 10^5 TCID50 at 2 dpi after ten passages (Fig. 3B).
These data indicate that A/H5N1HA 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 1-4 dpi throughout the ten serial passages with A/H5N1wildtype 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 [323]. The genetic composition of the viral quasispecies present in the nasal washes of ferrets after ten passages of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform (Table S1, S2). The mutations introduced in A/H5N1HA Q222L,G224S PB2 E627K by reverse genetics remained present in the virus population after 10 consecutive passages at a frequency >99.5% (Table S1, Fig 4). Numerous additional nucleotide substitutions were detected in all viral gene segments of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K after passaging, except in segment 7 (Table S1, 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 A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K was T156A in HA. This substitution removes a potential N-linked glycosylation site (Asn-X-Thr/Ser) in HA and was detected in 99.6% of the A/H5N1wildtype sequences after ten passages. T156A was detected in 89% of the A/H5N1HA Q222L,G224S PB2 E627K sequences after ten 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 SOM), we investigated if airborne transmissible viruses were present in the heterogeneous virus population that was generated during virus passaging in ferrets (Fig. S4). Nasal wash samples, collected at 3 dpi from ferrets at passage 10, were used in transmission experiments to test if 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 A/H5N1wildtype and four for passage 10 A/H5N1HA 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 allowed airflow from a donor ferret to a neighboring recipient ferret (Fig. S1) [150]. Although mutations had accumulated in the viral genome after passaging of A/H5N1wildtype 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

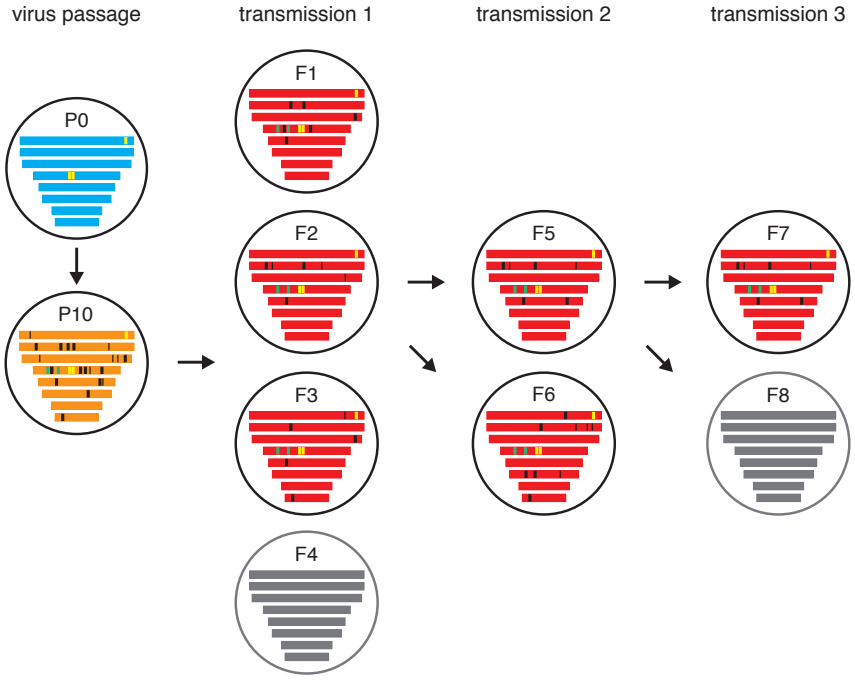

Figure 4. Summary of the substitutions detected upon serial passage and airborne transmission of A/H5N-1HA Q222L,G224S PB2 E627K virus in ferrets. The eight influenza virus gene segments and substitutions are drawn approximately to scale (top to bottom PB2, PB1, PA, HA, NP, NA, M, NS). Viruses in blue, orange, and red represent the initial recombinant A/H5N1HA Q222L,G224S PB2 E627K virus (P0), ferret passage ten virus (P10), and P10 virus after airborne transmission to recipient ferrets respectively. Viruses in grey indicate that virus was not transmitted to the recipient ferret. First, we tested if 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, F2 and F3). Next, we took a throat swab sample from F2 (this sample contained the highest virus titer among the positive recipient ferrets) and this virus 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 out of two recipient ferrets (F7). The genetic composition of the viral quasispecies 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 in genes indicate amino acid and nucleotide substitutions respectively. Substitutions introduced by reverse genetics are shown in yellow. Substitutions detected in passage ten and all subsequent transmissions are shown in green.

ten A/H5N1wildtype virus (Fig. 5A, 5B). By contrast, we did detect virus in recipient ferrets paired with those inoculated with passage ten A/H5N1HA Q222L,G224S PB2 E627K virus. Three (F1 - F3) out of four (F1-F4) naïve recipient ferrets became infected as confirmed by the presence of replicating virus in the collected nasal and throat swabs (Fig. 5C, 5D).

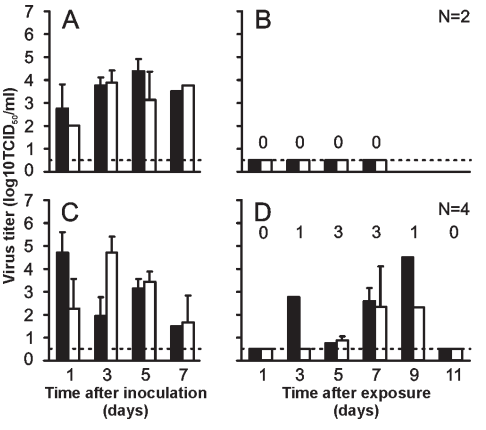

Figure 5. Airborne transmission of A/H5N1 viruses in ferrets. Transmission experiments are shown for A/H5N1wildtype (A, B) and A/H5N1HA Q222L,G224S PB2 E627K (C, D) after 10 passages (P10) in ferrets. Two or four ferrets were inoculated intranasally with nasal wash samples collected from P10 virus of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K respectively, and housed individually in transmission cages (A, C). A naïve recipient ferret was added to each transmission cage adjacent to a donor ferret at 1dpi (B, D). Virus titers in throat (black bars) and nose swabs (white bars) were determined by end-point titration in MDCK cells and geometric mean titers and standard deviations of positive samples are shown. The number of animals infected via airborne transmission is indicated in panel D for each time point after exposure; the drop from 3 animals infected at day 7 to 1 animal at day 9 and 0 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 line indicates the lower limit of virus detection.

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 and both of these animals, when placed in the transmission cage set up (Fig. S1), again transmitted the virus to the recipient ferrets (F5, F6)(Fig. 6A, 6B). 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, F8) in transmission cages, one (F7) of which subsequently became infected (Fig. 6C, 6D).

Conventional Sanger sequencing was used to determine the consensus genome sequences of viruses recovered from the six ferrets (F1-F3, F5-F7) that acquired virus via airborne transmission (Fig. 4, 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; H103Y that forms part of the HA trimer interface and T156A that is proximal, but not immediately adjacent to the RBS (Fig S8). Although several other mutations were observed, their occurrence was not consistent among the airborne viruses. This indicates 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, since, 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

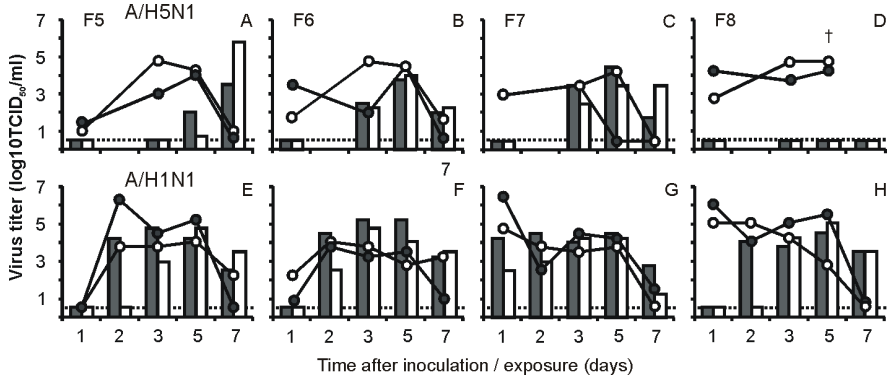

Figure 6. Comparison of airborne transmission of experimental passaged A/H5N1 and 2009 pandemic A/H1N1 viruses in individual ferrets. A throat swab sample from ferret F2 at 7 dpe (Fig. 5D) was used for the transmission experiments shown in (A, B) and a virus isolate obtained from a nose swab collected from ferret F5 at 7 dpi (Fig. 6A) was used for the experiments in (C, D). For comparison, published data on transmission of 2009 pandemic A/H1N1 virus between ferrets is shown (E-H) (27). 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 A/H5N1 (A - D), nose or throat swabs were not collected at 2 dpi and 2 dpe. Open circles and bars represent shedding from the nose and closed circles and bars represent shedding from the throat. The asterisk indicates the inoculated animal that died five days after intranasal inoculation.

in HA and one in PB2, may be sufficient to confer airborne transmission of HPAI A/H5N1 virus between mammals. The airborne-transmissible virus with the least number of amino acid substitutions, compared to the A/H5N1wildtype, was recovered from ferret F5. This virus isolate had a total of nine amino acid substitutions; in addition to the three mutations that were introduced (Q222L and G224S in HA and E627K in PB2) this virus harbored H103Y and T156A in HA, H99Y and I368V in PB1 and R99K 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 wildtype A/Indonesia/5/2005 virus at a dose of 1 x 10^6 TCID50, showed neurological disease and/or death [222,324]. It should be noted that inoculation of immunologically naïve ferrets with a dose of 1 x 10^6 TCID50 of A/H5N1 virus, and the subsequent course of disease is not representative for 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 [325,326]. It has been shown that mice and ferrets previously infected with an A/H3N2 virus are clinically protected against intranasal challenge infection with an A/ H5N1 virus [327,328].

Table 1. Lethality of wildtype and airborne-transmissible A/H5N1 virus in ferrets upon inoculation via different routes.

Inoculation route	Virus	Dead or moribund (No. dead/No. tested)	Day of death post inoculation (No.)
Intratracheal	**A/H5N1$_{wildtype}$**	6/6[1]	2(N=2), 3(N=4)
	A/H5N1/F5	6/6	3(N=6)
Intranasal	**A/H5N1$_{wildtype}$/P10**	2/2[2]	6(N=2)
	A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$/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/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$/P10	0/3	N.A.
	A/H5N1/F2	0/2	N.A.
	A/H5N1/F5	0/1	.N.A.

1. These data refer to a published study [211].
2. These ferrets were inoculated with passage 10 H5N1wildtype virus, but data are consistent with previous studies using larger groups of animals inoculated with the original strain [222,324].
N.A.; not applicable.

After intra-tracheal inoculation (experiment 5, Fig. S9), six ferrets inoculated with 1 x 10^6 TCID50 of airborne-transmissible virus F5 in a 3 ml volume of PBS died or were moribund at day three. Intratracheal inoculations at such high doses do not represent the natural route of infection, and are generally only used to test the ability of viruses to cause pneumonia [211], as is done for vaccination-challenge studies. At necropsy, the 6 ferrets revealed macroscopic lesions affecting 80-100 % of the lung parenchyma with average virus titers of 7.9 x 10^6 TCID50/gram lung (Fig. S10). These data are similar to those described previously for A/H5N1wildtype 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 a sensitivity to the antiviral drug oseltamivir similar to that of A/H5N1wildtype (Table S4). In experiment 7, we evaluated the recognition of the airborne-transmissible virus by antisera raised against potential A/H5N1 vaccine strains. Since 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, to allow 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 pre-pandemic vaccine strains (Table S5). In fact, the presence of the four HA mutations increased the reactivity with H5 antisera by two-fold or more. We subsequently used the same PR8/H5 chimeric virus in experiment 8 to evaluate the presence of existing immunity against the

Table 2. Receptor specificity of the different mutant A/H5N1 viruses as determined by a modified turkey red blood cell (TRBC) hemagglutination assay. Introduction of Q222L and G224S in the A/H5N1 HA resulted in a receptor binding preference switch from the avian α2,3- to the human α2,6-sialic acid receptor. Subsequent substitution of H103Y and T156A resulted in an increased affinity for α2,3- and α2,6-sialic acids, in agreement with glycan array studies [93]. For details, see SOM experiment 9.

		HA titer (HAU/50µl)		
Virus	**Subtype**	**TRBC**	**α2,3-TRBC**	**α2,6-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

airborne-transmissible virus in sera obtained from human volunteers over 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 A/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., α2,3-linked SA) to that of human strains (i.e., α2,6-linked SA)[90,92,329,330]. Amino acid position 103 is distal from the RBS, forms part of the trimer interface, and is unlikely to affect receptor specificity (Fig. S8). T156 is part of a N-glycosylation sequon and T156A (as well as N154K) would delete this potential glycosylation site (Fig. 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 A/H1 [331,332] and the virulence of A/H5 virus [333]. 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 α2,3-linked SA and α2,6-linked SA) and modified TRBC with either α2,3-linked SA or α2,6-linked SA on the cell surface was evaluated and compared to two reference viruses with known receptor binding preference: avian A/H5N1 and human A/H3N2 viruses. As expected and shown before, introduction of the Q222L and G224S mutations in the HA of A/H5N1 changed the receptor binding preference from α2,3-linked SA to α2,6-linked SA [90]. Furthermore, in our hands, the introduction of substitutions H103Y and T156A not only enhanced binding of A/H5N1HA Q222L,G224S PB2 E627K to α2,6-linked SA as expected from glycan array studies [93], but also increased the affinity for α2,3-linked SA. When these two mutations were introduced in the A/H5N1wildtype HA, the affinity for α2,3-linked SA also increased.

Substitutions Q222L and G224S have previously emerged in avian A/H2 and A/H3 viruses in nature [13,89] and mutations associated with similar changes in receptor binding specificity have been detected repeatedly in A/H5 viruses, e.g., substitu-

tion N182K has been reported nine times [93,322], 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 A/H5N1 viruses circulating in the field [334]. Only a minor fraction of the A/H5N1 viruses that have circulated in outbreaks have been sequenced (estimated to be <0.001 %) [334,335]. 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 [334], 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 [334]. Substitution E627K in PB2 has been found in ~27% of avian A/H5N1 virus sequences and in ~29% of human A/H5N1 viruses [334]. 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 [334].

Investigations of viral quasispecies during a massive avian influenza A/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 [95,336,337]. Given the large numbers of HPAI A/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 be simply a matter of chance and time for a human-to-human transmissible A/H5N1 virus to emerge.

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 or S590G/R591Q in PB2 yield a similar phenotype as E627K [110]. N182K and other substitutions in the RBS of HA may yield a similar phenotype to Q222L/G224S [90]. Such mutations should be considered for A/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 A/H5 virus [259]. This airborne virus contained seven genes of the 2009 pandemic A/H1N1 virus (which has S590G/R591Q in PB2 rather than E627K), with the HA of A/H5N1 virus A/Vietnam/1203/2004 [259]. These data indicate that different lineages of A/H5N1 virus and different amino acid substitutions that affect particular biological traits (receptor binding, glycosylation, replication) can yield airborne transmissible A/H5N1 viruses.

Although our experiments showed that A/H5N1 virus can acquire a capacity for airborne transmission, the efficiency of this mode remains unclear. Previous data has indicated that the 2009 pandemic A/H1N1 virus transmits efficiently among ferrets, and that naïve animals shed high amounts of virus as early as 1 or 2 days after exposure [150]. When we compare the A/H5N1 transmission data with that of reference [150], and keeping in mind that our experimental design for studying transmission

is not quantitative, the data shown in Fig. 5 and Fig. 6 suggest that A/H5N1 airborne transmission was less robust, with less and delayed virus shedding compared with pandemic A/H1N1 virus.
Although airborne transmission could be tested in a second mammalian model system such as guinea pigs [289], 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 A/H5N1 virus lineages [338], 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 A/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 [321].

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Supporting Material for

Airborne transmission of influenza A/H5N1 virus between ferrets

Sander Herfst, Eefje J.A. Schrauwen, Martin Linster, Salin Chutinimitkul, Emmie de Wit, Vincent J. Munster, Erin M. Sorrell, Theo M. Bestebroer, David F. Burke, Derek J. Smith, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus, Ron A.M. Fouchier

Risk assessment, Biosafety, Occupational health
Materials and Methods
Figures S1 to S10
Table S1 to S6

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 [339]. 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 [340].

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 [320]. 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 (-30Pa) 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 (< -200Pa). 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 A/H5N1 influenza vaccines with informed consent. Additional immunizations with A/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 (A/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 [171,208]. 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 (Aligent, 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 [171].

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, 2mM glutamine, 1mM sodium pyruvate, and non-essential amino acids.

Virus titration in MDCK cells

Virus titrations were performed as described previously [150]. 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 200µl of infection media, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodium bicarbonate, 10mM 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 [233].

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 FIGURES AND DATA

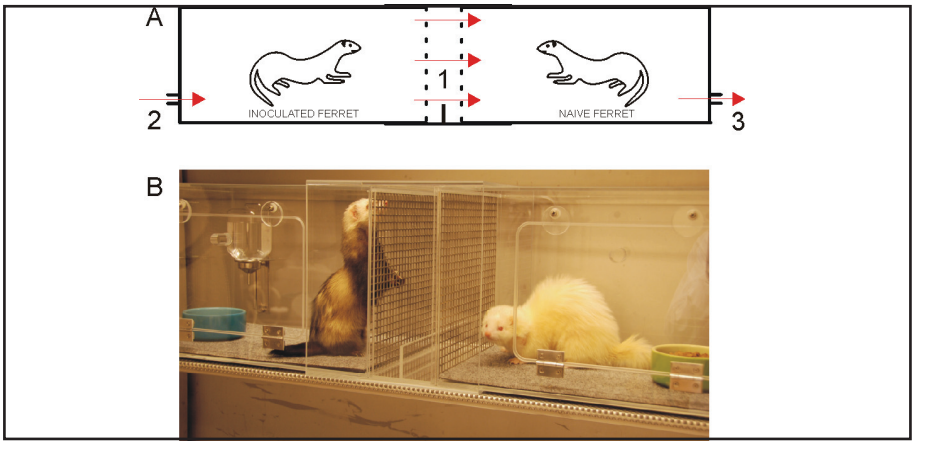


Figure S1. Schematic presentation (A) and a photograph (B) of paired transmission cages. The transmission cages were specifically designed to allow transmission experiments to be conducted in negatively pressurized isolator cages (1.6m x 1m x 1m) in the ABSL3+ facility. The ferrets are housed in clear Perspex cages, in which each inoculated animal was housed individually next to a naive ferret. Each ferret cage was 30 cm x 30 cm 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.

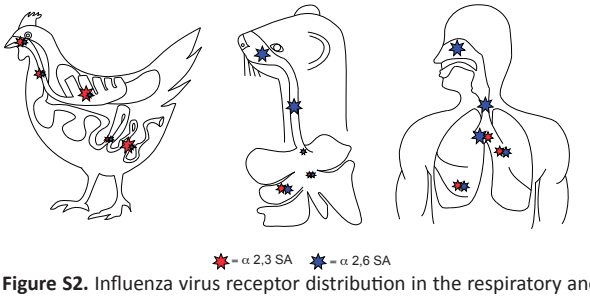


Figure S2. Influenza virus receptor distribution in the respiratory and enteric tract of chickens, and the respiratory tract of ferrets and humans. Avian and human influenza viruses preferentially bind to α-2,3-linked (red) and α-2,6-linked (blue) sialic acid (SA) receptors, respectively. In chicken, 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 [319]. 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 A/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 106 TCID50 of A/H5N1wildtype, A/H5N1HA N182K, A/H5N1HA Q222L,G224S, or A/H5N1HA N182K,Q222L,G224S (2 × 250 µL, divided over both nostrils), respectively (Fig. 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.

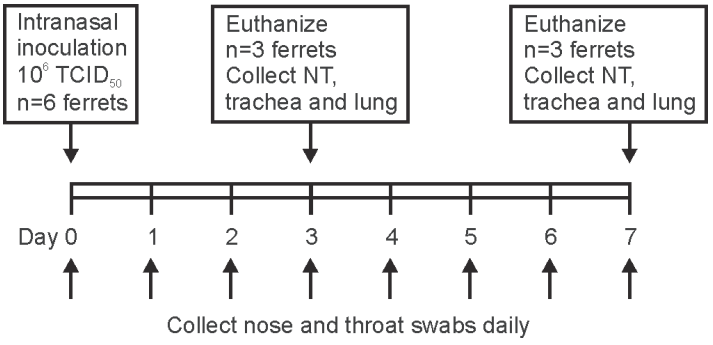


Figure S3. Schematic overview of experiment 1 to evaluate replication of A/H5N1wildtype, A/H5N1HA N182K, A/H5N1HA Q222L,G224S, or A/H5N1HA N182K,Q222L,G224S in ferrets. NT; nasal turbinates.

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 A/H5N1 virus with specificity for the human α2,6-linked SA receptor, we introduced this E627K mutations in virus A/H5N1HA Q222L,G224S that was used in experiment 1. Airborne transmission experiments were performed as described previously [150]. In short, four female adult ferrets were inoculated intranasally with 1 x 106 TCID50 of A/H5N1HA Q222L,G224S PB2 E627K by applying 250µL of virus suspension to each nostril. Each donor ferret was then placed in a transmission cage (Fig. 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.

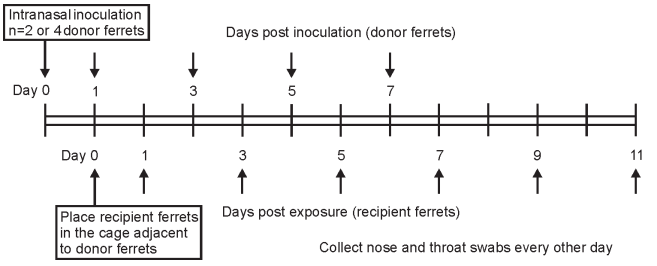


Figure S4. Schematic overview of experiments to test airborne transmission of influenza virus in experiments 2 and 4.

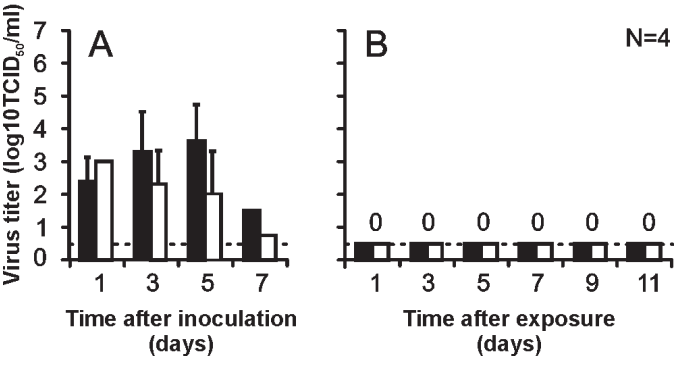


Figure S5. Shedding of influenza virus A/H5N1HA Q222L,G224S PB2 E627K in ferrets in transmission experiments (experiment 2). (A) Nose (white bars) and throat swabs (black bars) were collected on days 1, 3, 5, and 7 after inoculation from ferrets inoculated intranasally and (B) on days 1, 3, 5, 7, 9, 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, A/H5N1wildtype and A/H5N1HA 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 106 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 (Fig. 2, Fig. 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 A/H5N1wildtype and A/H5N1HA 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).

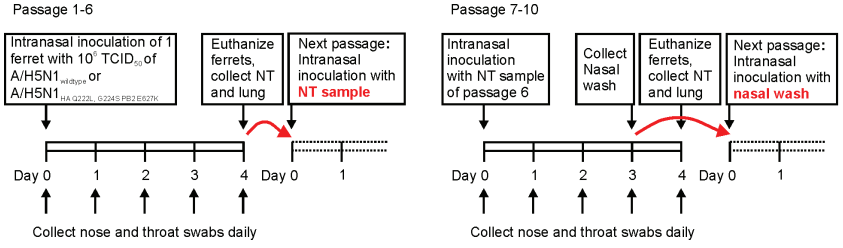


Figure S6. Schematic overview of experiment 3 to allow adaptation for efficient replication in the upper respiratory tract by serial passaging in ferrets. NT; nasal turbinates.

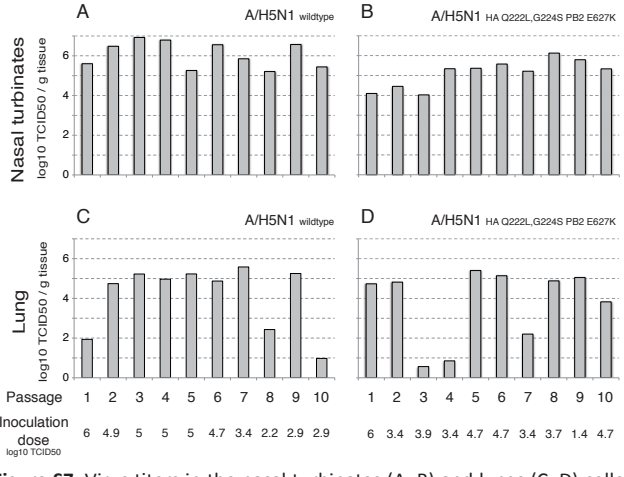


Figure S7. Virus titers in the nasal turbinates (A, B) and lungs (C, D) collected on day 4 from ferrets inoculated with A/H5N1wildtype (A, C) and A/H5N1HA Q222L,G224S PB2 E627K (B, D) throughout the ten serial passages as numbered on the x axes. Virus titers were determined by end-point titration in 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 A/H5N1wildtype, virus titers in the nasal turbinates were variable but high, ranging from 1.6 x 105 to 7.9 x 106 TCID50/gram tissue (panel A), with no further increase observed with repeated passage. After inoculation with A/H5N1HA Q222L,G224S PB2 E627K, virus titers in nasal turbinates averaged 1.6 x 104 in the first three passages, 2.5 x 105 in passage four to seven and 6.3 x 105 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 A/H5N1wildtype and A/H5N1HA 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 [341]. 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).

Table S1. Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with A/H5N1HA 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 A/H5N-1wildtype 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.

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).

Table S2. Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with A/H5N1wildtype 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 A/H5N1wildtype 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 (Fig. S4). Four and two donor ferrets were inoculated intranasally with nasal wash samples collected from passage 10 (P10) of the A/H5N1HA Q222L,G224S PB2 E627K and A/H5N1wildtype 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 A/H5N1HA 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 [342] using 32 primer sets that cover the full viral genome [341] 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, Fig. 4).

Table S3. Sanger sequence analysis of full viral genomes of airborne transmitted influenza viruses. Data are shown for individual ferrets (F1-F3, F5-F7) upon three subsequent transmission experiments, as described in figure 4 of the main text. The consensus sequence was determined for viruses isolated from the following samples: F1: throat swab 7 dpi, F2: throat swab 3 dpi, F3: throat swab 5 dpi, F5: throat swab 5 dpi, F6: nose swab 5 dpi and F7: throat swab 5 dpi. Substitutions detected in the virus samples that differ from the wildtype A/Indonesia/5/2005 virus are marked with "x". The introduced mutations are underlined.

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	

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

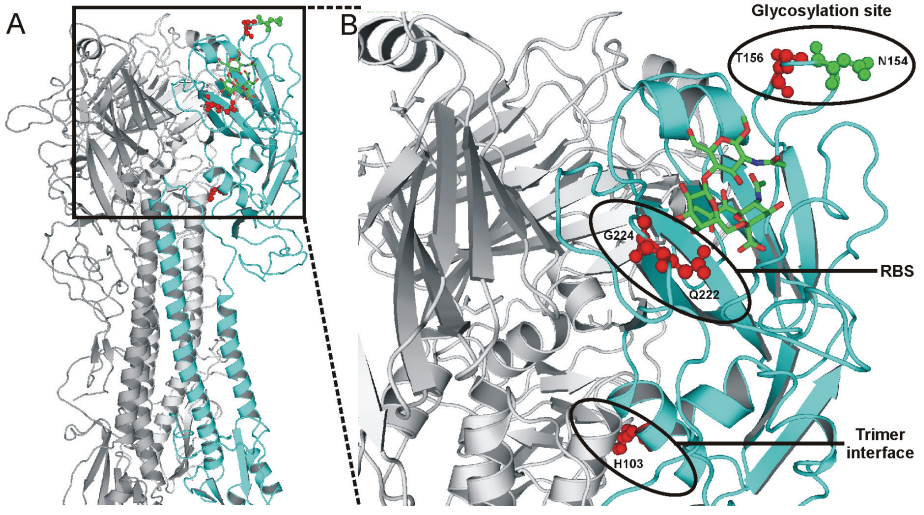


Figure S8. Cartoon representation of a model of the trimer structure HA of A/Indonesia/5/2005 (68) (PDB ID: 1JSM). One monomer is colored cyan for clarity. The structure of the three-sugar glycan NeuAcα2,6Galβ1-4GlcNAc is docked into the receptor binding site (RBS). The substitutions discussed in the 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 [343]. Loss of N-glycosylation sites at the tip of HA has been shown to enhance virulence of strains of H1 [331,332] and H5 [333]. Mir-Shekari et al. [332] 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 A/H5N1wildtype sequences after ten passages. While T156A was detected in only 89% of the A/H5N1HA Q222L,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 [90,92,329,330] 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 α2,6 linkage [329]. 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 106 TCID50 of virus in a 3 ml volume (Fig. 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).

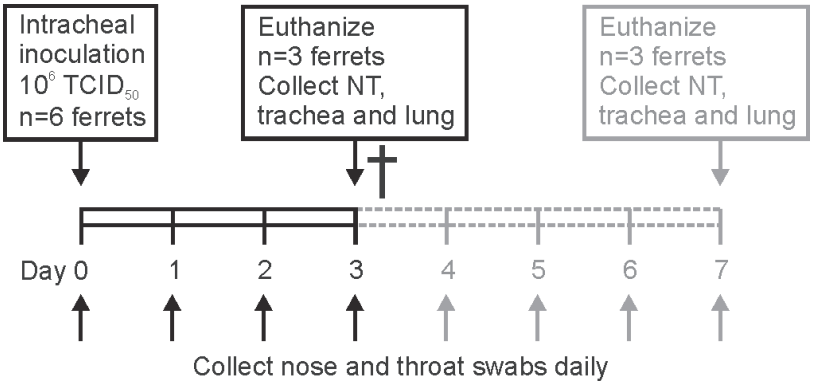


Figure S9. Schematic overview of experiment 5, to test whether airborne-transmissible H5N1 virus caused pneumonia upon high dose intra-tracheal inoculation. The experiment was halted at day 3.

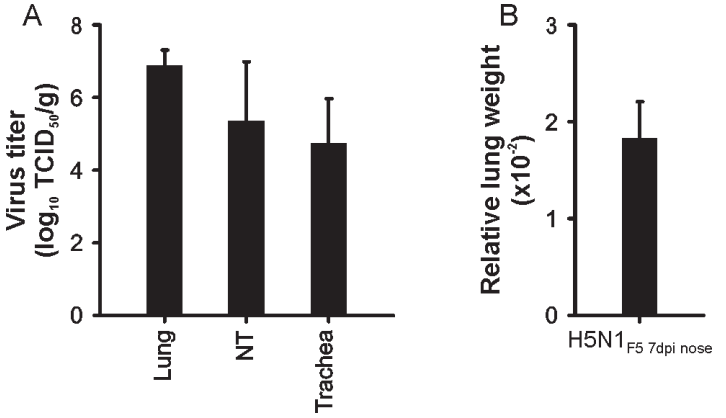


Figure S10. (A) Virus titers and (B) relative lung weight in six ferrets inoculated intra-tracheally with 1 x 106 TCID50 (in 3 ml) airborne-transmissible H5N1 virus obtained from the nose swab of transmission ferret F5 (A/H5N1F5 7dpi nose), which was passaged once in MDCK cells and used as inoculum. 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. Intra-tracheal inoculation with the wild-type A/H5N1 A/Indonesia/5/2005 virus had a similar effect [211]. 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 A/H5N1 virus obtained in this passage experiment (6.9 ± 0.4 log10 TCID50/gram) were slightly but not significantly higher than titers found in A/H5N1wildtype-inoculated animals (6.2 ± 0.6 log10 TCID50/gram) (p=0.093, Mann-Whitney). The relative lung weights of animals inoculated with A/H5N1wildtype were slightly higher compared to those of the A/H5N1F5 7dpi nose-inoculated animals (0,024 ± 0,006 and 0,018 ± 0,004 respectively, p=0.180, Mann-Whitney) [211].

Experiment 6

MDCK cells were seeded at 3x104 cells per well in 96-well plates (Greiner Bio-One) and cultured overnight. Virus stocks of interest were diluted to 1 x 103 TCID50/ml,

and added to cells in a volume of 100 µl per well. After incubation for one hour at 37 ºC, 100 µl of serially diluted oseltamivir carboxylate was added (range 170pM - 50µM) in four replicates. After three days of incubation at 37ºC, 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).

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

Table S4. Evaluation of the 50% inhibitory concentration (IC50) of oseltamivir for A/H5N1wildtype, A/H5N1HA H103Y,T156A,Q222L,G224S PB2 E627K and A/H5N1HA Q222L,G224S PB2 E627K. The 2009 pandemic A/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 [344]. Introduction of mutations found in airborne-transmissible A/H5N1 viruses did not affect the sensitivity of A/H5N1 virus to oseltamivir.

Experiment 7

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 [19]. 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 µL) were incubated with four hemagglutinating units of virus (in 25 µL) and incubated at 37 ºC for 30 minutes. Next, 25 µL of turkey RBCs were added, followed by one hour incubation at 4 ºC. 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 A/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 [90]. Due to this genetic modification, the HI assays could be performed under BSL2 conditions.

Experiment 8

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

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 A/H5N1wildtype, A/H5N1HA H103Y,T156A,Q222L,G224S PB2 627K and A/H5N1HA 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 A/H5N1 virus, compared with A/H5N1wildtype (indicated in italic), presumably owing to changes in the receptor binding site and loss of the potential glycosylation site [343].

	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

more likely to have had a history of exposure to pandemic influenza viruses), to the HA of the airborne-transmissible A/H5N1 and to the currently circulating 2009 pandemic A/H1N1, seasonal A/H1N1 and seasonal A/H3N2 viruses was investigated (Table S6).

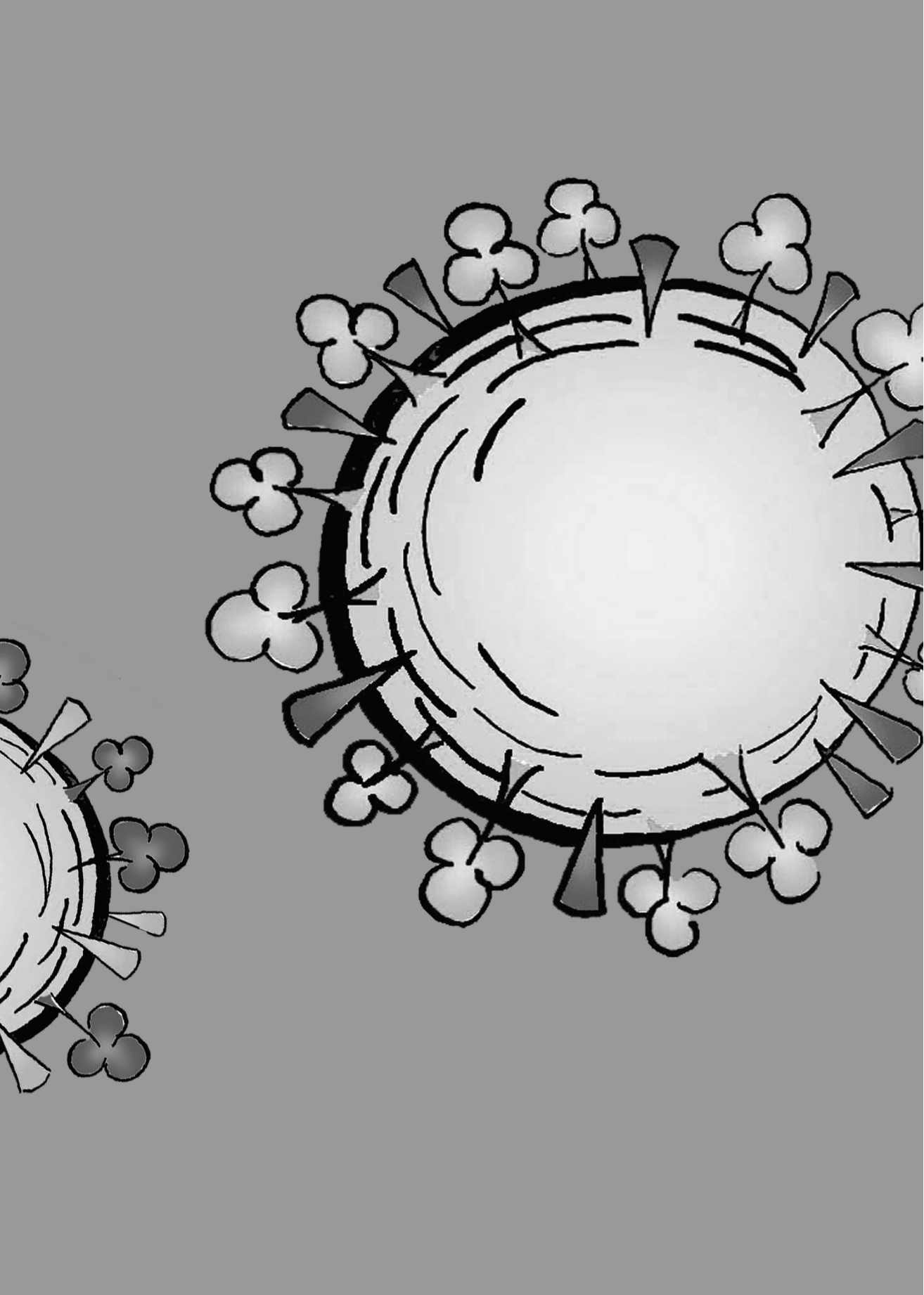
Experiment 9

For the experiment shown in Table 2 of the main text, modified turkey red blood cells (TRBC) were prepared as described previously [345]. Briefly, all α2,3-, α2,6-, α2,8-, and α2,9-linked sialic acids (SA) were removed from the surface of TRBC by incubating 62.5 µl 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 α2,3-(N)-sialyltransferase (Calbiochem, San Diego, CA) or 2mU of α2,6-(N)-sialyltransferase (Japan Tobacco, Inc., Shizuoka, Japan)

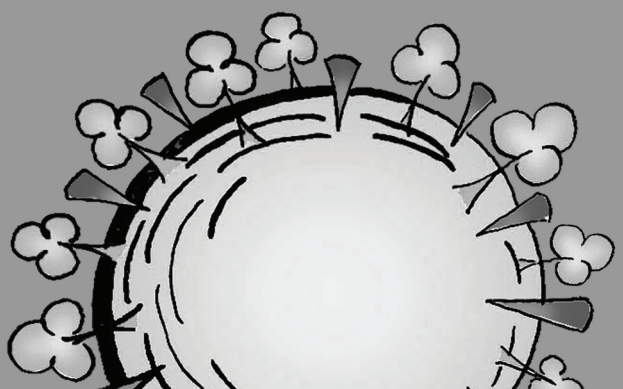
and 1.5 mM CMP-sialic acid (Sigma-Aldrich, Zwijndrecht, Netherlands) at 37°C in 75 µl for 2 h to produce α2,3-TRBC and α2,6-TRBC, respectively. After a washing step, the TRBC were resuspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5% TRBC. Resialylation was confirmed by hemagglutination of viruses with known receptor specificity; chimeric PR8 viruses with the HA of A/Vietnam/1194/2004 (H5N1, no MBCS; affinity for α2,3-TRBC) or A/Netherlands/213/2003 (H3N2; affinity for α2,6-TRBC). The receptor specificity of recombinant viruses with six gene segments of PR8 and the HA and PB2 gene segments of A/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 µl volume; 50 µl of 0.5% TRBC was added, followed by incubation for 1 hour at 4°C before determining the hemagglutination titer.

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 A/H5N1HA H103Y,T156A,Q222L,G224S PB2 E627K. Titers of human sera against the vaccine strains NYMC-IVR-148 (seasonal A/H1N1, A/Brisbane/059/2007), NYMC X-181 (pandemic A/H1N1, A/California/7/2009) and NYMC X-187 (seasonal A/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 A/H1N1 and A/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**



CHAPTER 5
SUMMARIZING DISCUSSION



SUMMARIZING DISCUSSION

The evolution of influenza viruses is an ongoing saga as (i) the RNA synthesis by the RNA polymerase is error-prone, (ii) the RNA genome of influenza A viruses is segmented thus allowing genome reassortment, and (iii) influenza A viruses circulate in multiple hosts.
Viral genome reassortment and the accumulation of point mutations contribute to the emergence of new influenza virus variants that might cause epidemics or pandemics. Moreover, the threat of an influenza A virus that can cross the species barrier is continuously present.

Subtype specific emergence of HPAI viruses

Avian influenza A viruses of the H5 and H7 subtypes have the ability to become highly pathogenic. The transition from low pathogenic avian influenza (LPAI) viruses to highly pathogenic avian influenza (HPAI) viruses has been observed only after introduction of LPAI viruses from wild birds into poultry. Despite extensive circulation in poultry of non-H5 and -H7 subtypes (e.g. H6N1 and H9N2), HPAI viruses have never emerged from these LPAI viruses. The highly pathogenic phenotype is the result of the introduction of a multibasic cleavage site (MBCS) in the HA gene. The absence of an MBCS in non-H5 or -H7 subtypes was at first thought to be due to compromised HA function. However, we describe in chapter 2.1 that the introduction of an MBCS in a non-H5 or -H7 avian influenza virus is not deleterious. We demonstrated that the insertion of an MBCS in an LPAI mallard H6N1 virus (H6N1MBCS) resulted in trypsin independency in vitro comparable to that of an HPAI H5N1 virus. An IVPI of 1.41 confirmed the highly pathogenic phenotype of H6N1MBCS, although, HPAI H5 and H7 viruses induce an even more pathogenic phenotype in chickens with IVPIs reaching the maximum of 3.00. After intranasal inoculation of chickens with H6N1MBCS virus, low virus shedding from the upper respiratory tract (URT) and low levels of systemic replication, indicated that further adaptation might be required for the H6 mallard virus to induce a full highly pathogenic phenotype in chickens. Other studies demonstrated that non-H5 and -H7 subtypes could support a highly pathogenic phenotype only in the appropriate HPAI viral background [346,347]. Veits et al. showed that the introduction of an MBCS in non-H5/H7 HAs, rescued in a low pathogenic chicken influenza virus backbone, was not sufficient to yield a highly pathogenic phenotype after oculonasal inoculation in chicken. However, an HPAI virus H5 HA in the same low pathogenic chicken influenza virus backbone did also not result in high virulence in chickens. This finding underlines the fact that other virulence and host-range factors or compensatory mutations need to be present to confer the highly pathogenic phenotype, which is in agreement with a study of Bogs et al [216]. However, it remains uncertain whether such additional virulence factors need to be obtained before or after the introduction of an MBCS.
For instance, when a modified H9N2 virus, with four basic amino acid residues at the HA cleavage site, was serially passaged in chickens, a highly pathogenic pheno-

type was acquired [348]. This was due to the loss of a glycosylation site near the cleavage site, which most likely contributed to the accessibility of HA to host proteases, as was shown previously [157,349-351].
We and others demonstrated that the restriction of HPAI viruses to the H5 and H7 subtypes is not the result of an inability of the HA to harbor an MBCS. Since the introduction of an MBCS into non-H5 and -H7 subtypes may result in a highly pathogenic phenotype, the emergence of HPAI viruses with other influenza virus subtypes is plausible. However, the observed HA subtype restriction in naturally occurring HPAI viruses indicates a unique predisposition of H5 and H7 HAs for an MBCS. This restriction might be due to a specific sequence and/or RNA structure of the H5 and H7 HAs. An MBCS acquisition does not always occur when LPAI viruses of H5 or H7 subtypes are introduced in poultry [352], suggesting that there are possible sequential and/or structural differences among viruses within one subtype.
The mechanism responsible for the incorporation of the MBCS in H5 and H7 is also unknown. However, several mechanisms have been proposed for the acquisition of an MBCS; (i) slippage of the influenza virus polymerase complex [179,353,354], (ii) stepwise introduction of amino acid substitutions [353,355] or (iii) recombination [356]. For H7 viruses, the manifestation of the highly pathogenic phenotype can be the result of recombination of the HA gene with ribosomal RNA [180]. Recombination between the HA gene and other viral genes, e.g. NP and MA, have also been described [181,356].
Under experimental conditions HPAI virus could be generated after sequential passaging of seal influenza virus in chicken embryo cells [357]. Similarly, after passaging of a wild swan LPAI H5N3 (R-E-T-R), a virus with a typical HPAI cleavage site (R-R-K-K-R) was obtained. However, this LPAI wild swan strain only became highly pathogenic in chickens after 24 consecutive passages by air sac inoculation, followed by five passages in the brain of one-day old chicks [358]. So far, HPAI viruses could only be obtained under experimental conditions that do not resemble the natural situation. Nevertheless, the mechanism by which the MBCS is acquired has still not been elucidated.
With the continuing emergence of HPAI viruses in poultry it is important to understand why and how the H5 and H7 subtypes have the ability to obtain an MBCS in nature.

HPAI viruses in mammals

Occasionally, HPAI viruses of the H5 and H7 subtypes are introduced from poultry into humans causing severe disease and even death. Other mammalian species including ferrets, mice, leopards, tigers, domestic dogs and cats, palm civets, stone marten, and foxes are also susceptible to HPAI H5N1 virus infection. In these species HPAI H5N1 virus can cause severe and/or fatal disease, with systemic viral replication [66,205]. In mice, removal of the MBCS from HA of an HPAI H5N1 virus resulted in a virus that caused only respiratory tract infection, in contrast to the systemic replication of the HPAI H5N1 virus, indicating that the MBCS is a major virulence

factor in mice [103]. However, experimental infection of non-human primates with HPAI H5N1 causes respiratory tract disease only [102]. The association between HA cleavability and replication outside the respiratory tract is less straightforward for influenza viruses in mammals than for poultry. In chapter 2.2, we demonstrate that the highly pathogenic phenotype is not supported by a human influenza virus in ferrets. Incorporation of an MBCS in the HA of a human influenza H3N2 virus (H3N2MBCS) resulted in trypsin independency of the virus in vitro, similar to that of HPAI H5N1 virus. However, the insertion of an MBCS did not result in increased virulence or a change in tissue tropism compared to the wildtype H3N2 virus. These findings suggest that, in addition to an MBCS, other factors are involved to induce systemic spread in ferrets. Groups of ferrets inoculated with either wildtype H3N2 or H3N2MBCS did not demonstrate virus replication in the lower respiratory tract (LRT), most likely due to the human receptor specificity of these viruses. Whether the LRT replication of HPAI H5N1 viruses contributes to systemic spread in ferrets remains to be determined. Since the avian receptor preference of the HPAI H5N1 virus is responsible for the robust infection of the LRT, it is plausible that the binding preference of influenza viruses plays an important role in the tissue tropism and subsequently the virulence of the virus.

In chapter 2.3, we determined by which routes HPAI H5N1 virus can spread systemically and demonstrated the role of the MBCS in extra-respiratory tract replication in ferrets. As the central nervous system (CNS) is the most common extra-respiratory site of replication in mammals, the olfactory route of infection was studied closely to show how HPAI H5N1 virus travels through the CNS. A clade 2.1 HPAI H5N1 virus replicated in the olfactory receptor neurons of ferrets which provided a direct route for H5N1 virus into the olfactory bulb from where it spread to the cerebrum and cerebellum. Infectious virus was isolated from cerebrospinal fluid (CSF) which is consistent with a case report in which HPAI H5N1 virus was detected in the CSF of a child [237]. Recently, Yamada et al. also showed the olfactory route as the main route of infection for ferrets that develop encephalitis [359]. They used a clade 1 HPAI H5N1 virus, demonstrating that virus entry via the olfactory route may be a general feature of HPAI H5 viruses. They also detected virus antigen in the epithelial cells of the Eustachian tube, the cochlea and the vestibulocochlear nerve, suggesting a second anterograde route of invasion, namely the vestibulocochlear pathway. The spread of HPAI H5N1 virus to the CNS via the olfactory route is a feature shared by the pandemic H1N1 virus. Van den Brand et al. observed virus replication in the olfactory mucosa, cerebrum and cerebellum suggesting that pandemic 2009 H1N1 also has the ability to become neurotropic and enter the CNS via the olfactory route [360]. This is consistent with findings in children with severe forms of encephalopathy associated with pandemic H1N1 virus [361,362]. However the pandemic H1N1 virus does not harbor an MBCS, indicating that the replication of this virus in the CNS is likely the result of another factor. In addition, some influenza viruses of the H1 subtype have this neurotropic feature by facilitating cleavage of HA through an additional mechanism which could depend on the viral NA [34,140].

In our study, HPAI H5N1 virus was also shown to spread via the hematogenous route in ferrets, as viral replication was observed in the pancreas, colon, liver and heart. These findings are consistent with other studies [214,359] and further investigation is needed to clarify the hematogenous spread in systemic infection with H5N1 virus. Suggested routes for the hematogenous spread of H5N1 virus are either via lymph, or directly into blood from a spill-over from alveolar capillaries.

The MBCS in HPAI H5N1 virus appears to be important for systemic spread in ferrets, since deletion of the MBCS from HPAI H5N1 (H5N1ΔMBCS) virus resulted in replication that was restricted to the respiratory tract. Replication of H5N1ΔMBCS virus in the URT of ferrets was reduced compared to wildtype H5N1; virus titers in the nose as well as the number of influenza virus-positive cells in the olfactory mucosa were lower in H5N1ΔMBCS-inoculated ferrets. As cleavage of HA of H5N1ΔMBCS virus is dependent on trypsin-like proteases, expression of these proteases is likely to be variable between different anatomical sites and cell types in the respiratory tract of ferrets. Although we cannot exclude that the H5N1ΔMBCS virus might be somewhat attenuated due to deletion of the MBCS.

The lack of systemic replication of H5N1ΔMBCS virus in mammals is most likely due to the absence of appropriate proteases outside the respiratory tract. The distribution of furin-like proteases and pro-protein convertases (PCs), which recognize multi basic motifs, plays an important role in pathogenicity and tissue tropism of influenza viruses [101,363,364]. These ubiquitously expressed proteases have the ability to cleave the consensus multi-basic motif present in HPAI viruses of subtypes H5 and H7, thus allowing these viruses to cause systemic infections. Novel type II transmembrane serine proteases, MSPL and TMPRSS13 have been recognized as HA processing proteases and are highly expressed in the lungs, brain and vascular epithelial cells of humans and chickens [365]. These proteases recognize a broader range of basic motifs from HPAI viruses. Further studies are required to determine the protease composition in extra-respiratory tract tissues and the role of proteases in cell tropism and pathogenicity of influenza viruses in mammals.

The extra-respiratory tract infection of HPAI H5N1 virus in mammals is most likely caused by multiple factors, in which the MBCS is essential. However, the presence of the appropriate receptors for virus attachment, presence of the proper proteases and the local innate immune response also influence the ability of influenza virus to replicate systemically. Additional studies are required to obtain a better insight into the expanded tissue tropism of HPAI H5N1 viruses in which the distribution of influenza virus receptors and proteases in extra-respiratory tract tissues should be determined.

Reassortment

The influenza A virus RNA genome is segmented, which allows the exchange of genes between different viruses when they co-infect a cell. This process, called reassortment, contributes to the emergence of new variants with potentially increased virulence and/or altered host range that might cause epidemics or pandemics. The

2009 pandemic H1N1 influenza virus (pH1N1) has a unique combination of gene segments originating from swine, avian and human influenza viruses. The viral PB2 and PA gene segments originate from north American avian viruses, the PB1 gene segment from a human H3N2 virus, the HA, NP and NS genes from classical swine viruses, and the NA and M genes from Eurasian avian-like swine viruses [50,272]. Since the pH1N1 virus co-circulated with seasonal H3N2 and H1N1 viruses, further reassortment with these viruses could possibly give rise to a more virulent virus.
After the emergence of pH1N1 virus, concurrent infections with the seasonal H1N1 and H3N2 viruses have been detected, thereby providing a possibility for reassortment to occur [23,24,252,366]. Reassortment between pH1N1 and seasonal H3N2 viruses has been observed in humans where co-infection resulted in reassortants that consisted of the HA and NA of seasonal H3N2 viruses and the remaining genes of pH1N1 virus [367]. However, these reassortant influenza viruses did not persist in the human population.
It is difficult to predict which potential reassortant variants could have a replication and/or transmission advantage over the wildtype pH1N1 virus as the generation and characterization of each reassortant (28=256 viruses) makes this approach cumbersome. To force reassortment, co-infections of ferrets with pH1N1 and either seasonal H1N1 or H3N2 virus can be conducted. However, when such experiments were performed, pH1N1 was able to outcompete the seasonal influenza viruses and to become the dominant virus [256]. To prevent the outgrowth of one dominant virus, we decided to design an in vitro selection method, as described in chapter 3.1 and 3.2. This method, using reverse genetics, allowed us to produce all gene segments at approximately similar copy numbers after transfection. Hereafter, newly formed reassortant influenza viruses with high replication efficiency were selected by serial passaging under limited dilution conditions.
In chapter 3.1, we describe that reassortment of pH1N1 with seasonal H3N2 virus can lead to an H1N2 virus, which is more virulent in ferrets compared to the pH1N1 virus. Reassortant viruses of pH1N1 with seasonal H1N1 PB2 (either with or without PA of seasonal H1N1) were found to be somewhat attenuated in ferrets compared to wildtype pH1N1 virus. Ferrets that were inoculated with a reassortant pH1N1 virus with seasonal H3N2 NA (H1N2) (either with or without the PB1 of seasonal H3N2) demonstrated higher virus antigen expression and more severe lesions in the lower respiratory tract compared to ferrets inoculated with wildtype pH1N1 virus. All reassortant pH1N1 viruses maintained the ability to be transmitted between ferrets. Altogether, this in vitro selection method allowed us to select reassortants between pH1N1 and seasonal H1N1 or H3N2 viruses and such reassortment events resulted in viruses with potentially increased virulence in ferrets.
Recently, Angel et al. attempted to overcome the problem that pH1N1 outcompetes the seasonal strains by performing co-infection studies in ferrets with viruses carrying the HA and NA genes of a seasonal H3N2 in the background of a pH1N1 virus and vice versa [368]. Surprisingly, after serial passage in ferrets, a dominant H1N2 virus population was observed where the NA and PB1 gene segments originated

from the seasonal H3N2 virus and the other six gene segments were derived from pH1N1 virus. Interestingly, by means of two different techniques, the pH1N1 virus was found to preferentially incorporate the NA and PB1 gene segments of a seasonal H3N2 virus.

The pH1N1 virus emerged as a result of reassortment of several influenza viruses from swine lineages where the PB1 gene segment was provided by a human H3N2 virus [50,272]. This could explain the selective replacement of the PB1 pH1N1 gene segment by its seasonal H3N2 virus counterpart.

The functional balance between the HA and NA proteins is essential for efficient replication of influenza virus [138,139]. It was shown that the pH1N1 HA and NA gene segments co-adapted, suggesting that a functional balance between HA and NA was required to initiate this pandemic. There is a functional match of low HA avidity and low NA activity of pH1N1 virus that corresponds to a similar balance of HA and NA activities observed for pandemic viruses of the last century [369]. Surprisingly, in the studies described above, another NA segment was preferentially selected to reassort with the pH1N1 virus. Although the functional balance of HA and NA is likely a required factor for efficient transmission of pH1N1 [370], transmission efficiency of the reassortant H1N2 virus was not altered. The interplay of the HA and NA genes from the reassortant H1N2 virus was likely to be enhanced, since both in vitro and in vivo replication of this virus was increased compared to that of the pH1N1 virus. To elucidate the exact mechanism that drives the enhanced interaction of the HA and NA gene segments derived from the H1N2 virus, functional studies should be performed.

Since the pH1N1 virus emerged after extensive reassortment in swine, it is important to continue the surveillance in swine to monitor novel swine influenza viruses which might have the ability to infect humans. A recent study showed rapid reassortment between co-circulating H1N1, H1N2, and H3N2 viruses in swine [371]. As a result, it is likely that further reassortment events will occur between currently circulating swine/human influenza viruses and the pH1N1 virus.

At least three of the previous pandemics emerged as the result of reassortment between animal and human influenza viruses. With the occasional HPAI H5N1 virus infection of humans, concerns have arisen of reassortment between avian H5N1 and human influenza viruses. In chapter 3.2 we describe the genetic compatibility between the gene segments of HPAI H5N1 virus and pH1N1, seasonal H3N2 or seasonal H1N1 viruses. Using the in vitro selection method as described above, only reassortant viruses that contained the H5 HA gene could be produced. We demonstrated that the NA and M gene segments of human influenza viruses were preferentially incorporated by H5 viruses. However, when the replication kinetics of these H5 reassortant viruses were studied in primary cell cultures, no increase in replication was observed. Previous in vitro or in vivo co-infection studies of HPAI H5N1 with either pandemic or seasonal H3N2 viruses also resulted in a preference for NA and M gene segments [154,264]. To study if reassortment between HPAI H5N1 and pH1N1 can be beneficial for virus replication in mammals and possibly confer air-

borne transmissibility, ferrets were inoculated with a mixture of reassortant viruses between HPAI H5N1 and pH1N1, from which the H1 HA was omitted. The genetic composition of reassortant viruses collected from infected ferrets, demonstrated the presence of the NA and M gene segments of pH1N1 virus at different time points during the course of infection. None of the viruses in the H5N1-pH1N1 reassortant mixture was found to be transmitted to the recipient ferrets via the airborne route.

Fortunately, HPAI H5N1 viruses have not reassorted with contemporary human influenza viruses in nature. This is partly contributed to the low probability of double infections of human and avian influenza viruses in one host. In addition, influenza viruses have to infect the same cell and similar level of adaptation to the host of are necessary to initiate gene reassortment. HPAI H5N1 viruses have undergone substantial reassortment with avian influenza viruses showing that they do have the capacity to reassort. This inter-subtype reassortment plays an important role in the evolution and variation of HPAI H5N1 virus and has been detected frequently. Since the emergence of HPAI H5N1 in 1997, reassortant influenza viruses have been detected in migratory birds, ducks, geese, chickens and other poultry, that contained the original H5 HA gene and the additional genes from other avian influenza viruses [372-374]. The extensive genetic reassortment of HPAI H5N1 viruses has lead to many H5N1 genotypes that resulted into multiple genetically distinct clades that have spread to at least 60 countries [260,375-378]. This inter-subtype reassortment is a characteristic shared by other influenza virus subtypes, like human H3N2 virus, since multiple reassortment events occurred between different lineages of H3N2 viruses [27,28]. This is in contrast to occasional reassortment events among different influenza virus subtypes, which suggests a preference for inter-subtype reassortment. Although a remarkable high rate of reassortment among avian influenza A viruses in wild birds was documented [379].

As both avian and human influenza viruses have the ability to infect swine, the risk of reassortant viruses arising from pigs is always present. Swine might play a critical role as a mixing vessel, since swine are susceptible to infection with both human and avian influenza viruses, as receptors for both viruses are present in swine trachea. However, the role of swine as mixing vessel in transmission of avian influenza viruses to humans may not be unique as humans can be infected with HPAI H5N1 and H7N7 viruses directly from an avian reservoir [380]. To further elucidate the potential role of swine in the emergence of human/avian influenza virus reassortants with pandemic potential, reassortment studies in ex vivo cultures from swine respiratory tract might be considered.

Airborne transmission of avian influenza virus

Human influenza A viruses are able to transmit via direct contact, indirect contact via fomites (contaminated environmental surfaces) and/or via aerosols or respiratory droplets (airborne transmission). Investigating the viral, host, and environmental factors responsible for transmission of animal influenza viruses between humans

is crucial. Only when we understand the underlying transmission mechanisms can we start to estimate the risk that animal influenza viruses pose for future human pandemics. The virus or virus subtype that will cause the next influenza pandemic is a highly debated topic in the field. Some scientist think that only influenza virus subtype H1, H2, and H3 can cause pandemics in humans, and therefore there is no need to be concerned about subtypes such as H5, H7, or H9.

Our knowledge of the viral traits required for host switching has increased significantly over the last decade. However, what exactly determines airborne transmission of influenza viruses in humans remains largely unknown. In chapter 4.1 we postulated the requirements for efficient airborne transmission of an animal influenza virus between humans [234]. We hypothesized that the minimal requirements for efficient transmission in mammals are: efficient virus attachment to (upper) respiratory tissues, replication to high titers in these tissues, and release and aerosolization of single virus particles.

The continuing spread of HPAI H5N1 viruses in poultry and the occasional transmission to humans resulting in severe disease has made HPAI H5N1 virus a top candidate to potentially cause a future pandemic. It is the lack of human-to-human transmission that has prevented an H5N1 pandemic so far. In chapter 4.2, we describe that a fully HPAI H5N1 virus can acquire the capacity for airborne transmission between mammals without the need of reassortment in an intermediate host. First, we genetically modified an HPAI H5N1 virus HA to obtain human receptor specificity (Q222L and G224S). Since the amino acid substitution E627K in PB2 has been associated with increased virus replication of avian viruses in mammalian cells, which might also be required for airborne transmission, this mutation was also introduced in the HPAI H5N1 virus. However, since the mutant virus harboring E627K in PB2 together with Q222L and G224S in HA did not transmit, we designed an experiment to force the virus to adapt to replicate in cells of the mammalian URT by repeated passage in ferrets. We found that the genetically modified HPAI H5N1 virus obtained additional mutations after passage and acquired the ability to transmit between ferrets. Surprisingly, besides the three mutations that were introduced by reverse genetics, only two additional mutations in HA (H103Y and T156A) were consistently detected in six airborne-transmissible virus isolates. The airborne-transmissible virus with the lowest number of amino acids substitutions had only nine mutations. Additional studies are necessary to identify which of the five to nine amino acid substitutions in this virus are essential to allow airborne transmission. In addition, it is important to determine whether these mutations would allow airborne transmission in other H5N1 genotypes and in other avian influenza A virus subtypes.

Around the same time, researchers at the university of Wisconsin-Madison reported that a virus containing the HPAI H5 HA (A/Vietnam/1203/2004) and the remaining seven gene segments from a pH1N1 virus, could confer airborne transmission in ferrets [259]. The HA of this airborne-transmissible virus had only four amino acid substitutions compared to the wildtype HA. The two publications on transmissible

H5N1 viruses indicate that different lineages of HPAI H5N1 viruses which contained functionally equivalent amino acid substitutions that affect receptor binding, glycosylation and replication, can become transmissible via the airborne route. The different amino acid substitutions from these studies and others that could confer the same biological traits are discussed below.

As we postulated in chapter 4.1, efficient virus attachment to cells in the URT might be required for airborne transmission between mammals. Therefore, as described above, we changed the binding preference of HPAI H5N1 virus from avian to human receptors, by introducing amino acid substitutions Q222L and G224S. From previous pandemics, we know that only two mutations in the HA receptor binding site may be required to confer binding preference for receptors on human URT cells [89]. When previous pandemic viruses were evaluated for their receptor recognition pattern, it was demonstrated that amino acid substitutions in HA resulting in a human receptor binding preference were a prerequisite for cross-species transmission and human adaptation of avian influenza viruses [88,152]. However, just changing the receptor specificity of HPAI H5N1 virus was not enough to confer airborne transmission in ferrets [92,259,261]. Inefficient transmission was observed between ferrets for a virus containing a clade 2.2 H5N1 influenza virus HA with human receptor specificity, a human NA and six other gene segments from a clade 1 HPAI H5N1 virus [92]. This study elaborated on the complexity of genetic changes in influenza viruses that could be responsible for the same biological traits and subsequently affect transmission. Imai et al showed that similar changes in the HA that affected the receptor binding preference (N220K and Q222L) could also yield an airborne-transmissible H5, however additional substitutions were present in HA [259]. To summarize, several studies have demonstrated that a human receptor preference is a prerequisite, but on its own not sufficient for an influenza A virus to become airborne-transmissible in the ferret model.

After serial passage in ferrets, two additional substitutions, H103Y and T156A, were consistently present in six H5 airborne-transmissible virus isolates [261]. The 103Y residue is part of the HA trimer interface and could alter the stability of the HA glycoprotein. Similarly, a T315I substitution was identified by Imai et al., which appeared to be essential for airborne transmission [259]. This amino acid substitution compensates for the altered stability of HA caused by the receptor binding mutations. Whether the H103Y substitution restores the same HA stability has not yet been elucidated, however preliminary data suggests that the H103Y residue is involved in HA stability as well. T156 is part of a putative N-glycosylation site that is located near the receptor-binding site, and mutation T156A would delete this glycosylation site. As shown previously, loss of the glycosylation site combined with acquisition of human receptor specificity enhances H5N1 virus binding to α-2,6–linked SA [343]. In addition, loss of this glycosylation site was found to be critical for H5N1 virus transmissibility in a guinea pig direct contact model [275] and H5N1 virus virulence in mice [333]. Interestingly, Imai et al. demonstrated that their H5 airborne-transmissible virus acquired a N154D substitution, that deletes the same

glycosylation site as T156A, suggesting that the loss of this glycosylation site is critical for H5 airborne transmission between mammals.
The E627K amino acid substitution is known to contribute to the host-range restriction of influenza A viruses. However, it has to be elucidated if this substitution is crucial for H5N1 airborne transmission and whether it can be substituted by functionally equivalent mutations. In the study of Imai et al, the airborne-transmissible virus contained the PB2 protein of an pH1N1 virus (which has G590S/Q591R rather than E627K) which also suggests the requirement of a human-adapted PB2 [259]. This was confirmed by a previous study on human H3N2 influenza virus, in which the introduction of the reverse mutation (K627E) resulted in less efficient transmission in guinea pigs. [108]. In addition, the K627E mutation was found to reduce transmission in a direct contact model for HPAI H5N1 virus. In contrast, introduction of D701N in combination with the K627E mutation resulted in more efficient transmission in mammals, a phenotype more similar to that of the wildtype viruses, suggesting that this residue can compensate for the absence of 627K. These data suggest that amino acids 627 and 701 in PB2 are determinants of mammalian transmission in diverse virus backgrounds; however we have to take into account that current transmission models are qualitative rather than quantitative.
In terms of virulence, it has been shown that E627K in PB2 is a critical virulence determinant for clade 2.3.4 H5N1 viruses, whereas D701N in PB2 and other unknown virulence determinants appear to be involved in the high pathogenicity of clade 1 H5N1 viruses [381].
The above-mentioned substitutions (E627K, D701N, and G590S/Q591R) have been shown to compensate for the lack of efficient polymerase activity of avian influenza A viruses in mammalian cells [104,110,115,382]. However, more studies are required to identify genetic determinants of efficient replication and transmission of avian influenza viruses in mammals. For this purpose we can use sequences of human and avian influenza viruses to identify species-specific amino acids. For example, 32 residues from PB2, PA, NP, M1 and NS1 were identified to be different between human and avian viruses where 13 were conserved among 1918, 1957 and 1968 pandemic influenza viruses [383]. These host-specific markers can help us identify the minimal requirements for virus adaptation and transmission of avian viruses to mammals and may have prognostic and diagnostic value for improving pandemic preparedness.
New research questions that arose after this research are important and should be addressed in the near future. An important question is whether the airborne H5N1 virus has retained its high virulence. Ferret experiments conducted with airborne H5N1 virus showed that it was highly virulent after intratracheal inoculation as ferrets were moribund or died by day 3. However, intratracheal inoculations at such high doses do not represent the natural route of infection and are generally only used to test the ability of viruses to cause pneumonia [211]. Noteworthy, the airborne H5N1 virus was not lethal after airborne transmission in ferrets. As shown previously, the route of inoculation determines the pathogenicity of H5N1 viruses

in ferrets [222]. Whereas the majority of ferrets will develop pneumonia after intratracheal inoculation, which was also shown in our experiments, ferrets that are inoculated intranasally with the wildtype H5N1 virus are likely to develop neurological symptoms, which indicates infection of the CNS as described in chapter 2.3. In addition, after intranasal inoculation of ferrets with wildtype HPAI H5N1 virus, blood-borne spread could be demonstrated. Inoculation of ferrets via an airborne route mimics the natural situation more closely, and should therefore be used to determine the virulence of airborne H5N1 virus. It is of importance to note that these studies have limitations since the virulence may vary with the dose of virus inoculation, and that virulence may be strain dependent. Innovative strategies are needed to further investigate the virulence of influenza viruses. For example, to study the virulence of airborne H5N1, ferrets could be inoculated using aerosol generators, to better mimic the natural situation.

The airborne H5N1 virus showed enhanced binding to α-2,6–linked SA but also retained some affinity for α-2,3–linked SA, most likely due to the loss of the putative N-glycosylation site. This affinity for both receptors will may result in a broader tissue tropism for the airborne H5N1 virus compared to H5N1 virus with a human receptor preference only. In chapter 2.3, we showed that wildtype HPAI H5N1 virus is able to infect the URT of ferrets. However, the majority of the infected cells were located in the olfactory epithelium that lines the caudal extremity of the nasal turbinates. It is plausible that the receptor specificity of airborne H5N1 virus facilitates replication in cells of the respiratory epithelium that line in the front of the nasal turbinates which would facilitate airborne transmission.

Another important determinant of the highly pathogenic phenotype of H5N1 virus is the MBCS. The airborne H5N1 virus has an MBCS, implying the ability to replicate systemically. However, we demonstrated in chapter 2.2 that an H3N2 virus with human receptor preference containing an MBCS was not able to induce systemic spread. Whether the human receptor specificity of airborne H5N1 virus will limit virus replication to the respiratory tract has yet to be elucidated.

The impact of the MBCS on transmissibility between mammals is unknown, since no highly pathogenic influenza viruses ever had the ability to be transmitted between mammals in nature. In chickens, it is plausible that the acquisition of an MBCS provides selective advantage for transmission via the respiratory tract since the oral-faecal route of transmission of LPAI viruses as observed in waterfowl may be suboptimal for poultry roaming in dry pens at high densities. A single amino acid substitution in the MBCS significantly reduced virulence without changing the transmission of an HPAI H5N1 virus in chickens [384]. It can be hypothesized that, in mammals, the MBCS might be disadvantageous for influenza virus transmission since the balance of the virulence and transmission should be optimal otherwise the basic reproduction number (R0) will decrease.

Whether an HPAI H5N1 virus with the ability of human-to-human transmission will ever emerge in nature remains unanswered. Several H5N1 strains with enhanced binding affinity to human-type α-2,6 receptors have been described in Indonesia

[322]. In addition, in Egypt new sublineages of HPAI H5N1 viruses have emerged in local bird populations with enhanced α-2,6–linked SA binding that retained binding affinity for α-2,3–linked SA [385]. These viruses expanded their receptor specificity from receptors in the human alveoli to the trachea. The emergence of these viruses was associated with an increase of human cases of H5N1 virus infection. In addition, some viruses that circulate in Egypt lost their putative N-glycosylation site and acquired the E627K substitution in PB2; both amino acid substitutions have been shown to be important in facilitating virus transmission in ferrets [259,261,386]. Phylogenetic analysis suggests that avian H5N1 viruses from Egypt lacking the glycosylation site transmit more readily from poultry to human than those that posses the glycosylation site [386]. These viruses, however, did not cause human-to-human transmission, which implies that these viruses require additional mutations. Together with our collaborators at the University of Cambridge and the University of Wisconsin-Madison, we assessed the likelihood that the substitutions, which confer airborne transmission between ferrets, could arise in nature [387]. A mathematical model of virus evolution in the host was used to study factors that could influence the probability of the remaining substitutions evolving after the virus infected a mammalian host. These factors combined with the presence of some of the substitutions in circulating H5N1 viruses, make a virus evolving in nature an actual potential threat. This data highlights critical research areas such as the importance of conducting routine surveillance and obtaining genetic data. Surveillance is the basis of infection control in the field. However, although genetic data is informative, the characterization of HPAI H5N1 viruses including antigenicity, pathogenicity, receptor binding preference and transmission is as important for investigating the evolution of HPAI H5N1 viruses. The continuous evolution of HPAI H5N1 virus, the increase in host range, and repeated infections of humans could eventually drive the virus to be transmitted between humans.

Identification and culling of infected and exposed flocks would limit the likelihood for humans and other mammals to become infected with HPAI H5N1 virus. However, this approach becomes difficult if the virus becomes enzootic in wild bird populations. China, Indonesia, Egypt, and Vietnam implemented vaccination after HPAI H5N1 became enzootic in domestic poultry [388]. Although vaccination helps to prevent virus transmission from infected animals, there are quite some drawbacks; (i) antigenically different groups of viruses, which are not cross-reactive, cocirculate in enzootic areas, (ii) H5N1 infects a variety of bird species and the optimal vaccination protocol may vary per species and (iii) vaccine driven evolution of avian influenza viruses may be stimulated [389].

The results of our work helps to better assess the risk posed by the current HPAI H5N1 epizootics for human health. In addition, it increases our fundamental understanding of the contribution of particular mutations or reassortments and their biological traits to transmission of the influenza virus. With the threat of avian-origin influenza viruses to cause future pandemics, this work has augmented value to estimate the potential risk. Recently, a novel reassortant avian origin influenza A

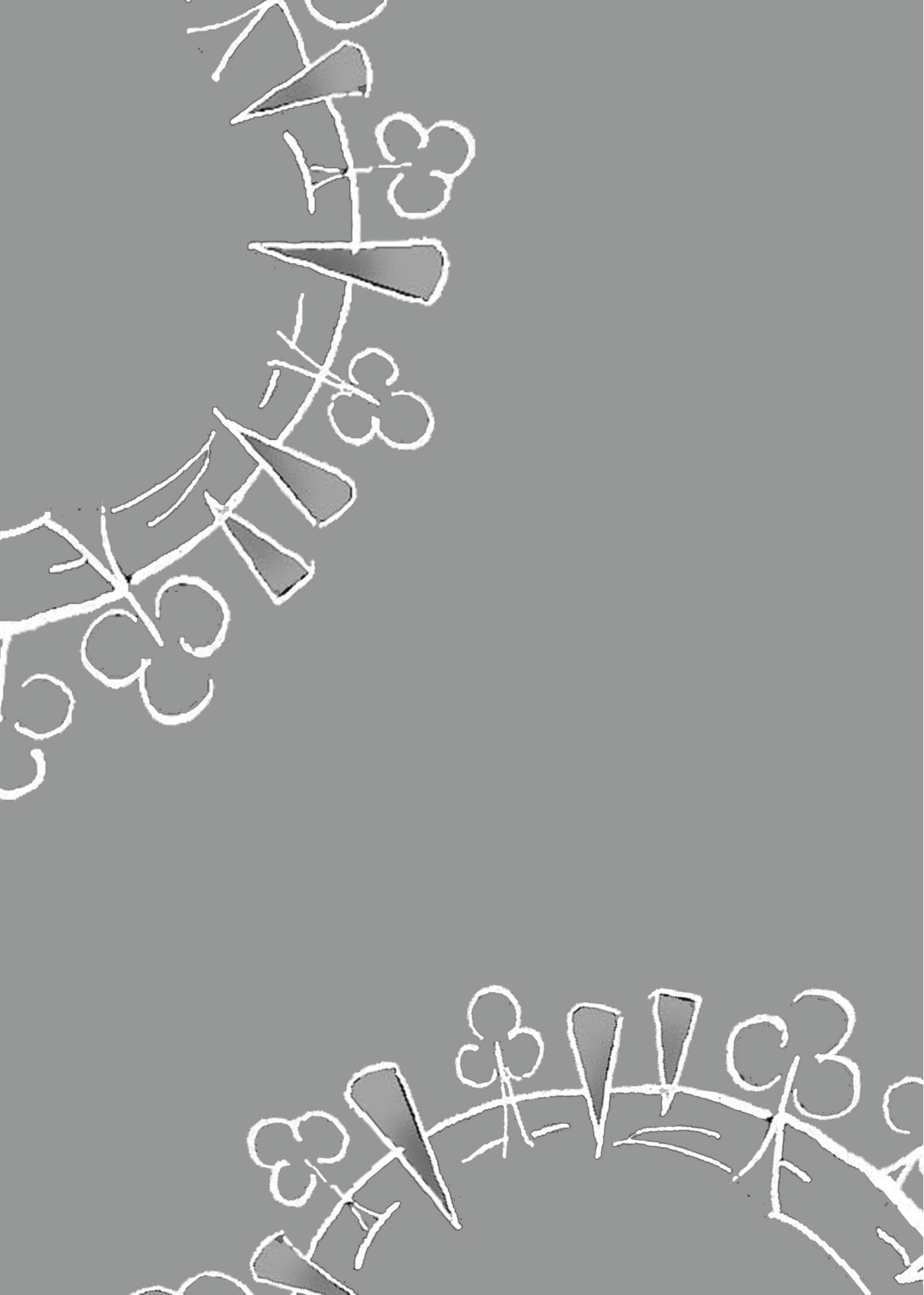
(H7N9) virus emerged in China. This novel reassortant H7N9 virus is associated with severe and fatal respiratory disease and is responsible for 108 confirmed human cases, which resulted in death of 22 cases up to now [70,390]. The introduction of this subtype to humans in Asia has not been observed previously. There is no indication thus far that it can be transmitted between people, however the pandemic potential should not be underestimated. Phylogentic analyses showed that there have been at least two introductions in human from different sources. The H7N9 virus has several characteristic amino acid substitutions in HA and PB2 which probably facilitate binding to the human receptor and efficient replication in mammals [391]. These viruses predominantly possess the Q222L residue that is responsible for human receptor specificity. Moreover, a T156A mutation leads to the loss of a glycosylation site, which results in increased virus binding properties. In addition, the human adaptation residue E627K was found in these human cases. Altogether, these H7N9 viruses possess several characteristic features of pandemic influenza viruses and airborne H5N1 virus, which may contribute to their ability to infect and transmit between mammals and therefore concerns rise regarding the pandemic threat. Our work on the H5N1 virus may help to better assess risks and may have value for prediction and prevention of a potential pandemic, and to evaluate intervention options.

Since it is impossible to predict which influenza viruses will preferentially emerge and may cause the next pandemic, it is key to continuously monitor the genetic composition of circulating influenza strains and to assess their infection and transmission potential in animal models. Some variables that are important for the probability of pandemic virus emergence upon reassortment are; (i) rate of reassortment, (ii) transmissibility of reassortant influenza viruses, (iii) the existence of cross-immunity acquired through previous infections [392]. Whether the next pandemic will be induced after genome reassortment only remains questionable, since a future pandemic might be triggered by direct transmission of a wholly avian virus to a human host without the requirement of reassortment.

Future perspectives

Here we provide insights into possible mechanisms of virulence, host adaptation, and airborne transmission of influenza A viruses. There is an increasing knowledge of the process by which influenza viruses emerge, although there is a need to better understand why, when, and where certain mutations and reassortments arise. With the public and animal threats posed by HPAI viruses, more knowledge is required about how and why these HPAI viruses emerge in poultry. Furthermore, additional information is needed on other virulence determinants and the factors that drive the natural selection of these biological traits in different hosts. Moreover, the process of gene reassortment is still incompletely understood and should be investigated in the near future. There is a tendency for more frequent reassortment within an influenza virus subtype compared to reassortment between different influenza virus subtypes. This may be related to compatibility of the packaging signals

and the functional balance between different gene segments, in particular between polymerase proteins and between HA and NA.
The studies on H5N1 virus transmission are of importance to elucidate mechanisms of transmission of influenza viruses. To identify the molecular basis for airborne transmission, reverse genetics will be needed to identify which amino acid substitutions in airborne H5N1 virus are essential. Second, the virulence and tissue tropism of the airborne H5N1 virus should be investigated in detail in ferrets and chickens. Third, the mutations we identified need to be tested for their effect on transmission in other genetic clades of H5N1 viruses. Importantly, the biological properties identified for the airborne H5N1 virus could be general characteristics of airborne viruses, and therefore mechanistic studies on the phenotypic traits associated with each of the identified amino acid substitutions from previous pandemics should be investigated.
Data on the biological properties of the transmissible H5N1 virus may be extrapolated to the new avian H7N9 virus in order to assess the risk of a new pandemic and to implement appropriate control measurements in time.
Transmission of influenza virus between humans may occur via respiratory droplets and/or aerosols. However, which sizes of exhaled particles are important for influenza virus transmission are unknown. More efforts should be dedicated to determine in which sizes of aerosols and/or droplets the infectious influenza virus is transmitted most effectively. In addition, it is conceivable that the stability of the influenza virus HA plays an important role in the airborne spread between humans. Monitoring exhaled virus aerosols, divided in size fractions, and their culturability will provide a better understanding of influenza virus transmission. Although it is known that influenza morphology affects virus production, the role in influenza virus transmission is still unclear. Studies showed that most influenza strains isolated from humans are predominantly filamentous and, upon continual passage in egg or tissue culture, adopt a more spherical morphology which correlated with an increase in virus titer [393]. Thus, it is possible that the increased levels of influenza virus production by spherical influenza strains will results in more efficient influenza virus transmission. The contribution of residues in the MA gene segment in virus morphology and consequently transmission should be evaluated.
Which influenza virus subtype will emerge and cause the next pandemic is still impossible to predict. Therefore, new influenza virus vaccine strategies are required that induce a broader immune response than the current influenza vaccines. The ultimate goal of this work should be to design a universal vaccine that would induce protection against all influenza virus subtypes. This may be achieved by targeting antibodies and T cells at more conserved parts of the influenza A virus than the globular head of the HA glycoprotein. In addition, better drugs should be designed to prevent or treat influenza. As influenza viruses replicate very rapidly, new drugs aimed to limit the disease or facilitate recovery from disease may be more feasible than better drugs to inhibit virus replication.



CHAPTER 6
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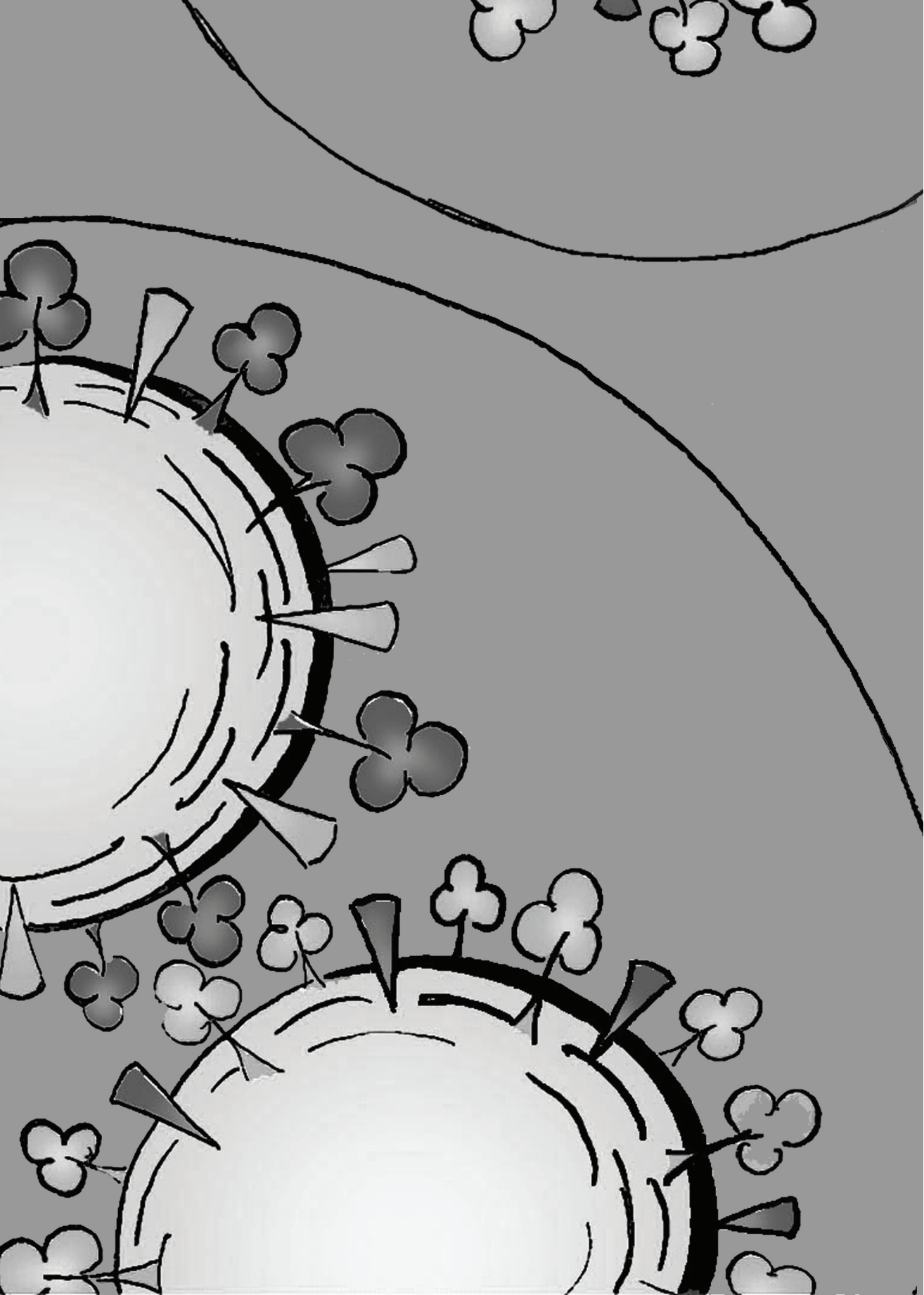

CHAPTER 7
NEDERLANDSE SAMENVATTING

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Introductie

Influenza A virussen, ook wel griepvirussen genoemd, worden onderverdeeld op basis van de oppervlakte-eiwitten hemagglutinine (HA) en neuraminidase (NA). Er zijn 17 HA subtypen en 10 NA subtypen bekend. De combinatie van de oppervlakte-eiwitten bepaalt de naam van het influenza A virus, bijvoorbeeld H3N2 en H5N1. Het influenza A virus is een negatief strengs RNA virus waarvan het genetisch materiaal verdeeld is over acht gensegmenten: PB2, PB1, PA, HA, NP, NA, M en NS.

De H1N1 en H3N2 virussen zijn momenteel verantwoordelijk voor de jaarlijks terugkerende influenza-epidemieën in de mens. Ouderen en risicogroepen worden aangeraden zich hiertegen te laten vaccineren. Veranderingen in HA en NA kunnen er echter voor zorgen dat de door het vaccin opgewekte antistoffen niet de influenza A virussen kunnen neutraliseren waardoor er toch een infectie optreedt. Doordat influenza A virussen regelmatig muteren, is het nodig het vaccin bijna jaarlijks aan te passen zodat gevaccineerde mensen beschermd blijven tegen de griep.

Influenza A virussen komen niet alleen voor in de mens maar ook in andere diersoorten. Met uitzondering van het H17N10 virus, komen alle influenza A virus subtypen voor in wilde vogels. H17N10 is alleen gevonden in vleermuizen. Sommige influenza A virussen die voorkomen in diersoorten kunnen de mens sporadisch infecteren. Dit zijn zogenaamde zoönotische infecties (of zoönosen). In pluimvee en andere vogelsoorten kunnen aviaire influenza A virussen ziekte veroorzaken; 'de vogelgriep' in de volksmond. Hoogpathogene aviaire influenza (HPAI) virussen van het H5 en H7 subtype veroorzaken enorme uitbraken in pluimvee en soms ook ziektegevallen in de mens, zoals de laatste 15 jaar in Zuidoost Azië. Meer dan zeshonderd humane gevallen van infectie zijn gemeld bij de Wereldgezondheidsorganisatie, van wie meer dan de helft aan de ziekte bezweek. Ook in andere zoogdieren kan infectie met HPAI H5N1 virus leiden tot een ernstig ziektebeeld en sterfte.

De introductie van een nieuw influenza A virus subtype in de mens waartegen geen immuniteit bestaat, kan leiden tot een pandemie. De voorwaarde voor een pandemie is dat het virus efficiënt wordt overgedragen tussen mensen. Omdat het influenza A virus uit acht gensegmenten bestaat, kan het infecteren van één cel door verschillende influenza A virussen resulteren in een onderlinge uitwisseling van het erfelijk materiaal, ook wel reassortering genoemd. Varkens kunnen een rol spelen in het reassorteren van influenza A virussen doordat zij zowel door aviaire, humane en natuurlijk varkens influenza A virussen geïnfecteerd kunnen worden. Het varken wordt daarom wel aangemerkt als 'mengvat' voor influenza A virussen, waarin uiteindelijk pandemische virussen kunnen ontstaan.

In de afgelopen honderd jaar hebben vier grieppandemieën plaatsgevonden. De ´Spaanse´ griep in 1918 was verantwoordelijk voor één van de meest ernstige pandemieën waaraan ongeveer vijftig miljoen mensen zijn gestorven. Het H1N1 virus dat deze pandemie veroorzaakte, is hoogstwaarschijnlijk direct afkomstig van vogels zonder tussenliggende gastheer. Een nieuwe pandemie werd veroorzaakt in 1957

door een H2N2 virus, de 'Aziatische' griep. Deze verdreef het H1N1 virus uit de mens. De H2N2 viruspandemie was het resultaat van een reassortering tussen het humane H1N1 virus en een aviair H2N2 virus, waarvan de HA, NA en PB1 gensegmenten afkomstig waren. Dit virus circuleerde in de mens tot 1968, toen reassortering plaatsvond van het humane H2N2 virus met een aviair H3 virus. Dit leidde tot de volgende pandemie die werd veroorzaakt door een H3N2 virus, de 'Hongkong' griep. Dit virus veroorzaakt jaarlijks nog steeds epidemieën. Het H1N1 virus keerde in 1977 terug om vervolgens tot 2009 naast het H3N2 virus in de mens te circuleren. De meest recente introductie van een nieuw influenza A virus in de mens vond plaats in het voorjaar van 2009. Een nieuw H1N1 virus was het resultaat van een reassortering tussen humane, aviaire en varkens influenza A virussen en werd overgedragen van varken op mens. Dit nieuwe griepvirus werd wereldwijd bekend als de Mexicaanse griep. Het pandemische H1N1 virus verving het 'oude' H1N1 virus en circuleert tot op heden samen met H3N2 in de mens.

Alle pandemische virussen van de laatste eeuw waren in staat efficiënt tussen mensen te worden overgedragen via aerosolen of luchtwegdruppels. De incidentele introducties van aviaire influenza A virussen van het H5 en H7 subtype vanuit kippen in de mens zorgt voor een dreiging van een nieuwe pandemie. Gelukkig zijn deze virussen vooralsnog niet efficiënt overdraagbaar gebleken tussen mensen.

Een influenza A virus vermenigvuldiging begint als het virale oppervlakte-eiwit HA zich bindt aan de receptor op een cel, waarna het virus de cel kan binnendringen. Humane influenza A virussen binden een andere receptor dan aviaire influenza A virussen. Voordat een influenza A virus een celreceptor kan binden, moet het oppervlakte-eiwit HA geknipt worden zodat er twee componenten van HA (HA1 en HA2) ontstaan. Een influenza A virus waarvan HA niet is geknipt, is niet in staat een cel te infecteren. De knip wordt uitgevoerd door de in de gastheer aanwezige proteases; eiwitten die specifieke ketens van aminozuren knippen. Humane en laagpathogene aviaire influenza A virussen hebben een zogenoemde enkelvoudige basische knipplek in HA. Deze wordt herkend door trypsine-achtige proteases, die zowel aanwezig zijn in de luchtwegen van zoogdieren en vogels als in de darmen van vogels. Door de aanwezigheid van de juiste proteases kan het virus zich op deze locaties vermenigvuldigen. Het hoogpathogene karakter van aviaire influenza A virussen van het H5 en H7 subtype wordt veroorzaakt door extra aminozuren die aanwezig zijn in de knipplek van HA. Deze virussen hebben een meervoudige basische knipplek (MBCS) die wordt herkend door in het lichaam veelvoorkomende proteases (furine-achtige proteases). Hierdoor zijn hoogpathogene influenza A virussen in staat om in organen buiten de luchtwegen en darmen te vermenigvuldigen.

Het ontstaan van een subtype specifiek HPAI virus

In de natuur komt een MBCS alleen voor bij influenza A virussen van het H5 en H7 subtype. De genetische verandering vindt hoogstwaarschijnlijk uitsluitend plaats in pluimvee. Naast influenza A virus subtypen H5 en H7 circuleren ook andere subtypen (bijvoorbeeld H6 en H9) in kippen maar deze ontwikkelen zich nooit tot een

hoogpathogeen virus doordat deze subtypen geen MBCS verkrijgen. In hoofdstuk 2.1 wordt een onderzoek beschreven waarmee is aangetoond dat een virus van het H6 subtype na de kunstmatige introductie van een MBCS (H6N1MBCS), de mogelijkheid heeft zich als een HPAI virus te gedragen. Het onderzochte H6N1MBCS virus was trypsine-onafhankelijk in cellen net zoals een HPAI H5N1 virus. Door het bepalen van een intraveneuze pathogeniteit index (IVPI) werd gekeken hoe virulent het virus is in kippen. Deze IVPI varieert van 0 (laagpathogeen) tot 3 (hoogpathogeen), waarbij een IVPI hoger dan 1.2 wordt geassocieerd met een hoogpathogeen influenza A virus. Het H6N1MBCS virus bleek een IVPI van 1.41 te hebben waarmee is aangetoond dat H6N1MBCS een hoogpathogeen virus is. Ook bleek het H6N1MBCS virus de mogelijkheid te hebben verkregen om buiten de luchtwegen en darmen van kippen te vermenigvuldigen. Met deze studie toonden we aan dat de afwezigheid van een MBCS in H6 influenza A virussen niet komt door een incompatibiliteit van het HA-eiwit. Waarom andere influenza A virus subtypen dan H5 en H7 zich in de natuur niet ontwikkelen tot een hoogpathogeen fenotype is daarmee echter nog niet opgehelderd.

HPAI in zoogdieren

Sporadisch worden mensen geïnfecteerd met HPAI H5 virussen. Dit kan leiden tot een ernstig ziektebeeld, soms met sterfte tot gevolg. Ook andere zoogdieren zijn vatbaar voor HPAI H5 virusinfectie, zoals fretten, tijgers, honden en katten. Fretten worden veel gebruikt in influenza A virusonderzoek (zo ook in dit onderzoek) om meerdere redenen: (1) fretten vertonen ongeveer hetzelfde ziektebeeld als mensen, (2) in de luchtwegen zijn dezelfde virusreceptoren aanwezig en (3) fretten hebben de mogelijkheid humane influenza A virussen over te dragen naar andere fretten via de aërogene route (via de lucht), terwijl aviaire influenza A virussen niet worden overgedragen via de aërogene route.

De HAs van humane influenza A virussen bevatten geen MBCS. In hoofdstuk 2.2 wordt beschreven dat het humane H3N2 virus na de kunstmatige introductie van een MBCS (H3N2MBCS) geen verhoogde virulentie vertoonde in fretten. Dit H3N2MBCS virus gedroeg zich net als een HPAI H5N1 virus voor wat betreft trypsine-onafhankelijkheid in celkweek. In fretten was vermenigvuldiging van het H3N2MBCS virus beperkt tot de luchtwegen net zoals het H3N2 virus zonder een MBCS. Uit deze studie bleek dat naast de MBCS ook andere factoren een belangrijke rol spelen bij de vermenigvuldiging van influenza A virussen buiten de luchtwegen. De voorkeur van het influenza A virus HA-eiwit voor de receptor op de cel speelt hier hoogstwaarschijnlijk een grote rol bij.

Een HPAI H5N1 virusinfectie in fretten wordt gekenmerkt door systemische vermenigvuldiging waarbij het virus meerdere organen buiten de luchtwegen infecteert, zoals de hersenen en de lever. Hoe het virus in zoogdieren zich exact een weg naar de hersenen baant en wat de rol van de MBCS is in de systemische vermenigvuldiging van het virus, was nog onbekend. In hoofdstuk 2.3 laten we zien dat de

verwijdering van een MBCS uit een HPAI H5N1 virus (H5N1ΔMBCS) een bepalende factor was voor de virusverspreiding buiten de luchtwegen in fretten. Na intranasale toediening van het H5N1ΔMBCS virus werd de virusverspreiding beperkt tot de luchtwegen. Dit in tegenstelling tot infectie met het wildtype HPAI H5N1 virus, dat resulteerde in systemische vermenigvuldiging van het virus. Het HPAI H5N1 virus verspreidde zich vanuit de olfactorische receptorneuronen in de neus van een fret naar de bulbus olfactorius om zich vervolgens verder te verspreiden naar de grote en kleine hersenen. Ook kon het HPAI H5N1 virus geïsoleerd worden uit hersenvocht. Hiermee werd aangetoond dat het olfactorische traject verantwoordelijk is voor een van de belangrijkste routes voor de ontwikkeling van encefalitis, ofwel een infectie van de hersenen. Ook bij een HPAI H5N1 virusinfectie van de mens wordt het virus soms teruggevonden in hersenvocht, wat duidt op een infectie van de hersenen. Of de virusverspreiding in de mens ook verloopt via de olfactorische route zal met behulp van verder onderzoek bepaald moeten worden.

Reassortering van influenza A virussen

Ten minste drie van de vier grieppandemieën van de laatste eeuw zijn ontstaan als het gevolg van reassortering van humane met dierlijke influenza A virussen. In 2009 manifesteerde een nieuw H1N1 virus (pH1N1) zich dat de eerste influenzapandemie van de 21ste eeuw veroorzaakte. Aangezien het pH1N1 virus niet beschikte over bekende virulentiefactoren, werd gevreesd dat dit virus zich verder zou aanpassen aan de mens via mutaties of reassortering. In het begin van de pandemie circuleerde het pH1N1 virus samen met de seizoensgriepvirussen H3N2 en H1N1 (sH1N1). In hoofdstuk 3.1 wordt de mogelijkheid van reassortering tussen pH1N1 en H3N2 of sH1N1 virussen beschreven. Een mix van verschillende pH1N1 reassortanten werd in cellen gecreëerd, waarna virussen met een verhoogde vermenigvuldigings capaciteit werden geselecteerd. Met behulp van “reverse genetics” werden deze geselecteerde reassortanten nagemaakt en de virulentie van deze virussen vergeleken met het pH1N1 virus in het frettenmodel. Reassortanten van pH1N1 met PB2 en PA of alleen PB2 van sH1N1 bleken zich minder goed te vermenigvuldigen in het frettenmodel dan het wildtype pH1N1 virus. Echter pH1 virus reassortanten met NA en PB1 of alleen NA van H3N2, vertoonden een verhoogde vermenigvuldigings capaciteit, wat geassocieerd was met ernstigere longlaesies dan die van fretten geïnfecteerd met wildtype pH1N1. Met deze studie toonden we aan dat het pH1N1 virus de mogelijkheid heeft om te reassorteren met de circulerende H3N2 en sH1N1 virussen. Reassortanten van het pH1N1 virus bleken virulenter te zijn dan pH1N1 en bleven aërogeen overdraagbaar. Omdat andere genetische samenstellingen ook zouden kunnen ontstaan in mens of dier werd aanbevolen om met behulp van surveillance programma’s reassortering van het pH1N1 virus nauwlettend in de gaten te houden tijdens de pandemie.

Door de incidentele infecties met hoogpathogene H5N1 virussen in de mens wordt gevreesd dat dit virus reassorteert met in de mens circulerende influenza A virus-

sen. Dit kan leiden tot influenza A virussen met een aërogene overdraagbaarheid. De mogelijkheid voor het H5N1 virus om te reassorteren met humane influenza A virussen wordt beschreven in hoofdstuk 3.2. Met behulp van dezelfde selectiemethode beschreven in hoofdstuk 3.1, werden reassortanten tussen hoogpathogene H5N1 en pH1N1, H3N2 of sH1N1 virussen verkregen. Deze geselecteerde H5 virus reassortanten bevatten voornamelijk de NA en M gensegmenten van de humane influenza A virussen. Geselecteerde reassortanten werden met behulp van "reverse genetics" nagemaakt. De vermenigvuldigings capaciteit van de reassortanten was niet hoger in zoogdier- en humane bronchiale epitheelcellen dan bij die van het wildtype H5N1 virus. In dit hoofdstuk toonden we aan dat H5N1 virussen de mogelijkheid hebben te reassorteren met humane influenza A virussen en dat de NA en M gensegmenten van de humane influenza A virussen de hoogste genetische compatibiliteit vertonen met het H5N1 virus. Een mix van reassortanten tussen hoogpathogeen H5N1 en pH1N1 werd geëvalueerd voor de mogelijke aanwezigheid van aërogeen overdraagbare influenza A virussen. In geïnfecteerde fretten werden voornamelijk H5 virussen gedetecteerd die de NA en/of M gensegmenten bevatten van pH1N1, maar deze influenza A virussen bleken niet aërogeen overdraagbaar te zijn tussen fretten. Het gebrek van aërogene overdraagbaarheid van deze H5 virus reassortanten heeft mogelijk te maken met een geringe uitscheiding vanuit de bovenste luchtwegen en de voorkeur van deze influenza A virussen voor een aviaire receptor.

Aërogene overdraagbaarheid van aviare influenza A virussen

De incidentele infecties met aviaire influenza A virussen in mensen dragen bij aan de dreiging van een nieuwe pandemie. Gelukkig zijn deze aviaire influenza A virussen tot op heden niet aërogeen overdraagbaar gebleken tussen mensen. Het is belangrijk om virale-, gastheer- en omgevingsfactoren te bestuderen om erachter te komen wat de aërogene overdraagbaarheid bepaalt van influenza A virussen in de mens. In hoofdstuk 4.1 wordt informatie over de eerdere pandemieën, zoönozen en epidemieën gebruikt om de minimale vereisten voor een influenza A virus om overdraagbaar te worden tussen mensen in kaart te brengen. We postuleren dat (1) sterke binding aan cellen van de bovenste luchtwegen, (2) verhoogde virusreplicatie in de bovenste luchtwegen en (3) efficiënte productie van enkelvoudige partikels die in aërosolen kunnen worden opgenomen, bijdragen aan efficiënte overdraagbaarheid van influenza A virussen tussen mensen. In hoofdstuk 4.2 werd deze hypothese getest door te onderzoeken of een aviair H5N1 virus de mogelijkheid heeft aërogeen overdraagbaar te worden tussen zoogdieren. Een HPAI H5N1 virus werd door middel van drie aminozuur veranderingen aangepast aan het zoogdier als gastheer. Eén van deze veranderingen was een glutaminezuur naar lysine op positie 627 van PB2. Dit is een mutatie die belangrijk is voor de adaptatie van influenza A virussen van vogels aan zoogdieren. Ook werd de voorkeur van het influenza A virus om te binden aan aviaire receptoren op cellen veranderd in een voorkeur voor humane receptoren door twee mutaties aan te brengen in HA. Deze drie muta-

ties zorgden er echter niet voor dat het influenza A virus aërogeen overdraagbaar werd tussen fretten. Het genetisch gemodificeerde H5N1 virus werd daarom vervolgens door middel van het herhaald infecteren van fretten aangepast aan replicatie in de bovenste luchtwegen van de fret. Het genetisch gemodificeerde H5N1 virus verkreeg extra mutaties tijdens het passeren in fretten. Daarna werd getest of in de verkregen “mix” van geadapteerde virussen aërogeen overdraagbare varianten aanwezig waren. In speciaal ontwikkelde transmissiekooien bleek inderdaad dat het H5N1 virus in staat was aërogeen overdraagbaar te worden. Alle aërogeen overdraagbare H5N1 virussen hadden vijf mutaties die overeen kwamen; de drie door ons geïntroduceerde mutaties en twee extra mutaties in HA. Eén van de extra mutaties is verantwoordelijk voor het verliezen van een potentiële glycosyleringsplaats met sterkere receptorbinding tot gevolg. De andere mutatie zou mogelijk van belang kunnen zijn voor de stabiliteit van HA. Aanvullende experimenten moeten uitwijzen of alleen deze gevonden mutaties daadwerkelijk zorgen voor aërogene overdraagbaarheid. Ook is het belangrijk te onderzoeken hoe virulent dit virus zal zijn in zoogdieren en kippen. In deze studie bleek dat fretten die het aërogene H5N1 virus verkregen via de aërogene route niet bezweken aan de ziekte. Deze bevindingen maken duidelijk dat aviaire H5N1 virussen de potentie hebben te evolueren tot aërogeen overdraagbare virussen in zoogdieren en dus een pandemische dreiging vormen.

CHAPTER 8
DANKWOORD

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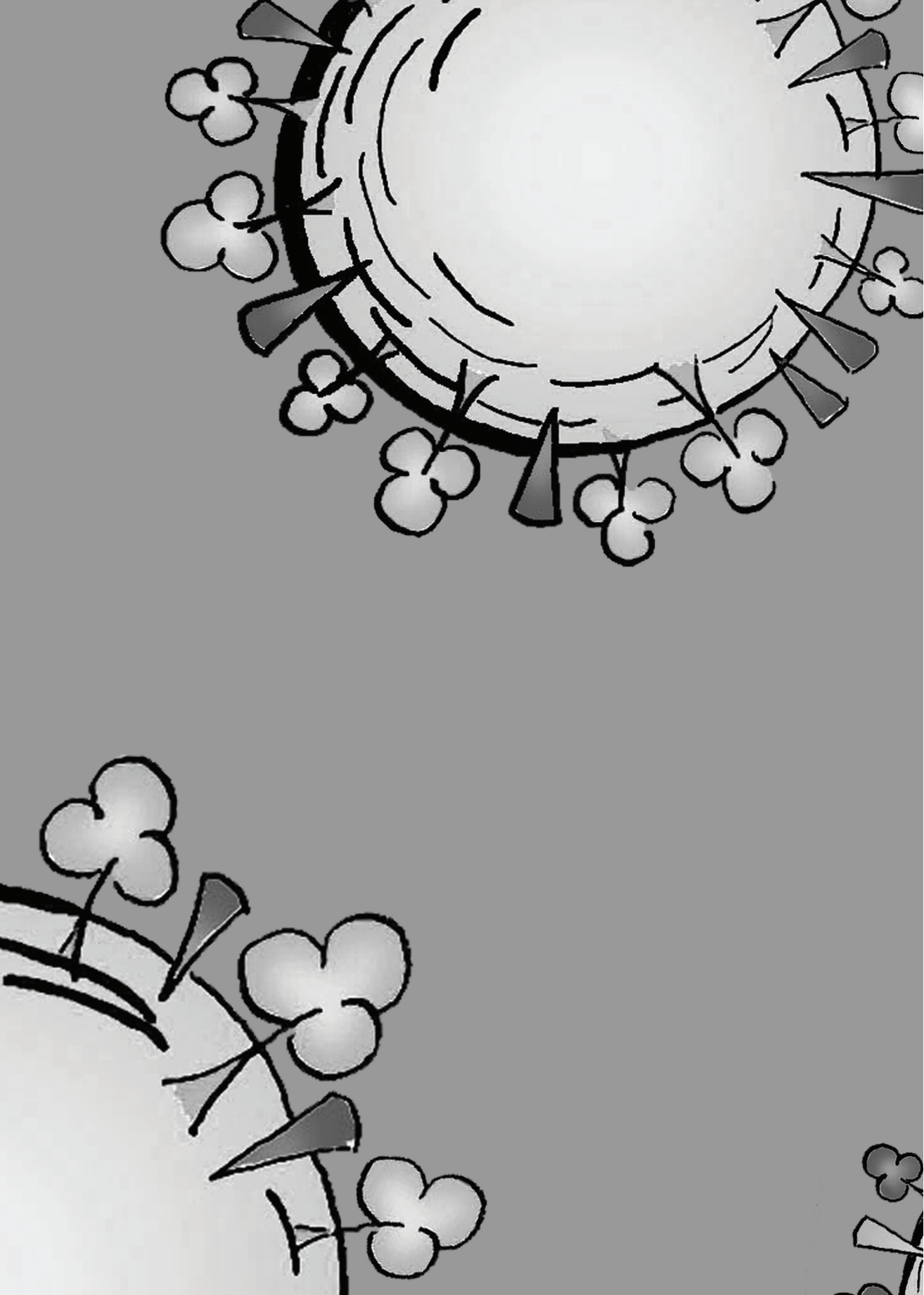
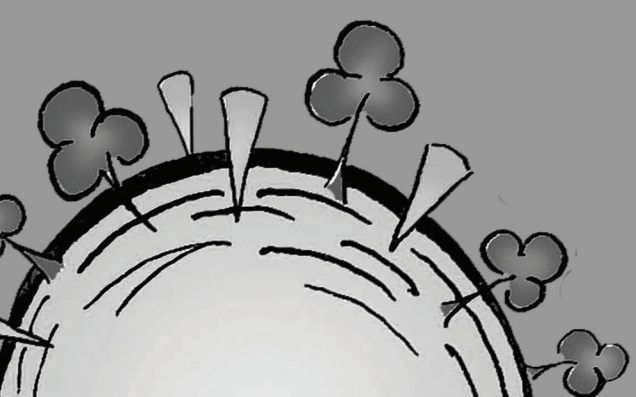


CHAPTER 9
ABOUT THE AUTHOR



CURRICULUM VITAE

The author of this thesis, Eefje Schrauwen, was born on 19th of July 1979 in Breda, The Netherlands. After she finished high school at Markenhage college in Breda in 1996, she started her study at the Hoge school Breda (HLO college) and obtained her bachelor of science degree. She specialized in medical microbiology and during her internship her research focused on expression of influenza A and B virus and respiratoir syncytial virus proteins from the baculovirus sytem in order to develop an ELISA system at the department of virology at the Erasmus medical center in Rotterdam. She graduated in 2000 and immediately started as a technician at the department of Viroscience of the Erasmus MC under supervision of Prof. Dr. Ab Osterhaus focusing on vaccins of feline immunodeficiency virus. From 2005 until 2009 she worked as a technician under supervision of Prof. Dr. Ab Osterhaus and Prof. Dr. Ron Fouchier performing fundamental and vaccin research on the human metapneumavirus. In 2009 she started her master study in Infection and Immunity at the Erasmus university from which she graduated in 2011. In 2009 she started her PhD at the department of Viroscience focusing on influenza A virus pathogenicity which resulted in this thesis. In 2010 she married Maurits Roos and in 2012 she had a child: Birgit Roos.

LIST OF PUBLICATIONS

Mathilde Richard*, **Eefje J.A. Schrauwen***, Miranda de Graaf, Theo M. Bestebroer, Monique Spronken, Sander van Boheemen, Dennis de Meulder, Pascal Lexmond, Martin Linster, Sander Herfst, Derek J. Smith, Judith M. van den Brand, David F. Burke, Thijs Kuiken, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus, Ron A.M. Fouchier. Limited airborne transmission of influenza A/H7N9 virus between ferrets. *Nature* 2013 Accepted for publication.

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* Both authors contributed equally to the results of this study

PHD PORTFOLIO

Name PhD student:	Eefje Schrauwen
Research group:	Department of Viroscience
Research school:	Post-graduate Molecular Medicine
PhD period:	2009-2013
Promotors:	Prof. dr. R.A.M. Fouchier Prof. dr. A.D.M.E. Osterhaus

Education:

2009-2013	PhD program, Erasmus MC, Rotterdam, The Netherlands. Title of thesis: Reassortments and Mutations Modulating Virulence and Transmission of Influenza A Virus
2009-2011	Master of Science, Erasmus MC, Rotterdam, The Netherlands. Study: Infection & Immunity
1996-2000	Bachelor of Science, Hogeschool Avans, Etten-Leur, The Netherlands. Study: Medical Laboratory Sciences. Specialisation: Medical Microbiology

In-depth courses:

Workshop Presenting Skills for Junior Researchers (MolMed)	2013
Course in Biomedial English writing (MolMed)	2011
Course in Grand Proposal writing (MolMed)	2011
Course in adobe Photoshop and Illustrator (MolMed)	2010

Presentations:

6th Orthomyxovirus research conference, Montreal (oral)	2012
16th Molecular medicine day, Rotterdam (poster)	2012
ESWI, 4th European Influenza Conference, Malta (oral)	2011
Keystone symposia meeting on Pathogenesis of Influenza: Host-Virus Interactions, Hong Kong (poster)	2011
15th Molecular medicine day, Rotterdam (poster)	2011
4th Annual CEIRS Network Meeting, Fairport, New York (poster)	2010
14th Molecular medicine day, Rotterdam (poster)	2010
5th Orthomyxovirus research conference, Freiburg (poster)	2009

Attended meetings:

Dutch annual virology symposium, Amsterdam	2012
Dutch annual virology symposium, Amsterdam	2011
Dutch annual virology symposium, Amsterdam	2010
Virology meeting, Dakar	2009
13th Molecular medicine day, Rotterdam	2009

Dutch annual virology symposium, Amsterdam	2009
Mini symposia Immunology, Rotterdam	2009

Supervision & teaching activities

Co-organisator “proefstuderen”	2013
Co-supervisor MSc student (AR)	2013
Coach “Viruskenner”	2012-13

Grants & Awards

Award for best poster presentation, Molecular Medicine day, Rotterdam	2012
ESWI young Scientist travel grant, 4th ESWI conference, Malta	2011
Award for best poster presentation, 5th Orthomyxovirus research conference, Freiburg	2009