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Swine Influenza Viruses – Evolution and Zoonotic Potential

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Publication date:
2014

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Swine Influenza Viruses – Evolution and Zoonotic Potential

PhD thesis by Kristina Fobian

2014

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Swine Influenza Viruses – Evolution and Zoonotic Potential

PhD thesis 2014 © Kristina Fobian

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PREFACE

This PhD thesis is based on the work performed at Section for Virology, National Veterinary Institute, Technical University of Denmark (DTU), including a two month research stay at Division of Virology, Department of Infectious Diseases, St. Jude Children Research Hospital, Memphis, USA. The work was performed in the period June 2010 to July 2014 and was partly funded by the EU concerted Action: European Surveillance Network for Influenza in Pigs 3 (ESNIP 3).

First of all I would like to thank my Supervisors Professor Lars E. Larsen for giving me the opportunity to perform this work as well as invaluable input to and discussions about thoughts and ideas of varying quality and to Solvej Ø. Breum for guidance in all practical and scientific aspects of the work, for always taking the necessary time and for encouraging me to always try to do better.

I would also like to thank Ramona Trebbien for discussions of many different topics and for the invaluable help commenting on this thesis. Thanks also to Jesper Schak Krog, Charlotte K. Hjulsager and Lise Kirstine Kvisgaard for always taking the time to answer my numerous questions and for many great talks and laughs.

Special thanks also to all the technicians, to Kristine Vorborg, Hue Thi Thanh Tran, Tine Hammer, Sari Mia Dose and Helene Ringvig for invaluable technical assistance as well as for teaching me new techniques - you are the cornerstones of this group.

I would also like to express my most sincere gratitude to Dr. Richard Webby at the Division of Virology, St. Jude Children Research Hospital for giving me the opportunity for a very educative and pleasant research stay and to Thomas P. Fabrizio for helping me in all aspects, practical as well as scientific both before, during and after my stay. Also thanks to everyone in the Flugroup at St. Jude, but most importantly to Sook-San Wong, Mark Zanin, Bryan Kaplan, John Franks, Min-Suk Song, Scott Krauss, Jeri Carol Crumpton, Trushar Jeevan, Jennifer DeBeuachamp, Sun-Woo Yoon and *Bindumadhav* M. Marathe for your great help and hospitality and for making my stay at St. Jude one of my best experiences. Thanks also to Robin Borzon and Tricia Satkowski for help with the many practical and more formal aspects of my research stay at St. Jude. I am also grateful to National Institute of Health (NIH) for funding the experimental work at St.Jude.

And special thanks go to my fellow PhD student Lif Rødtness Vesterby Knudsen with whom I have shared frustration and progress during the arrangements of our research stays in the US.

Preface

LIST OF ABBREVIATIONS

APC   Antigen presenting cell

B cells   Bone marrow-derived lymphocytes

BI   Bayesian inference

BSA   Bovine serum albumin

ccdB   Control of cell death

$CD4^+$ Cluster determinant 4 positive T cells

$CD8^+$ Cluster determinant 8 positive T cells

CMAH   Cytidine monophosphate-N-acetylneuraminic acid hydroxylase

CPE   Cytopathic effect

cRNA   Complementary RNA

CTL   Cytotoxic T lymphocyte

DC   Dendritic cells

ELISA   Enzyme-linked immunosorbent assay

Gal   Galactose

GalNAc   N-acetylgalactosamine

GlcNAc   N-acetylglucosamine

HA   Hemagglutination assay

HI   Hemagglutinin inhibition

HPAI   High pathogenic avian influenza

IAV   Influenza A virus

IC_{50} 50% of maximal inhibitory concentration

IFN   Interferon

LPAI   Low pathogenic avian influenza

List of abbreviations

MAA	*Maackia Amurensis*
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NAI	Neuraminidase inhibitor
NEAA	Non-essential amino acids
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NGS	Next generation sequencing
NHBE	Normal human bronchial epithelial cells
PAMP	Pathogen-associated molecular patterns
PDZ	An acronym combining the first three letters of the proteins PSD95, Dlg1 and zo-1
Pol I	DNA-dependent RNA polymerase I
PRDC	Porcine respiratory disease complex
PRR	Pattern recognition receptors
pSREC	Primary swine respiratory epithelial cells
RBS	Receptor binding site
RDE	Receptor destroying enzyme
RdRp	RNA-dependent RNA polymerase
RIG	Retinoic acid inducible gene I
SA	Sialic acid
SIV	Swine influenza virus
T-cell	Thymus-derived lymphocytes
$TCID_{50}$	50% tissue culture infectious dose

TLR   Toll-like receptor

tMRCA   Time of most recent common ancestor

TRIG   Triple-reassortant internal gene

TRS   Triple reassortant swine

vRNA   Viral RNA

vRNP   Viral ribonucleoprotein

SUMMARY

Influenza A virus (IAV) is an important respiratory pathogen with a broad host range. The natural reservoir for IAV is waterfowls, but both human and swine are considered natural hosts. During the past century IAV has caused severe pandemics as well as seasonal epidemics in the human population. In pigs, swine influenza virus (SIV) is endemic worldwide and is associated with economic losses for the farmer due to the impact on pig health causing lowered production. Swine has been shown to be susceptible to infection with IAVs of different host origin and has hence been considered as potential mixing vessels of new IAVs. Furthermore, transmission of IAVs from swine to human and vice versa has been documented on several occasions and further classifies this virus as a highly important zoonosis. This aspect enhances the possibility of the formation and establishment of new and potentially more virulent viruses with the capacity to cause severe pandemics. Therefore, it is important to gain a deeper understanding of the evolution of SIVs, their zoonotic potential as well as host-range characteristics and this PhD project aimed at elucidating parts of these important points.

The PhD thesis begins with a presentation of the aims and a brief introduction of the situation of SIV in Denmark. In the background section an extensive review on IAVs with emphasis on SIV is provided. The results obtained during the PhD are presented in two complete manuscripts and one work in progess, followed by a joint discussion of the main results.

Manuscript I analyze the genetic and antigenic evolution of two of the most prevalent SIVs circulating in Denmark. In total, 78 sequences of the H1N1 and H1N2 subtypes, collected in the period 2003-2012, was analyzed. The genetic analysis was based on several computational methods for estimation of phylogeny, selection pressure, evolutionary rates and time of most recent common ancestor for the surface glycoproteins, HA and NA. The antigenic relationship of the Danish H1 SIVs was determined by antigenic cartography. High evolutionary rates of HA and NA compared with low evolution suggests that evolution is primarily controlled by purifying selection. Further, a high level of genetic relatedness and of low evolution was observed for the Danish H1 sequences, this observation was supported by both phylogeny and antigenic cartography. Antigenic cartography also revealed few antigenic outliers that potentially indicated drift away from current H1 viruses. The time of most recent

common ancestor for H1 was estimated to be markedly earlier than previously suggested. Phylogenetic analysis of the Danish N2 gene revealed that two different lineages are circulating in Denmark.

Manuscript II describes the biological characterization of four different Danish SIVs and includes an experimental pathogenesis study performed in ferrets, which are regarded as the most appropriate small animal model for human IAV infections. The viruses chosen for this study were two enzootic SIVs (H3N2 and H1N2) and two new SIV reassortants (H1avN2hu and H1pdmN2sw). The two reassortants were detected for the first time in 2011 and have since then become established and are now circulating in Danish pigs. Viral replication in nasal wash samples and viral load in respiratory organs were determined. Growth kinetics of the four SIVs were determined *in vitro* using respiratory swine and human cell lines. The affinity of HA of the four SIVs for α2,3- and α2,6-receptors were assessed as well as receptor kinetics and antiviral susceptibility of NA. This study showed that all four SIVs were able to infect and transmit efficiently and to high titers via direct contact and H3N2 was found also to transmit efficiently via the airborne route. H3N2 and H1pdmN2sw were found to induce the most severe lung lesions, consistent with these two viruses expressing the highest viral load in lung tissue samples. Growth kinetics demonstrated that all four SIVs were able to infect and replicate to high titers in both swine and human respiratory cell lines.

Receptor studies showed a high preference for binding to α2,6-receptors for the Danish SIVs. NA kinetics revealed a high enzyme activity for H1pdmN2sw compared to the remaining viruses, suggesting that NA activity alone is not sufficient for the observed airborne transmission of H3N2. Furthermore, it was revealed that the Danish SIVs were found to be sensitive to all of the neuraminidase inhibitors tested. Based on the findings in this study it was proposed that viruses with a human-like HA play a more significant role in transmission compared to viruses with only a human-like NA. Furthermore, this study also underlined the importance of continued surveillance of SIVs in order to detect new reassortants as well as the necessity of assessing their zoonotic potentials.

Manuscript III describes the establishment of a reverse genetics system based on a backbone from the Danish H1N2 SIV, which is one of the two most prevalent subtypes in Denmark. Recently, a variant of a North American swine H3N2 virus containing a pandemic M gene was transmitted to humans in the US and on few occasions human-to-human transmission was observed. These events underline the need

Summary

for a reverse genetics system to be used for an analysis of the behavior of a pandemic M gene in a Danish SIV.

SAMMENDRAG (DANISH SUMMARY)

Influenza A virus (IAV) er et vigtigt respiratorisk patogen med bred værtsspecificitet. Det naturlige reservoir for IAV findes i svømmefugle, men både mennesker og svin betragtes også som naturlige værter. I løbet af det sidste århundrede, har IAV både forårsaget alvorlige pandemier og sæson-betingede epidemier hos mennesker. I svin, er svineinfluenza virus (SIV) endemisk og er forbundet med økonomiske tab for landmanden grundet påvirkningen af svinenes generelle helbred, hvilket derved forårsager en nedgang i produktionen. Det er tidligere blevet vist at svin er modtagelig for infektion med IAVs der stammer fra forskellige værter og er derfor blevet foreslået som værende potentielle "blande-kar" for nye IAVs. Herudover er det i flere tilfælde blevet dokumenteret at IAV er i stand til at smitte fra svin til mennesker og vice versa, hvilket yderligere klassificerer dette virus, som en meget vigtig zoonose. Dette aspekt fremmer muligheden for dannelse og etablering af nye og potentielt mere virulente virus med potentiale for at forårsage alvorlige pandemier. Derfor er det vigtigt at opnå en større dybere forståelse af SIV evolution, det zoonotiske potentiale såvel som værts-specificitets karakteristika og formålet med dette PhD projekt er at belyse dele af disse vigtige aspekter.

PhD afhandlingen starter med en præsentation af formålet og en kort introduktion til SIV situationen i Danmark. I baggrundsafsnittet gives et dybdegående litteraturstudie af IAV med vægt på SIV. Resultater opnået under dette PhD projekt præsenteres i form af to komplette manuskripter, samt et igangværende studie, hvilket efterfølges af en generel diskussion af resultaterne.

Manuskript I analysere den genetiske og antigene evolution af to af de hyppigst forekommende SIV subtyper der cirkulerer i Danmark. I alt blev 78 sekvenser, af H1N1 og H1N2 subtyperne indsamlet i perioden 2003-2012, analyseret. Den genetiske analyse var baseret på flere forskellige beregningsmetoder for at estimere fylogeni, selektionstryk, evolutionære rater og tidspunktet for den seneste fælles forfader for overfladeproteinerne HA og NA. Det antigene forhold mellem de danske H1 SIV blev bestemt ved antigen kartografi. Høje evolutionære rater for HA og NA sammenholdt med lav evolution tyder på at evolutionen primært er kontrolleret af vedligeholdende selektion. Ydermere blev der observeret en tæt genetisk relation og lav evolution for de danske H1 sekvenser, en observation der blev understøttet af både fylogeni og antigen kartografi. Den antigene kartografi synliggjorde også få antigene afvigere, der potentielt kan indikere at de pågældende afvigere bevæger sig væk fra de

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nuværende H1 virus. Estimatet af tidspunktet for den seneste fælles forfader for H1 blev fundet til at være forekommet længe før tidligere foreslået. Fylogenetisk analyse af det danske N2 gen viste at to forskellige linjer cirkulerer i svin i Danmark.

Manuskript II beskriver den biologiske karakterisering af fire forskellige danske SIV og inkluderer et eksperimentelt patogenese studie i fritter, der anses for at være den bedst mulige smådyrsmodel for humane IAV infektioner. De virus der blev udvalgt til dette studie var to enzootiske SIV (H3N2 og H1N2) og to nye SIV reassortanter (H1avN2hu og H1pdmN2sw). De to reassortanter blev påvist for første gang i 2011 og har siden da etableret sig og cirkulerer nu i danske svin. Virus replikation i næsevask prøver, samt virusmængde blev bestemt. Vækstkinetik for de fire virus blev bestemt *in vitro* i respiratoriske svine og humane celle-linier. Affiniteten af hver af de fire virus' HA for α2,3- og α2,6-receptorer blev også undersøgt, såvel som receptorkinetik og anti-viral modtagelighed for NA. Dette studie viste at alle fire SIV var i stand til effektivt og med høje titer-værdier, at inficere og overføres mellem fritter via direkte kontakt og det viste sig at H3N2 også effektivt kunne overføres mellem fritter ved luftbåren smitte. H3N2 og H1pdmN2sw gav de mest alvorlige læsioner i lungerne, hvilket var i overensstemmelse med at disse to virus også udviste den højeste virusmængde i vævsprøver fra lungerne. Vækstkinetikken demonstrerede at alle fire SIV var i stand til at inficere og replikere til høje titer-værdier i både svine og humane respiratoriske celle-linier.

Receptor studier viste, at alle fire danske SIV udviste en høj præference for binding til α2,6-receptorer. NA kinetikken viste at enzym aktiviteten for H1pdmN2sw var høj i sammenligning med de resterende virus, hvilket tyder på at NA aktiviteten ikke alene er afgørende den luftbårne smitte der blev observeret for H3N2. Yderligere blev det fundet at de danske SIV var modtagelige for alle de testede neuraminidase inhibitorer. Baseret på fundende i dette studie kunne det tyde på at virus med et HA af human oprindelse spiller en større rolle i overførslen mellem fritter, sammenlignet med virus hvor NA er af human oprindelse. Ydermere, understregede dette studie også betydningen af en fortsat overvågning af SIV for at kunne detektere nye reassortanter, såvel som nødvendigheden af at være i stand til at undersøge disse nye reassortanters zoonotiske potentiale.

Manuskript III beskriver etableringen af et kloningssystem, der er baseret på en dansk H1N2 SIV, der er en af de mest almindelige subtyper i Danmark. For nyligt blev en variant af et nordamerikansk svine H3N2, indeholdende et pandemisk M gen, fundet i stand til at smitte til mennesker i USA og i få

tilfælde blev smitte mellem mennesker også konstateret. Disse begivenheder understreger behovet for et kloningssystem der kan anvendes til analyse af det pandemiske M gen i en dansk SIV.

INTRODUCTION

The circulation of SIV in pig herds is of economic concern to farmers due to production impacts, such as retained growth, increased mortality and use of antibiotics, as well as decreased animal health welfare. In addition, the circulation of SIVs in the herds also represents the risk of zoonotic transmissions to humans, which could have a pandemic potential. Pigs have been shown to be susceptible to infection with both human and avian IAVs, which have led to the hypothesis that pigs could serve as an intermediate host in the generation of new reassortant viruses. The importance of pigs in IAV ecology was further underscored by the emergence of the pandemic H1N1 in 2009 - an event that also highlighted the significance of understanding the evolution of these highly diverse viruses.

In 1981 the first SIVs were detected in Danish pigs, and have been circulating endemically since. However, since then no further detailed studies have been conducted concerning the evolution of SIV in Denmark and there has been an almost complete lack of systematic surveillance.

In 2011 a more detailed passive surveillance of SIV in Denmark was established supported by the Danish Veterinary and Food Administration (DVFA). In this program, SIV detected in clinical samples from pigs with respiratory symptoms were subtyped (HA and NA genes) and selected isolates were full genome sequenced. This more systematic and thorough surveillance provided an overview of the prevalence of the different subtypes circulating in Danish pigs. Furthermore, several new SIV reassortants were detected, some of which appear to have become established and now circulates in pigs, albeit at low levels. However, the surveillance does not consider the evolution of these viruses and hence no studies regarding the genetic and antigenic evolution of Danish SIVs have been conducted. The surveillance program did not, however, support further characterization of the genetic and antigenic evolution of the established subtypes, nor did it support biological and zoonotic characterization of the new reassortants.

In the US, the reassortant H3N2 SIVs containing the H1N1pdm09 matrix (M) gene has been shown to be able to efficiently transmit to humans on several occasions, highlighting the need for assessment of the zoonotic potential of new reassortant viruses. Furthermore, also the need for specific tools for

investigation of the various barriers and factors involved in the successful adaptation of reassortant viruses to new hosts, are highly needed.

The main aims of this PhD project were to improve the knowledge on the evolution of circulating SIV strains in Danish pigs and to characterize the new reassortant viruses. Furthermore, the aim was to establish a reverse genetics system to be used as a fast and functional tool for future analysis of host-range restrictions of potential new IAV reassortants.

BACKGROUND

Influenza is one of the most common respiratory diseases in humans and one of the most significant, due to the generally high morbidity and the increased mortality of infants, elderly and chronically ill persons (Nicoll *et al.*, 2012;Taubenberger and Morens, 2008). In humans, influenza is an acute respiratory disease with symptoms including high fever, coryza, cough, headache, prostration, malaise, and inflammation of the upper and sometimes the lower respiratory tract. The symptoms usually persist for 7 to 10 days, but weakness and fatigue can be experienced for weeks after clearance of the infection. Outbreaks often occur in the winter period in the northern hemisphere (Cate, 1987;Taubenberger and Morens, 2008).

The first reports of possible influenza pandemics can be dated back to 412 BC in early Greek writings and symptoms resembling those of influenza were frequently described from that point onward. It was not until the 18th century, that both quality and quantity of data were sufficient to be reliable as possible descriptions of influenza epidemics and/or pandemics. First from 1957, where the virus was available for further analysis, the terms pandemic and epidemic could be used without questioning (Potter, 2001).

Only three HA subtypes of IAV (H1N1, H2N2 and H3N2) have caused epidemics in humans in modern times (Fig 1). The most severe pandemic in the past century was caused by the H1N1 subtype in 1918, also known as "the Spanish flu", which resulted in approximately 546,000 deaths alone in the United States (Taubenberger and Morens, 2006) and killed up to 50 million people worldwide (Johnson and Mueller, 2002). In 1957, a pandemic was caused by an IAV of H2N2 subtype, known as the "Asian flu" due to its origin in China. The H2N2 subtype was replaced by the H3N2 subtype in 1968, which was the cause of the next pandemic known as the "Hong Kong flu". In 1977, a re-emergence of a descendant of the 1918 H1N1 IAV occurred, which was found to be genetically similar to the viruses that circulated before 1957. Since it was thought highly unlikely that a virus could have circulated for 20 years without being detected or having accumulated mutations, it was therefore suggested that the re-emergence had to be caused by an accidental release of a frozen version of an early 1950 strain (Nakajima *et al.*, 1981;Taubenberger and Kash, 2010).

In 2009 a new pandemic H1N1 virus (H1N1pdm09) occurred which differed from the earlier known H1N1 viruses (Smith *et al.*, 2009a;Smith *et al.*, 2009b) and since then, this IAV has gradually replaced the seasonal H1N1 virus (Neumann and Kawaoka, 2011) and began co-circulating with H3N2 causing seasonal influenza epidemics in humans.

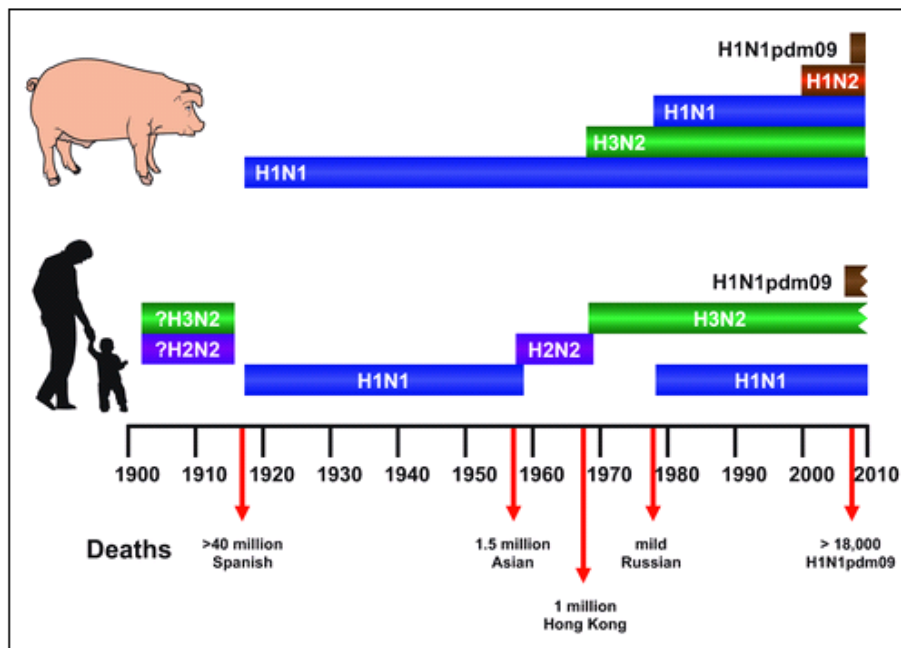**Figure 1** An overview of the different influenza A virus (IAV) subtypes circulating in both humans and pigs during the last century. The jagged ends on the bars of the H1N1pdm09 and H3N2 bars indicate that these viruses continue to circulate in the human population. In pigs the IAV subtypes are more diverse and different variants can be found dependent on country and continent. Modified from (Webster and Govorkova, 2014).

INFLUENZA A VIRUS IN SWINE

In swine, influenza is an important disease resulting in compromised health of pigs, and also has a significant impact on the production economy. SIV is also included in the porcine respiratory disease complex (PRDC), a term used for describing respiratory symptoms and poor growth in finishing pigs. PRDC is a multifactorial syndrome caused by a combination of several pathogens and environment (Bochev, 2007).

Swine influenza was described for the first time in the Midwestern US in 1918 (Koen, 1919), this description coincided with the human influenza pandemic. It was not until 1930 that the first SIV, belonging to the H1N1 lineage, was isolated from North American pigs, which also happened to be the first IAV ever to be isolated (Shope, 1931a). The clinical signs of swine influenza resemble those also observed for humans and are characterized by an acute onset of the disease, fever, inactivity, inappetence, respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge (Zell *et al.*, 2012). Virus is shed from nasal discharges and is transmitted via pig to pig contact by droplets and aerosols (Brown, 2000b). Usually the course of disease is mild and sometimes also asymptomatic (Loeffen *et al.*, 1999). The morbidity is high (up to 100%) and mortality has been estimated to be low (around 1-4%) (Koen, 1919;Shope, 1931b), however secondary bacterial infections is a common sequelae and can exacerbate the severity of the clinical condition. The incubation period varies from 1-3 days and full recovery is usually obtained 4-7 days after onset of the disease (Ma *et al.*, 2009). In European pigs the virus has been found to circulate throughout the year (Zell *et al.*, 2012), which could be due to modern farming facilities having a high number of pigs, where fatteners are slaughtered every six months, leading to a continuous introduction of new and susceptible animals into the herds (Brown, 2000b).

European and Danish swine influenza viruses

The IAV observed for the first time in pigs back in 1918 of H1N1 subtype was later termed classical swine H1N1(Brown, 2000b). Several years later, sequencing of both human IAV and SIV revealed that the virus most probably spread from humans to pigs (Reid *et al.*, 1999).

The classical swine H1N1 virus (Fig. 2) was demonstrated in Europe in 1976, in pigs imported to Italy from US (Nardelli *et al.*, 1978). Accordingly, the classical swine H1N1 was isolated during epidemics

from several European countries, including Denmark in the period 1981-1984 (Johnsen, 1985;Sorensen *et al.*, 1981). In 1979 outbreaks of influenza among swine in the Netherlands and Germany were caused by a new H1N1 subtype which differed from the classical H1N1 (Pensaert *et al.*, 1981). The new H1N1 virus was thought to be the result of a transmission event from birds to pigs and created a new lineage, afterwards referred to as the avian-like H1N1 (Fig. 2) (Scholtissek *et al.*, 1983). The avian-like H1N1 spread throughout Europe, causing severe disease in swine and gradually replaced the classical swine H1N1 in Europe (Abusugra *et al.*, 1987;Schultz *et al.*, 1991).

Following the "Hong Kong flu" pandemic in 1968, a human-like H3N2 virus was isolated from pigs in Asia (Miwa *et al.*, 1987). This human-like H3N2 spread and became adapted to swine and started to circulate, though at low levels, in Europe (Ottis *et al.*, 1982;Tumova *et al.*, 1980). Around 1984, a reassortment event between the human-like H3N2 and the avian-like H1N1 took place. The human-like H3N2 acquired the internal genes of the avian-like H1N1, but kept the HA and NA from the human-like H3N2 subtype (Castrucci *et al.*, 1993). This created the reassortant human-like H3N2 virus subtype (Fig. 2) which induced severe clinical symptoms in pigs compared to the original human-like H3N2 (Campitelli *et al.*, 1997;Castrucci *et al.*, 1993). Since its detection in the early 1980s, the reassortant human-like H3N2 gradually replaced the original human-like H3N2 in Europe and during the 1990s it was identified in several European countries including Denmark (Castro *et al.*, 1988;Yus *et al.*, 1992;Zhang *et al.*, 1989) (Bøttner, personal communication).

In 1987, an H1N2 virus was isolated in Brittany in France, with its HA originating from the avian-like H1N1 and its NA originating from the reassortant human-like H3N2 (Gourreau *et al.*, 1994). This H1N2 did not spread in Europe, but in 1994 another H1N2 was isolated in UK. The HA of this virus was found to originate from a human-like H1N1 that had been circulating in pigs since the 1977 Russia pandemic (Brown *et al.*, 1993) and NA originating from the European reassortant human-like H3N2. This virus now had HA and NA of human-like origin, whereas all internal genes were of avian-like origin (Brown *et al.*, 1998) and was therefore named reassortant human-like H1N2 (Fig. 2). This virus quickly spread to the central Europe (Kuntz-Simon and Madec, 2009), but has not been detected in Denmark (Bøtner, 1994;Trebbien *et al.*, 2013). In 2003 a new reassortant H1N2 was detected in Denmark, containing an H1 of avian-like H1N1 origin and a N2 of reassortant human-like H3N2 origin (Fig. 2) (Trebbien *et al.*, 2013). This subtype has since become established in Denmark and is now one

of the two most prevalent subtypes circulating in the Danish swine herds (unpublished data). The reassortant avian-like H1N2 virus has also been detected in Sweden, Germany and Italy (Balint *et al.*, 2009;Harder *et al.*, 2013;Metreveli *et al.*, 2011). Several different reassortants of the H1N2 subtype have been detected in different parts of Europe (Kyriakis *et al.*, 2013;Marozin *et al.*, 2002), which has made naming and interpretation complicated.

In January 2010, the first H1N1pdm09 was detected in Danish pigs and from this first detection, the H1N1pdm09 started to co-circulate with the remaining enzootic SIVs in Denmark (unpublished data). The H1N1pdm09 which caused the latest pandemic in the human population has a unique gene constellation with six of its segments (PB2, PB1, PA, HA, NP and NS) derived from the North American triple reassortant swine (TRS) IAV and two segments (M and NA) derived from the Eurasian SIV lineage (Fig. 3) (Dawood *et al.*, 2009).

Since 2011 a more detailed passive surveillance of SIVs has been conducted in Denmark, revealing that the avian-like H1N1 and H1N2 SIVs are the two most prevalent subtypes circulating in Danish swine, followed by the H1N1pdm09. Since 2009, the H3N2 virus has only been detected few times in the surveillance (unpublished data). But more importantly, since the more detailed surveillance began, several new reassortant viruses have been detected, one of these contains seven segments derived from the avian-like H1N1 and a N2 most closely related to that of a human seasonal H3N2 virus that circulated in the mid-1990s (Breum *et al.*, 2013). This subtype has been termed H1avN2hu (Fig. 2), to distinguish it from the H1N2. Another subtype containing seven genes from the H1N1pdm09 and an N2 gene similar to the one found in the H1avN2hu, termed H1pdmN2hu (Fig. 2), has also been detected, as well as a subtype containing the seven segments from H1N1pdm09 and the N2 from the reassortant avian-like H1N2 (termed H1pdmN2sw, Fig. 2). The H1pdmN2sw has also been detected in Germany (Starick *et al.*, 2012). Reassortants different from those already mentioned have also been detected in Denmark, but common for the three mentioned here, are that they have all been detected in several submissions in the recent years, indicating that they may have become established in Danish pigs, albeit presumably at low levels (unpublished data).

Segment								
Subtype	HA	NA	M	NP	NS	PA	PB1	PB2
Classical H1N1								
Avian-like H1N1								
Reassortant human-like H3N2								
Human-like H1N1								
Reassortant human-like H1N2								
Reassortant avian-like H1N2								
H1N1pdm09								
H1avN2hu								
H1pdmN2hu								
H1pdmN2sw								

Origin	Color
Classical	Pale red
Avian-like	Blue
Human-like (H3N2)	Pale green
Human-like (H1)	Green
Human-like (N2)	Pale orange
H1N1pdm09	Purple

Figure 2 Overview of the different influenza A virus (IAV) subtypes circulating in Europe.

North American swine influenza viruses

Since the first isolation of SIV virus in 1930 in US (Shope, 1931a) and until 1998-1999, only the classical swine H1N1 virus was isolated in the US pig population. In 1998, severe influenza outbreaks were observed in several states in the US. These outbreaks were found to be caused by two different H3N2 reassortants (Fig. 3), one was a double reassortant containing the NS, NP, M, PB2 and PA segments from the classical swine H1N1 and PB1, HA and NA from a seasonal human H3N2. The other virus was a triple reassortant containing the NP, NS and M segments from the classical swine H1N1 and PB1, HA and NA segments from the same seasonal human H3N2 as the double reassortant and also PB2 and PA segments from an avian virus (Zhou *et al.*, 1999). The double reassortant H3N2 virus disappeared from the pig population, whereas the triple reassortant became established in the US pig population (Webby *et al.*, 2000). This triple reassortant H3N2 virus later reassorted with the classical swine H1N1 and evolved further through antigenic drift and different introductions of human H3 genes. Of the different introduction of H3 genes, three became established and gave rise to the phylogenetic clusters I, II and III. The H3N2 belonging to the phylogenetic cluster III has become dominant in North America (Gramer *et al.*, 2007). Common for all these H3N2 reassortants are their constellation of internal genes; classical swine H1N1-like NP, M and NS, avian-like PB2 and PA and

human H3N2-like PB1, which have been termed the triple reassortant internal genes (TRIG) and appear to provide the virus with an advantage upon infection of the pig host (Vincent *et al.*, 2008).

Further reassortants have been identified and among these, the reassortant H1N1 (rH1N1, Fig. 3), with its surface genes originating from the classical swine H1N1 and the internal genes constituted by the TRIG cassette and also a virus of H1N2 subtype (Fig. 3) containing the TRIG cassette with the N2 gene from the triple reassortant H3N2 and the H1 from the classical swine H1N1 (Karasin *et al.*, 2002;Webby *et al.*, 2004). Based on the H1s derived from classical swine H1N1s, these viruses have been divided phylogenetically into three different clusters termed α, β and γ. In 2003 a new H1N2 subtype of human origin (Karasin *et al.*, 2006) and in 2005 yet another subtype with human-origin H1 and N1 and the TRIG cassette was identified (Fig. 3) (Vincent *et al.*, 2009), representing separate introductions of human-origin H1 viruses into the US pig population. These two new lineages have since become endemic in US pigs and are now one of the major subtypes isolated and characterized from swine respiratory outbreaks (Lorusso *et al.*, 2012) and have been assigned phylogenetically as belonging to cluster δ1 and δ2, respectively (Vincent *et al.*, 2009).

Subtype	Segment							
	HA	NA	M	NP	NS	PA	PB1	PB2
Classical H1N1	Pale red	Pale red	Pale red	Pale red	Pale red	Pale red	Pale red	Pale red
Triple reassortant H3N2	Green	Green	Pale red	Pale red	Pale red	Yellow	Green	Yellow
Double reassortant H3N2	Green	Green	Pale red	Pale red	Pale red	Pale red	Green	Pale red
rH1N1	Pale red	Green	Pale red	Pale red	Pale red	Yellow	Green	Yellow
H1N2	Yellow	Green	Pale red	Pale red	Pale red	Yellow	Green	Yellow
H1N2	Light green	Green	Pale red	Pale red	Pale red	Yellow	Green	Yellow
H1N1	Light green	Light green	Pale red	Pale red	Pale red	Yellow	Green	Yellow
H1N1pdm09	Yellow	Blue	Blue	Pale red	Pale red	Yellow	Green	Yellow

Origin	Color
Classical	Pale red
Eurasian avian-like	Blue
Human-like (H3N2)	Green
Human-like (H1 and N1)	Light green
Avian-like	Pale yellow

Figure 3 Simplified overview of the subtypes that have been isolated from the US pig population.

Asian swine influenza viruses

Movement of live pigs has a high impact on the introduction and establishment of new strains to immunologically naïve pig populations and thereby also the potential development of new SIVs. In this respect, Asia is very important due to the frequent import of live pigs from both Europe and the US.

The classical SIV was detected in Asia for the first time in the mid-1970s (Shortridge and Webster, 1979) and human H3N2 viruses were found to circulate in different variants among pigs in Asia, containing the entire human-like structure (Peiris *et al.*, 2001;Shortridge and Webster, 1979). In the late 1990s, the reassortant human-like swine H3N2 circulating in Europe, was introduced in Asia (Gregory *et al.*, 2001), and a few years later there was an introduction of European avian-like swine H1N1 which became dominant (Vijaykrishna *et al.*, 2011). Other co-circulating viruses are the American triple reassortant H1N2 and the H1N1pdm09 as well as a wide range of transient reassortants. The only reassortant virus that has become established is a strain containing seven segments from the European avian-like swine lineage and a NS segment from the TRS lineage and this lineage is now the predominant virus of the European avian-like swine lineages (Vijaykrishna *et al.*, 2011).

Interspecies transmission between human and swine

During the last decades, swine farming practices have significantly changed from small-scale backyard farming to large-scale modern-production facilities. This has also significantly increased the interface between human and swine, which thereby also has enhanced the probability of interspecies transmission. In the first line is the personnel working at these large-scale farming facilities, they present a naïve population in terms of SIVs and they can also be involved in the introduction of human IAV to pigs – a situation that potentially represents the possibility for the formation and establishment of new reassortant viruses in both species (Krueger and Gray, 2012).

The first well-documented human case of infection with SIV occurred in 1958 (Gray *et al.*, 2012;Krueger and Gray, 2012) and since then sporadic infections with SIV have continually occurred (Krueger and Gray, 2012). Among the cases of SIV transmissions to humans were the incidence at Fort Dix, Missouri, in 1976, where several soldiers were infected with classical swine H1N1 (Gaydos *et al.*, 1977). In 2009 an IAV of H1N1 subtype containing segments of swine-origin, caused the first pandemic of this century (Garten *et al.*, 2009;Smith *et al.*, 2009b) and most recently cases where a variant of the North American H3N2 SIV, containing a H1N1pdm09 M gene, have been transmitted to humans on several occasions (2012;2011;Nelson *et al.*, 2012). A general trait of the SIV transmission to humans is that sustained human-to-human transmission has only been observed in rare cases (Krueger and Gray, 2012).

The introduction of IAVs from human to swine has likewise occurred on several occasions with the first described introduction being the classical H1N1 that was transmitted from human to swine (Koen, 1919). This was followed by the introduction of H3N2 originating from the 1968 pandemic (Miwa *et al.*, 1987;Ottis *et al.*, 1982;Tumova *et al.*, 1980) and lately reverse zoonotic events introduced the H1N1pdm09 into the pigs as well (Howden *et al.*, 2009;Moreno *et al.*, 2010). Recently, a phylogenetic study also estimated that IAVs from humans have frequently been introduced to the pig population during the last century. In this time period at least 20 introductions of human IAVs showed sustained transmission in pig population for at least a year after the introduction (Nelson *et al.*, 2014).

These findings clearly show that human IAVs as well as SIVs both play a substantial role in the overall ecology of IAVs.

BIOLOGY OF INFLUENZA VIRUSES

Taxonomy and structure

IAV belongs to the family *Orthomyxoviridae*, including five genera, Influenza A, B and C, Thogoto- and Isa virus. IAVs have been isolated from a wide range of species, including humans, swine, birds, seals, cats, horses and dogs, but aquatic birds are considered the natural reservoir of IAV (Webster *et al.*, 1992). Influenza B viruses have been isolated from humans and seals and influenza C viruses have been isolated from humans and swine and usually only causes mild disease in the upper respiratory tract. Influenza B viruses can cause a wide variety of disease, but generally clinical symptoms are similar to those of IAV (2014;Baigent and McCauley, 2003).

The IAV genome consists of a total of 13588 nucleotides and virions are enveloped and spherical or pleomorphic with a size ranging from 50-120 nm in diameter (Fujiyoshi *et al.*, 1994).

The genome of IAV is divided into eight negative sense RNA segments encoding at least 13 proteins. These proteins include polymerase basic proteins 1 and 2 (PB1 and PB2), polymerase acidic protein (PA), nucleocapsid protein (NP), surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), matrix proteins (M1 and M2) and non-structural proteins 1 and 2 (NS1 and NS2, also known as nuclear export protein; NEP) (Table 1).

Table 1 Influenza A virus genome

Segment	Protein	Size (nucleotide)	Function
1	PB2	2341	Polymerase subunit: host cap binding and endonuclease
2	PB1	2341	Catalytic subunit of polymerase
	PB1-F2		Pro-apoptotic protein
	PB1-N40		Unknown function
3	PA	2233	Subunit of polymerase, active in vRNA synthesis
	PA-X		Represses cellular gene expression
4	HA	1778	Receptor binding to sialic acids and fusion with host cells
5	NP	1565	Nucleocapsid
6	NA	1413	Neuraminidase, cleaves sialic acid linkages
7	M1	1027	Matrix protein
	M2		Ion channel
8	NS1	890	Interferon response inhibitor
	NEP (NS2)		RNP nuclear transport

The envelope covering the virion is made of a lipid bilayer derived from the host cell membrane and contains the three viral proteins; HA, NA and M2 (Fig. 4). The HA protein is the most abundantly expressed of the three and constitutes approximately 80% of the surface proteins, NA constitutes approximately 17% and M2 as the least abundantly expressed protein with just 16-20 M2 proteins per virion (Nayak *et al.*, 2009). On the inside of the viral membrane is M1, where it makes up a matrix that holds the viral nucleoproteins (vRNPs). The vRNPs are comprised of viral RNA (vRNA) and are wrapped around NP and small amounts of NEP and at one end of the vRNPs are the polymerase proteins, PA, PB1 and PB2 (Nayak *et al.*, 2009).
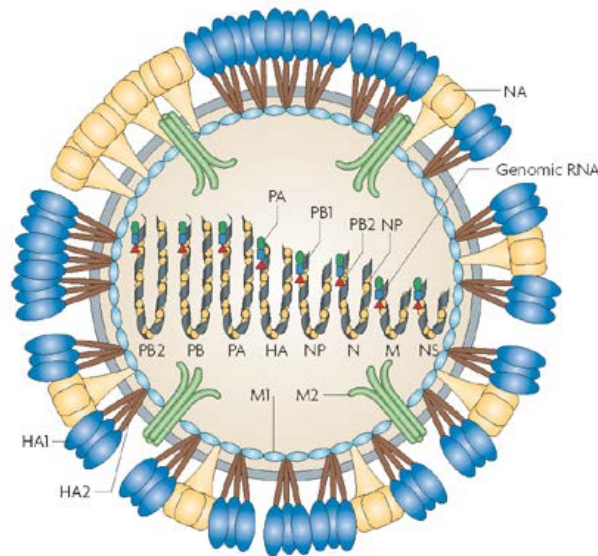
Figure 4 A schematic presentation of an influenza A virus. The surface glycoproteins hemagglutnin (HA) and neuraminidase (NA), as well as the ion channel matrix 2 (M2) are located on the surface of the virion. On the inside of the membrane, the matrix (M1) protein forms a matrix holding the viral ribonucleoproteins (vRNPs) containing the viral polymerase complex (PA, PB1 and PB2) and NP, as well as small amounts of NEP (Karlsson Hedestam *et al.*, 2008).

IAVs are subtyped according to their surface glycoproteins HA and NA and to date, 18 HA and 11 NA subtypes have been isolated, where the H17N10 and H18N11 are the most recently discovered subtypes and have only been isolated from bats (Fouchier *et al.*, 2005;Tong *et al.*, 2013;Tong *et al.*, 2012;Webster *et al.*, 1992). HA subtypes are further divided into two groups based on phylogenetic relationship, where group 1 includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18; group 2 includes H3, H4, H7, H10, H14 and H15 (Fig. 5) (Air, 1981;Nobusawa *et al.*, 1991;Wu *et al.*, 2014). Also NA can be divided into two groups, here group 1 contains N1, N4, N5 and N8 and group 2 contains N2, N3, N6, N7 and N9 (Russell *et al.*, 2006), recently N10 and N11 have been proposed to form group 3 (Fig. 5) (Wu *et al.*, 2014).
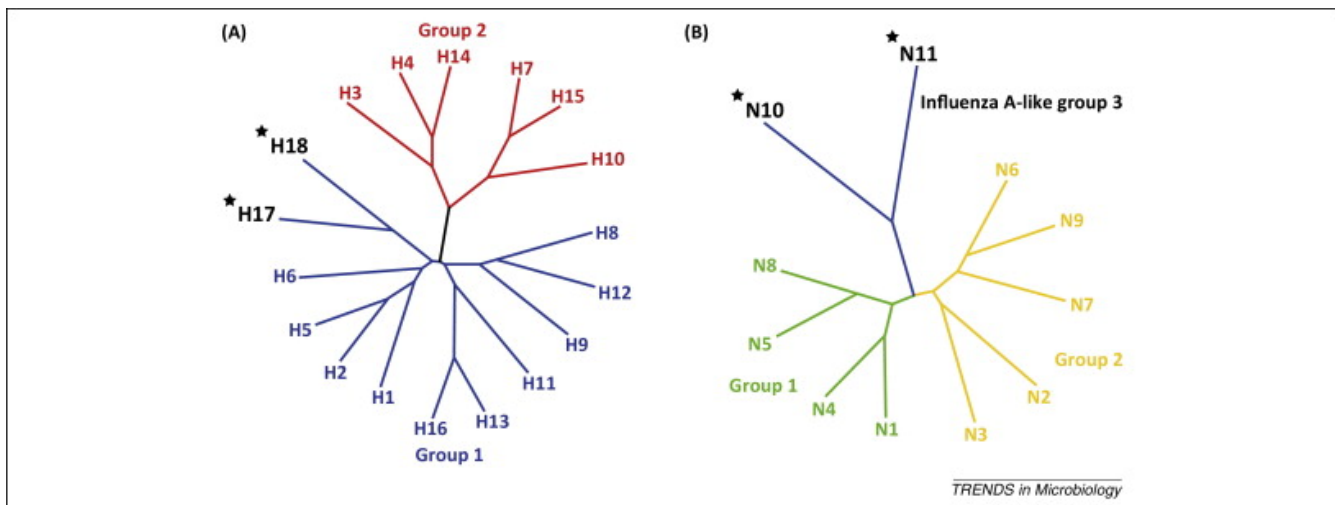
Figure 5 Phylogenetic relationship among hemagglutinins and neuraminidases. (A) HAs can be grouped into two groups, group 1 and 2. The bat-derived H17 and H18, belonging to group 1, are marked with an asterisk. (B) NAs are originally grouped into two groups, but the bat-derived N10 and N11 (marked with asterisks) could not be placed in either group and were therefore proposed to form a third group (Wu *et al.*, 2014).

Influenza A evolution

Antigenic variation is the evolutionary advantage of the IAV which can be divided into two phenomenons, namely antigenic shift and antigenic drift. Antigenic shift can occur when a host is co-infected with two or more different IAVs and gene segments are interchanged during virion packing. This event is termed reassortment and is defined by the exchange of intact segments between two differing influenza A viruses (Brown, 2000a).

Antigenic drift is due to the high mutational rate of the polymerase, where the lack of proofreading of the RNA polymerases contribute to a mutation rate of the order of 10^{-5} to 10^{-6} substitutions/site/replication (Nobusawa and Sato, 2006;Stech *et al.*, 1999), and the selective pressure of the immune system of the host. Most nucleotide substitutions are silent, meaning that they do not affect the virus. The majority of amino acids residues in the IAV are negatively selected, a process termed purifying selection. This means that substitutions are only accepted under certain selection pressures, since they otherwise would affect viral fitness. When amino acid residues are positively selected, it is termed diversifying selection and only very few residues are positively selected. The combination of the lack of proofreading of the polymerase and the immune pressure exerted by the host

will favor the situation where the virus obtains a substitution that permits its escape from the immune system and thereby provides a fitness advantage to the virus. The accumulation of these substitutions will eventually lead to the development of an antigenically different virus strain which is more fit and able to replace existing strains (Zell *et al.*, 2012).

SURFACE GLYCOPROTEINS

Hemagglutinin

HA is synthezised from RNA segment four as a precursor protein, HA0, consisting of a single polypeptide chain of 549 amino acids (for H1) (Sriwilaijaroen and Suzuki, 2012). Subsequent cleavage of HA0, as well as glycosylation, occurs during the co-translational process. The precursor protein is cleaved by cellular proteases into two chains, HA1 (327 amino acids) and HA2 (222 amino acids), respectively (Fig. 6). Cleavage of the precursor protein happens for most HA subtypes at a single basic amino acid residue which most often is arginine and rarely a lysine (Laver, 1971) and subsequent to cleavage, the two subunits are still linked through a disulphide bond (Sriwilaijaroen and Suzuki, 2012;Wiley and Skehel, 1987). In poultry, virulence of H5 and H7 subtypes are determined by the number of basic amino acids in the cleavage site. IAVs containing a single basic amino acid at the cleavage site are said to be low pathogenic avian influenza (LPAI), whereas IAVs containing a multi-basic amino acid sequence are said to be a high pathogenic avian influenza (HPAI). The difference in virulence of these two types is due to the cleavage of HPAI by specific proteases present throughout the host giving rise to a systemic infection (Alexander, 2000).

The functional HA protein is a homotrimeric type I integral membrane protein (Fig. 6), which carries multiple glycosylation sites. The HA is involved in viral attachment and entry into host cells during virus infection, by binding to sialic acid receptors on the host cell surface. The three monomers of HA each consists of conserved structures, including a globular head domain and a stem region. The globular head region is comprised of a pocket at the distal tip, acting like the receptor binding site (RBS) and surrounded by antigenic sites. At the edges of the receptor binding site, three conserved secondary structures exists, the 130 (including residues 130-138) - and 220 (residues 221-228)-loops and the 190-helix (residues 190-198). A set of conserved residues, 98Y, 153W, 183H and 195Y (H3-numbering is used throughout this thesis) forms the base of the RBS (Skehel and Wiley, 2000). The

antigenic sites of HA was determined by analysis of acquired mutations upon growth of the viruses in the presence of monoclonal antibodies and antigenic sites are defined as antibody recognizing sites. For H1, five antigenic sites were determined and designated accordingly: Sa (residues 128-129, 156-160, 162-167) and Sb (residues 187-198), Ca1 (residues 169-173, 206-208, 238-240) and Ca2 (residues 140-145, 224-225) and Cb (residues 74-79) (S: strain-specific; C: common) (Caton *et al.*, 1982;Gerhard *et al.*, 1981). For H3, these sites are designated A (122, 133, 137, 143-146), B (155-156, 158-159, 188-189, 193), C (53-54, 275, 278), D (201, 205, 207-208, 217, 220) and E (Jahangir *et al.*, 2012;Perez *et al.*, 2013;Shin and Seong, 2013;Skowronski *et al.*, 2013;Tharakaraman *et al.*, 2013;Webster and Laver, 1980;Wiley *et al.*, 1981). Specific amino acids have previously been shown to be critical determinants of receptor binding specificity. Amino acids 190 and 225 have been shown to be of high importance for receptor binding and host specificity of H1 viruses. For H3 viruses the residues shown to be of major difference between human and avian viruses are located at residues 226 and 228 (Matrosovich *et al.*, 2000).
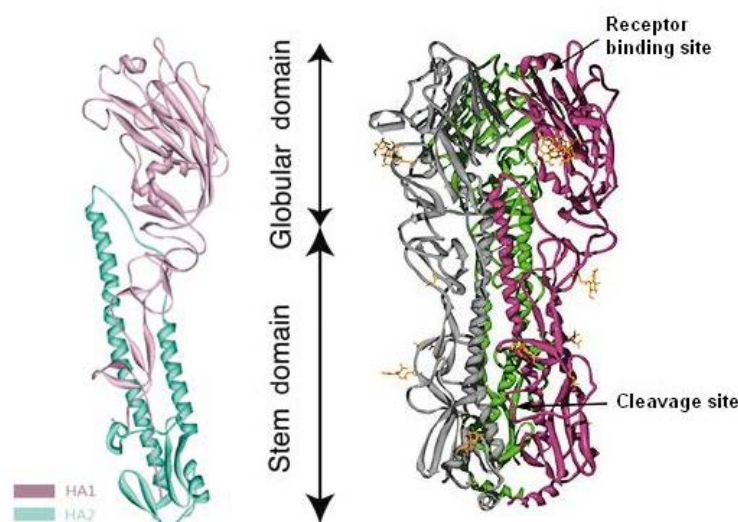
Figure 6 Structure of influenza A virus hemagglutinin. The monomeric form of hemagglutinin showing the globular domain and the stem domain, where HA1 is shown in pink and HA2 in cyan. In the active trimeric form of HA, each of the monomers are colored and arrows depict the site of cleavage and receptorbinding site. Glycosylation sites are shown in yellow. Modified from (Ge *et al.*, 2010) and (http://www.accessexcellence.org/WN/SU/avianflufeb04.php).

Antigenic cartography is a method used for visualization of the antigenic relationship among IAVs. This method is based on the relative distances between antigens and selected antiserums. In this way it

is possible to predict when an eventual vaccine update is necessary, since viruses drifting away from vaccine strains will indicate a beginning antigenic diversity. Recently it has also been shown that substitutions at certain amino acid residues were associated with cluster transitions and/or antigenic outliers in antigenic cartography. In the human H3N2, these amino acids were 145, 155, 156, 158, 159, 189 and 193 (Fig. 7)(Koel *et al.*, 2013). Except for residue 193, the exact same amino acid residues were defined in North American swine H3N2 viruses (Lewis *et al.*, 2014). For human H1N1 viruses, substitutions at amino acid residue 145, were also shown cause cluster transitions (Koel *et al.*, 2013).
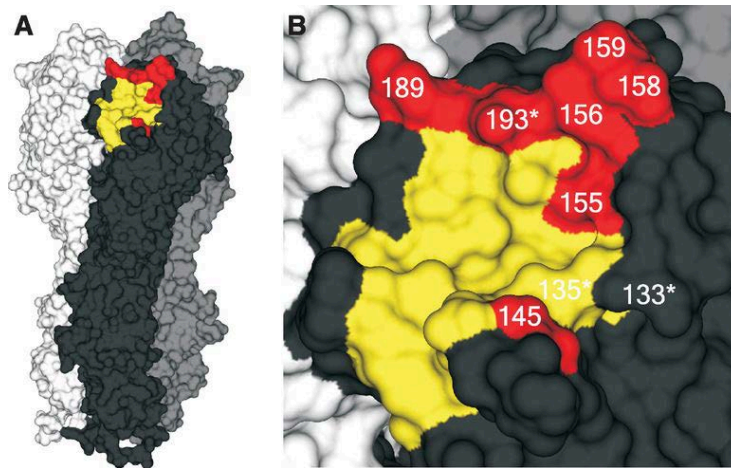
Figure 7 The hemagglutinin molecule of A/Aichi/2/1968 (H3N2) influenza virus. Here, amino acid residues crucial in antigenic drift are indicated. (A) The trimeric model of the HA molecule. The three monomers are shown in black, grey and white. Amino acids involved in antigenic drift are shown in red and the receptor-binding site is shown in yellow. (B) Residue 145 is located in antigenic site A, and residues 155, 156, 158, 159 and 193 are in the antigenic site B. These are considered "cluster-transition" amino acids. Asterisks indicates accessory substitutions and position 193 is both a cluster transition substitution and an accessory substitution. Modified from (Koel *et al.*, 2013).

Neuraminidase

NA is a type II integral membrane protein that cleaves sialic acid linkages, including those between hemagglutinin and cell surface receptors, which thereby releases newly formed IAVs from the cell surface (Fig 8) (Palese and Compans, 1976). Structural studies have shown that NA projects slightly further from the membrane compared to HA and that the cell membrane of the virion is covered with approximately 40-50 NA spikes. These spikes are situated in clusters of approximately 300-400 HA spikes on an average sized virion (around 120 nm) (Harris *et al.*, 2006). The functional NA protein consists of four identical monomers with a size of approximately 470 amino acids (Fig. 8). Each monomer consists of four structures; an N-terminal sequence, a transmembrane domain and a stalk domain of varying length ending in the globular head domain carrying the active site of the enzyme. The stalk of the NA protein has been found to vary in both length and sequence, dependent on the subtype (Blok and Air, 1982). A short stalk in NA has been associated with progeny viruses that are unable to detach from the host cell membrane, due to the inability of the active site to access the substrate efficiently and furthermore, stalk length has also been associated with host range restrictions (Castrucci and Kawaoka, 1993).

The active site consists of a pocket on the surface of each subunit formed like a six-bladed propeller and constituted by 15 amino acids shown to be conserved in all IAVs (Colman *et al.*, 1983;Varghese *et al.*, 1983).

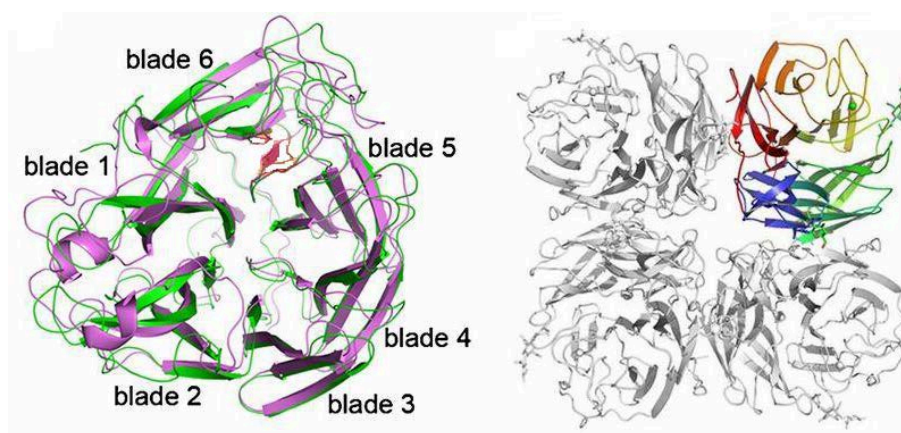**Figure 8** Structure of neuraminidase. The monomer shows a propeller-like arrangement of six-bladed β sheets where the active site is located in the middle of this arrangement, surrounded by antigenic sites. The active protein consists of four monomers, of which one of the monomers has been colored. Modified from (Li *et al.*, 2012).

Antigenic sites of NA have been shown to surround the enzyme active site (Malby *et al.*, 1994;Tulip *et al.*, 1992;Venkatramani *et al.*, 2006), but the extent of antigenic sites of NA is not as well characterized as for HA, even though it has been shown that the antigenic drift in NA is similar for NA and HA and that the NA has been mutating faster, compared to HA, in the recent years. The active site of NA is also the target of the anti-influenza antivirals oseltamivir and zanamivir (Air, 2012).

Glycosylation of hemagglutinin and neuraminidase

N-linked glycosylation is a common post-translational process, in which the addition of glycans to glycoproteins takes place. Glycans are added to a specific glycosylation motif N-X-S/T, where T and X is any amino acid except P (Kornfeld and Kornfeld, 1985). Due to this highly conserved glycosylation motif, potential glycosylation sites can be predicted from sequence analysis, but cannot be used as the sole source of identification of glycosylation sites. Potential glycosylation sites can be situated in very close structural proximity and hence are not glycosylated due to potential restrictions in the surrounding amino acid sequence or steric hindrance (Blake *et al.*, 2009;Sun *et al.*, 2011).

In general, N-linked glycans carry critical information in terms of correct folding, maturation and transport or degradation of proteins (Aebi *et al.*, 2010), and the addition of glycans to both HA and NA has been shown to be critical for correct folding of the functional protein (Roberts *et al.*, 1993;Wu *et al.*, 2009). The biosynthesis and modification of glycans occurs in the endoplasmic reticulum and the Golgi apparatus of the host cell and therefore, the structure of the glycans are thereby also determined by the particular host cell (Deom and Schulze, 1985).

The addition of glycans to both HA and NA are found both in globular and in stem regions. These glycosylation sites appear to have a large variation in the globular head region of HA1, whereas the stem regions are found to be more conserved (Wang *et al.*, 2009). The presence of glycosylation sites in globular head regions is thought to provide the IAVs with a selective advantage for escaping the host's immune system (Sun *et al.*, 2011;Vigerust and Shepherd, 2007). Furthermore, glycosylation has also been shown to interfere with receptor binding (Gambaryan *et al.*, 1998;Ohuchi *et al.*, 1997), regulation of the catalytic activity of NA as well as prevention of proteolytical cleavage of the NA stalk (Matsuoka *et al.*, 2009;Wu *et al.*, 2009).

HOST FACTORS AND VIRULENCE

Receptors

The first stage in the viral infection cycle is the recognition and binding of HA to receptors on the surface of the host cell. The cell surface of mammalian cells are covered by a variety of glycans implicated in various physiological and pathological processes and plays an essential role in the attachment of different pathogens, like IAVs (de Graaf and Fouchier, 2014;Varki and Varki, 2007). Glycans are linked to cell-surface glycoproteins and glycolipids and at the outermost ends of the glycans, sialic acids (SA) can be found. SAs are a class of nine-carbon monosaccharides and the two most common forms of these found in mammals are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), both ligands for IAVs (Suzuki *et al.*, 1986). The distribution of Neu5Gc and Neu5Ac has been found to vary among tissues and animals species, but Neu5Gc is absent in humans due to the lack of the CMP-Neu5Ac hydroxylase (CMAH) responsible for its synthesis (Chou *et al.*, 1998). Further, it has been found that IAVs prefer binding to Neu5Ac over Neu5Gc (Suzuki *et al.*, 1986). In swine, both Neu5Ac and Neu5Gc have been found to be present, with the highest abundance of Neu5Ac (Bateman *et al.*, 2010;Sriwilaijaroen *et al.*, 2011).

SAs are bound at the terminal sugar of glycoproteins and the sugar residues linked to SAs can vary in structure and includes galactose (Gal), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) (Nicholls *et al.*, 2008). Linkage of the SA to the sugar residue is generated by different α-linkages from the 2-carbon on the SA (Nicholls *et al.*, 2008).

Receptor distribution

Viruses from different host species usually display different binding preferences and this preference is believed to be a major determinant of IAV host range and species specificity. Typically, human IAVs recognize α2,6-linked SAs. It has previously been shown that α2,6-linked SAs are the most abundant receptor in the upper respiratory tract in humans (Shinya *et al.*, 2006), but accordingly another study showed that the distribution of α2,3-linked SAs is much more widespread in the respiratory tract of humans than first anticipated (Nicholls *et al.*, 2007) (Fig. 9). The same study also found that the expression level of α2,3-linked SAs in children was higher than their expression level of α2,6-linked SAs compared to that of adults.

Structure studies have demonstrated that the RBS of human and pig IAVs shows a slight increase in the receptor binding pocket site compared to that of avian influenza viruses that could increase the binding affinity for α2,6-SA receptors (Ha *et al.*, 2001;Stevens *et al.*, 2004).
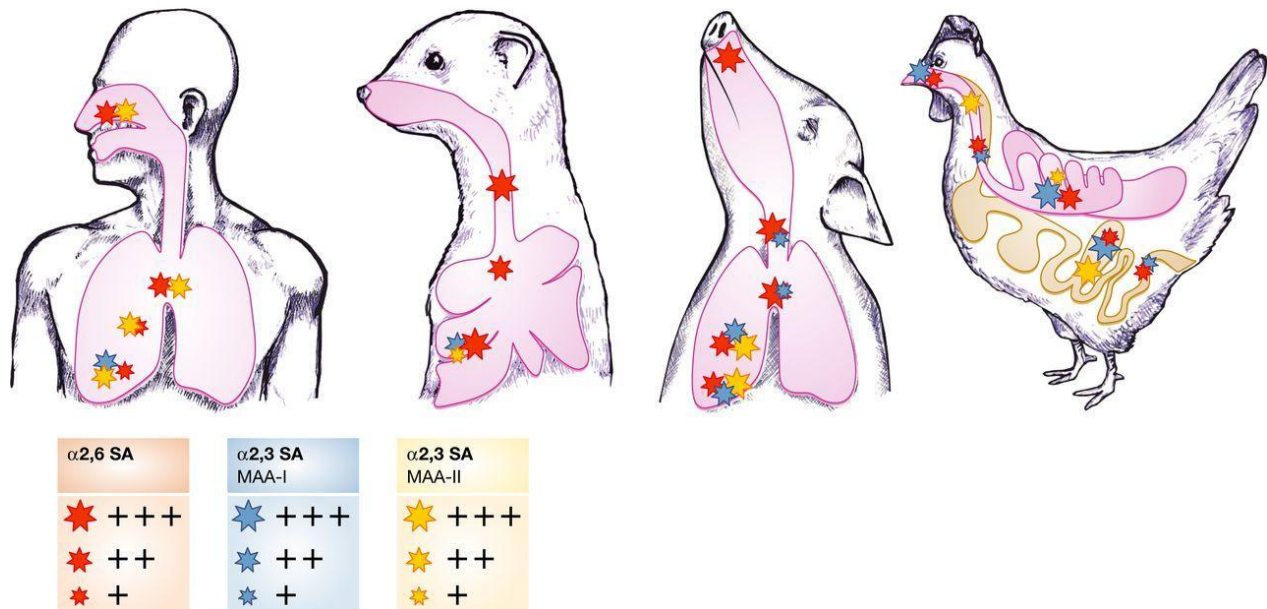
Figure 9 The distribution of α2,6-sialic acid (red stars) and α2,3-SA based on *Maackia amurensis* agglutinin (MAA)-I (blue stars) and MAA-II staining (yellow stars), where star size illustrate receptor abundance. Distribution was analyzed in nasal cavity, trachea, bronchus, bronchiole, and alveoli of humans (Nicholls *et al.*, 2007), ferrets (Xu *et al.*, 2010), pigs (Nelli *et al.*, 2010;Trebbien *et al.*, 2013), and chicken, as well as the small and large intestine of chickens (Costa *et al.*, 2012;Trebbien *et al.*, 2013). Receptor distribution between the different studies may vary, due to the use of specific lectins, methods, or a difference in receptor distribution between animals. Lectin staining was not performed for the human trachea, nasal cavity and bronchioles of ferrets in the cited studies. Modified from (de Graaf and Fouchier, 2014).

Avian IAVs have been shown to prefer binding to α2,3-linked SAs (Nobusawa *et al.*, 1991;Rogers and Paulson, 1983), but recently it was found that some avian species have both α2,3- and α2,6-linked SAs in both the respiratory and intestinal tracts, though the abundance of these receptors were found to vary among avian species (Franca *et al.*, 2013).

Previous studies have reported pigs to be possible mixing vessels for new pandemic IAVs, due to the presence of both α2,6- and α2,3-linked SAs in the trachea (Ito *et al.*, 1998;Suzuki *et al.*, 2000), but recent studies have shown that α2,6- linked SAs were present in high amounts in all areas of the respiratory tract of pigs whereas α2,3- linked SAs were only detected in the lower respiratory tract

(Trebbien *et al.*, 2011). Taken together, these recent findings indicate that the receptor biology of IAVs is more complicated than just the α2,3 over α2,6 binding and that the role of the pig as being a "mixing vessel" of new influenza viruses may not be unique for this specie.

Virulence

The host specificity and virulence of IAVs has been found to be linked to several factors. As mentioned previously, both HA and NA play a large role in this aspect, but are not sole determinants. Host specificity and virulence can be affected by several molecular determinants of the IAV, of which several have been identified.

One of the virulence factors is the PB1-F2 protein, which has been found to contribute to secondary bacterial infections (McAuley *et al.*, 2007) and furthermore has been shown to induce apoptosis of infected cells (Gibbs *et al.*, 2003). The PB1-F2 protein induces apoptosis by localizing to the inner and outer mitochondrial membrane, where it is forming holes in the mitochondrial membrane and thereby releases cytochrome C (Zamarin *et al.*, 2005).

Residue 627 in PB2 has been found to be a major determinant of host specificity and pathogenicity. The avian consensus at this residue is glutamic acid, whereas this residue in human IAVs predominantly possesses a lysine and it has been shown that the E627K mutation both increases the pathogenicity in mammalian hosts (Shinya *et al.*, 2004) and viral polymerase activity (Gabriel *et al.*, 2005).

Especially the NS1 protein has been shown to play a wide range of roles in infection and has a major role in circumventing host cell immunity. The most significant function of NS1 is to antagonize IFN-α/β mediated antiviral response of host cells, which is done by preventing activation of transcription factors and in this way limiting the induction of IFN-β (Hale *et al.*, 2008;Wang *et al.*, 2000). NS1 also interacts with a cellular protein, retinoic acid-inducible gene product-I (RIG-I). This protein is a cytoplasmic pathogen sensor that recognizes IAV ss RNA (Hale *et al.*, 2008). The C-terminus of NS1 has also been identified as a determinant of virulence, but acts in a way independent of the IFN-antagonistic properties of the protein (Jackson *et al.*, 2008). The NS1 C-terminus of avian IAVs has the sequence of a PDZ domain ligand. The PDZ domain is a common structure in proteins involved in the arrangement of signaling complexes at the cellular membrane. Hence, the PDZ domain recognizes and

binds to the NS1 C-terminus, which is thereby able to disrupt the signaling pathway and thus cause increased virulence (Jackson *et al.*, 2008;Obenauer *et al.*, 2006).

Other examples of features exhibited by NS1 in order to circumvent host immunity is by blockage of the 2'-5'-oligoadenylate synthetase (a cytoplasmic antiviral protein) activation of RNaseL, which otherwise inhibits viral replication by degrading RNA (Min and Krug, 2006;Silverman, 2007). Further, it has been shown that NS1 also prevents the maturation of dendritic cells, which thereby limits the activation of T-cells in the host and in this way, NS1 acts by limiting the virus-clearance exerted by the host (Fernandez-Sesma *et al.*, 2006).

REPLICATIVE CYCLUS

Entry

Initiation of the replication cycle of the IAV, begins by binding of the viral HAs to SA receptors at the surface of the host cell and subsequent receptor-mediated endocytosis (Pleschka, 2012). The low pH in the late endosomes causes two actions; first, protons from the endosome enters the virion via the M2 ion-channel causing the interior of the virus to acidify. This acidification helps the viral ribonucleoproteins (vRNPs) to dissociate from the viral matrix. Second, the low pH also causes a conformational change of HA, by which the fusion peptide of the protein is exposed. This allows the formation of a fusion pore between the viral and the endosomal membrane (Harrison, 2008), after which the vRNPs are released into the cytoplasm of the cell for further transportation into the nucleus, where viral replication takes place (Bouvier and Palese, 2008) (Fig. 10).
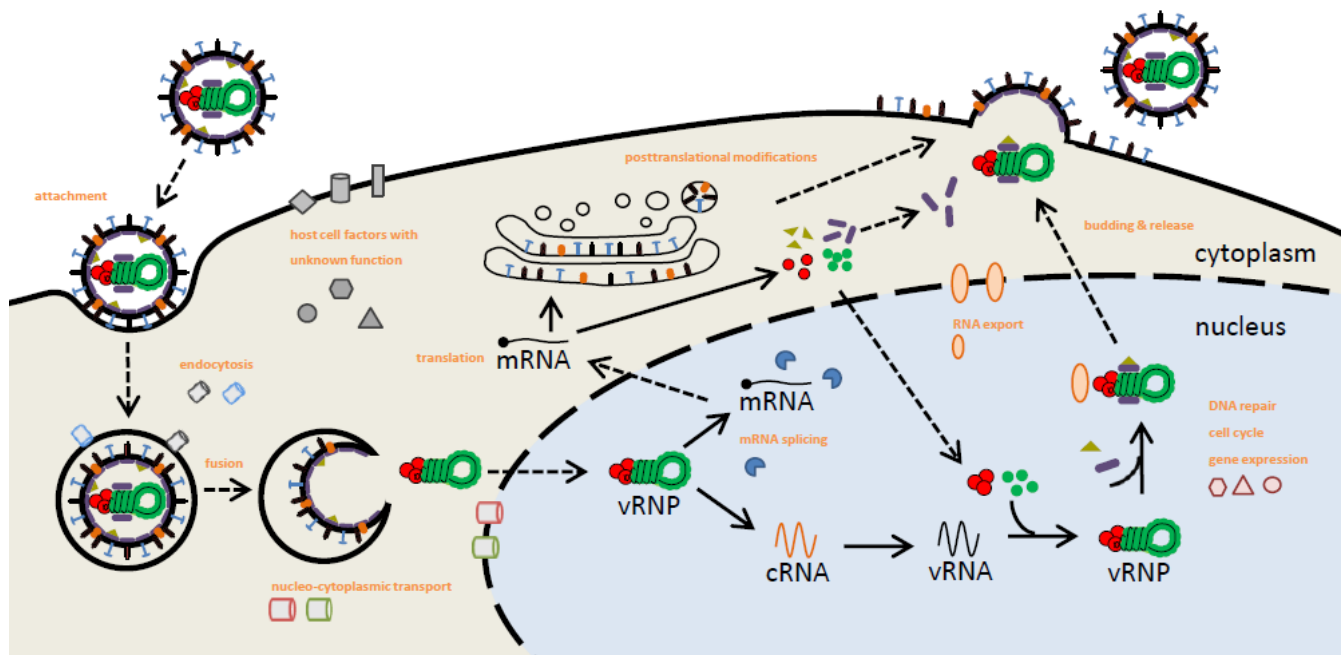
Figure 10 Viral replication cycle. Influenza A virus attaches to sialic acid receptors at the surface of the host cell and enters the cell via endocytosis. Acidic environment in endosomes facilitates the release of vRNPs to the cytoplasm, after which the complex is transported into the nucleus. In the nucleus transcription and replication takes place, mRNA is transported to ribosomes for translation and vRNPs are packaged and transported to the site of assembly at the membrane, where cleavage of sialic acid receptors by NA releases new virions (http://www.mpi-magdeburg.mpg.de/660933/MB.png).

Replication

Transportation of vRNPs into the nucleus, is thought to be mediated by nuclear localization signals present in both PB2 and NP. Once inside the cell nucleus, replication and transcription of vRNA takes place. Transcription is performed by the RNA-dependent RNA-polymerase (RdRp) complex, comprised of the viral PB1, PB2 and PA and vRNA is transcribed into 5'- end capped and 3'-end polyadenylated messenger RNA (mRNA) (Pleschka, 2012). Polyadenylation is obtained by reiterative copying of five to seven uracils near the 5' end of the vRNA. Capping of the 5'-end requires cap-snatching from host cell mRNAs, which are recognized and bound by PB2 and cleaved by the endonucleolytic activity in the PA subunit (York and Fodor, 2013). Subsequent to capping and polyadenylation, the mRNA is exported to the cytoplasm for translation into viral proteins. Further, vRNA is also used as template for synthesis of the positive complimentary RNA (cRNA) used for the replication of new negative sense vRNA (Fig. 10). These new vRNAs can then be used for

amplification or for packaging into progeny virions. The new vRNA for packaging is co-transcriptionally encapsidated by free NP and thus forms a new vRNP. Initially both transcription and replication occurs at a high rate, but later in the infection cycle transcription decreases, whereas replication continues (Shapiro *et al.*, 1987). The shift from transcription to replication has been found to implicate, at least partly, NP which is thought to play a regulatory role in the process (Portela and Digard, 2002;Shapiro and Krug, 1988). Further, also M1 has been implicated in the down regulation of the mRNA transcription through accumulation in the late stage of infection (Perez and Donis, 1998).

Assembly and release

HA, NA and M2 proteins are synthesized from viral mRNA in ribosomes and into the endoplasmic reticulum, where they are folded and accordingly transferred to the golgi-apparatus for further post-translational modifications, before they reach the cell membrane, where they are incorporated into the lipid bilayer (Bouvier and Palese, 2008). Subsequent to the transcription in the nucleus, vRNPs are transported through nuclear pores to the site of assembly on the cell membrane, a transport thought to be mediated by a NEP-M1 complex (Hutchinson and Fodor, 2013). The exact mechanism of packaging of vRNPs is not fully understood, but it is suggested that all eight segments contains packaging signals, thus ensuring correct packaging in the majority of progeny virions (Bouvier and Palese, 2008). At the cytoplasmic site of the cell membrane, the accumulation of M1 initiates budding and subsequent to budding, the new virion still remains bound to the cell surface via SA receptors until the binding has been cleaved by the enzymatic activity of the NA protein (Kuiken *et al.*, 2006).

Following successful replication and budding of the new virion, infection of new cells has to be initiated in order to maintain infection. A step potentially arrested by innate immune responses exerted by the host (Kuiken *et al.*, 2006).

IMMUNOLOGY

Successful infection by the influenza A virus is dependent of a wide spectrum of factors. Initiation of infection depends on the ability of the virus to infect epithelial cells, lining the respiratory tract, which are the primary target of the virus. Here the innate immune system is the first defense meeting the virus – a fast general defense system that lacks specificity and memory.

Innate immunity

Innate immunity can be divided into two steps, the physical barriers and the cellular immune response. The physical barriers include mucus found in the airways and is composed of cells, cellular debris, polypeptides and mucins. Mucins are a family of glycoproteins that are highly sialylated, a feature resulting in water being retained at the surface and thus creates a viscous gel and thereby a physical barrier for the virus (Nicholls, 2013). Having crossed this physical barrier successfully, the IAV initiates infection which is recognized by specialized pattern recognition receptors (PRRs). These PRRS recognize pathogen-associated molecular patterns (PAMPs) present in the pathogen or produced during infection (Iwasaki and Pillai, 2014). The IAV is recognized by at least three distinct classes of PRRs being the Toll-like receptors (TLRs), the RIG-I receptors and the NOD-like receptor family member termed NLRP3 (van de Sandt *et al.*, 2012). The TLRs are both found present on the mucosal surface, where they recognize HA and NA proteins of the virus, as well as intracellularly where they recognize double stranded (ds) and ss RNA. RIG-I receptors and NLRP3 are present in the cytoplasm, where RIG-I receptors recognizes replicating vRNAs and NLRP3 recognize cellular damage (Iwasaki and Pillai, 2014). In the end, the activation of these PRRs leads to the production of IFNs, cytokines and chemokines, which in turn recruits neutrophils, activates macrophages and induces the maturation of dendritic cells (DCs) (van de Sandt *et al.*, 2012).

The DCs play an important role linking the innate and adaptive immune response, since they act as antigen presenting cells (APCs) (Fig. 11). The presentation of antigens can be accomplished in two ways, either by the direct infection of DCs, where antigens are presented by major histocompatibility complex (MHC) class I or by phagocytosis of apoptotic cells or virions, where antigens are presented by MHC class II complexes. The presentation by MHC class I complexes on the cell membrane are

recognized by CD8^{+} T cells, whereas presentation by the MHC class II complexes are recognized by CD4^{+} T cells (Fig. 11) (van de Sandt *et al.*, 2012).

Adaptive immunity

The adaptive immune response represents the second line in the defense against infection with IAV. The CD4^{+} T cells, activated by MHC class I complexes, can be divided into T helper 1 and T helper 2 cells. These helper cells produces cytokines, as well as assists in the proliferation and differentiation of B cells into antibody producing plasma cells and of CD8^{+} cells into cytotoxic T lymphocytes (CTLs). Hence, activation of CD8^{+} cells can take place through both antigen presenting MHC class II complexes and by the activated CD4^{+} T helper cells (Fig. 11) (van de Sandt *et al.*, 2012;Van Reeth and Ma, 2012). The CTLs kills virus-infected cells and the antibody producing plasma cells (originating from B cells), produces antibodies mainly directed against HA, NA, M and NP (Van Reeth and Ma, 2012).
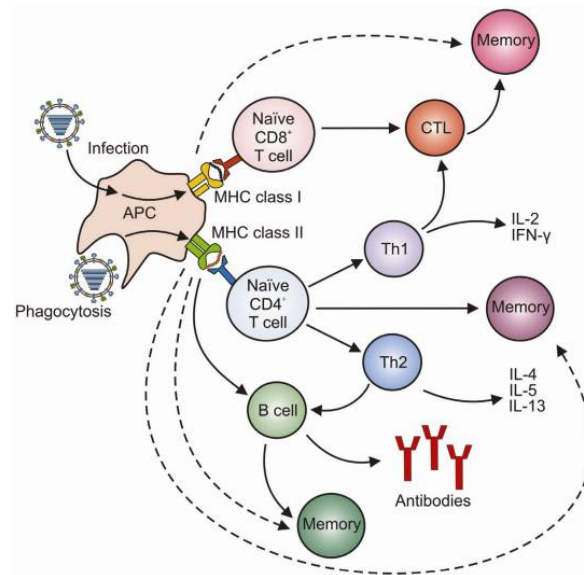
Figure 11 The adaptive immune response. Influenza virus particles are phagocytosed by antigen presenting cells (APCs), which accordingly presents small parts of the virus in MHC I or II complexes and are recognized by CD4^{+} and CD8^{+} T cells. Activated CD4^{+} T cells can be divided into T helper 1 or T helper 2 cells, assisting in the maturation and proliferation of CD8^{+} T cells and B cells, as well as producing cytokines. CD8^{+} T cells mature into cytotoxic T lymphocytes (CTLs), which kill virus particles and B cells mature into antibody-producing plasma cells. Both CD4^{+}, CD8^{+} T cells and B cells participates in the establishment of memory formation preventing reinfection with the same influenza virus subtype (van de Sandt *et al.*, 2012).

Antibodies directed against HA are able to block the binding of viruses to host cells and are thereby termed neutralizing antibodies, whereas antibodies directed against NA prevents the enzymatic activity of the protein and thereby limits the spread of new virions. Antibodies in pigs are usually detected after 7-10 days of infection where antibody levels peak approximately after 2-3 weeks (Heinen *et al.*, 2000;Van Reeth *et al.*, 2006). The usual time course of infection is relatively fast and virus is cleared approximately within a week. Following SIV infection, immunological memory is formed and reinfection with the same virus, results in a faster and stronger immune response. The circulation of HA-specific antibodies in serum provides immunity against homologues viruses, but under experimental conditions pigs have also been shown to be partially protected against different viruses of the same subtype and in few cases also against different subtypes. This observed partial protection is important information for the development of future SIV vaccines (Van Reeth and Ma, 2012).

VACCINATION

Due to the lower immunological pressure exerted by pigs on circulating IAVs, vaccines have not had to be updated at the same regular intervals as necessary for the human influenza A viruses and hence no formal system for the update of SIV vaccines exists. The large diversity of SIVs across countries and continents also complicates such an update (Van Reeth and Ma, 2012).

Due to the great variability in the strains circulating in North America and Europe, vaccines are produced locally and contain different strains. Further, in US the production of autogenous vaccines has gained popularity. In this setting a virus isolated in a farm is inactivated and used as a vaccine on that specific farm now containing a herd-specific strain. Vaccination in US is in general weighted higher compared to Europe, as well as the update of vaccine strains (Van Reeth and Ma, 2012).

For the last decade, the only commercial vaccine, registered for use in Europe has been Gripovac VET (MERIAL, NORDEN A/S), containing the A/New Jersey/8/1976(H1N1) and A/Port Chalmers/1/1973(H3N2) strains. This vaccine was in 2010 replaced by Gripovac 3 - a trivalent vaccine containing the strains A/swine/Bakum/IDT1769/2003(H3N2), A/swine/Haselünne/IDT2617/2003(H1N1) and A/swine/Bakum/1832/2000(H1N2). Both vaccines are adjuvanted and inactivated vaccines propagated in eggs or in cell culture, but Grippovac 3 (MERIAL, NORDEN A/S), contain strains closer related to European contemporary circulating strains (Van Reeth

and Ma, 2012). It has been found that the bivalent commercial vaccine has proven more efficient and also induce a higher cross protection compared to the newer trivalent strain, an efficacy that was found to be linked to the adjuvant used in the former vaccine (Kyriakis *et al.*, 2010). The old bivalent vaccine contained an oil-in-water emulsion adjuvant, whereas the newer trivalent vaccine contained a carbomer based adjuvant (Van Reeth and Ma, 2012) and it has been shown that the replacement of the carbomer with the mineral oil provided better cross protection against the H1N1pdm09 (Durrwald *et al.*, 2010). Thus, not only the selection of appropriate strains for the vaccine, but also the choice of adjuvant plays a role in the efficacy of the vaccines against SIVs. The general view on inactivated vaccine is that it reduces disease symptoms, but does not prevent the infection, replication or shedding of the virus, although a reduction in nasal shedding has been observed in vaccinated pigs (Romagosa *et al.*, 2011).

A common strategy has been to vaccinate sows in order to protect against reproductive problems and to secure that maternally derived antibodies are transferred to the piglets (Kitikoon *et al.*, 2006). Maternally derived antibodies wean after 3-4 months which mean that there is a consistent supply of immunological naïve pigs in the herds which gives the virus the opportunity to circulate or even persist in the pig herds. The natural infection of pigs with SIVs efficiently protects the pig from reinfection with the same or similar strains and it has been shown that this preexisting immunity effectively increases the antibody response upon vaccination with the inactivated vaccine (Van Reeth *et al.*, 2006).

METHODS

The four major methods used in this PhD project are described in the following sections.

Antigenic cartography

Traditionally viruses have been antigenically characterized by hemagglutination inhibition (HI) assay, in which the ability of HA to agglutinate red blood cells is measured in the presence of specific antisera. These antisera are raised against the same or related strains and the resulting HI titer provide information about the affinity of the antisera to the virus and hence the antigenic relationship between the tested strain and the one used to raise the antiserum. Testing of several viruses against several antisera results in many HI values and the interpretation is accordingly difficult and laborious (Cai *et al.*, 2011;Smith *et al.*, 2004).

To overcome this problem, a computational method has been developed. The method, termed antigenic cartography, resembles much geographical cartography in which, for an example, the distances between cities in an area, are arranged in a map according to their mutual distances. In antigenic cartography, each antigen and antiserum is assigned to a point in a 2 or 3 dimensional (2/3D) map in relation to their antigenic distances from each other, measured by their HI titers (Fig. 12) (Cai *et al.*, 2011). The application of specific algorithms also allows for the identification of antigenic clusters of viruses with antigenic properties resembling those of one another (Lewis *et al.*, 2014;Smith *et al.*, 2004).
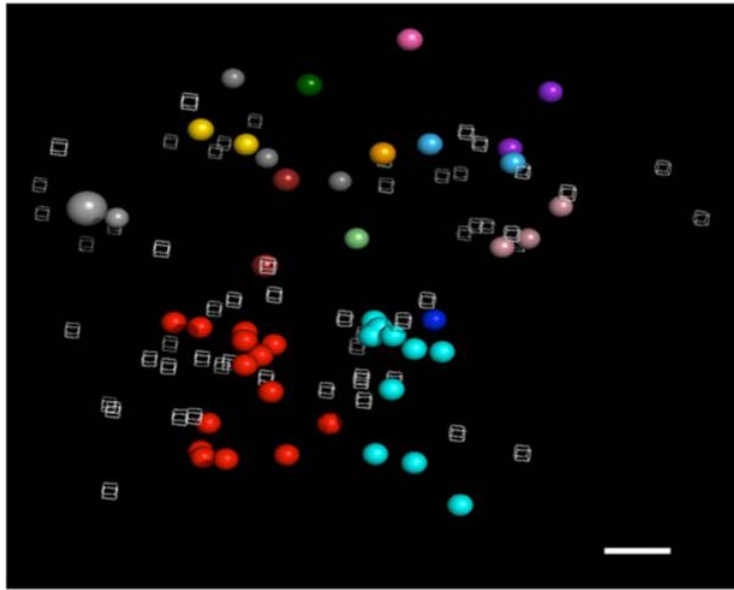
Figure 12 Antigenic cartography map of SIVs and human H3N2. A 3D map of the antigenic relationship between SIVs isolated across the US and human H3N2. Antigens are represented as spheres and antisera as open cubes. Colors of the swine isolates represent the antigenic clusters to which they belong and contemporary human H3N2 viruses are shown as grey spheres, where the large grey sphere represents A/Victoria/361/2011. The scale bar represents one antigenic unit or a twofold change in HI titer. Modified from (Lewis *et al.*, 2014).

Antigenic cartography maps are invaluable tools for the identification of human IAVs drifting away from currently circulating strains or vaccine strains. When the distance between viruses expected to circulate in forthcoming influenza seasons is at least 2 antigenic units away from the vaccine strains, a vaccine update is recommended by WHO (Smith *et al.*, 2004).

Phylogenetic analysis

Mapping the genetic relationship among RNA viruses is best delineated in a phylogenetic tree. Phylogenetic analysis is a widely used method for depicting the evolutionary relationship among sequences from related species. Coding sequences are compared by multiple sequence alignment, where similarities and differences among the sequences are used for the estimation of a common ancestor in a phylogenetic tree. The phylogenetic trees are constructed using computational methods for which three different methods exist (mentioned after the simplest and least demanding computational methods); Neighbour joining (NJ), Maximum likelihood (ML) and Bayesian inference (BI). The starting point of the algorithm behind the NJ method is an unresolved tree with a star-like

topology and based on a distance matrix, the program adjust branch lengths according to this matrix, until all branch lengths in the tree is known (Saitou and Nei, 1987). The ML method uses a statistical approach for determining the tree topology and the tree estimated using this method is the tree with the highest probability of generating the given data (Felsenstein, 1981). The BI method is different from the other two, in that it estimates many trees, where NJ and ML estimates only one tree, and the resulting phylogenetic tree is based on all of these trees by means of the posterior probability. This means that during the estimation of the resulting phylogenetic tree, relevant evidence for construction of this particular tree has been examined (Holder and Lewis, 2003;Rannala and Yang, 1996). Estimation of phylogenetic trees has the advantage, other than predicting evolutionary relationship between sequences, that they can also be used for the prediction of selection on the genes (Yang and Nielsen, 2000), as well as for the estimation of evolutionary rates and time of divergence in relation to the most recent common ancestor for a group of sequences (Holder and Lewis, 2003).

Animal models

The use of animal models for studying IAV pathogenesis as well as for the assessment of zoonotic potential of some subtypes is of very high importance. Furthermore, the choice of animal model is crucial in order to ensure that the obtained results will be as closely comparable as possible to those expected for humans. For these purposes, several animal models have been established and among these are the most widely used mice, rats, guinea pigs, ferrets, non-human primates and pigs (Barnard, 2009).

The usage of mice and rats as model animals for IAV infection is compromised by the need to adapt the virus strains for obtaining effective replication, but the mouse model is widely used, due to the low costs of housing and its availability (Barnard, 2009;Belser *et al.*, 2011). The guinea pig model has been found to support the transmission of influenza viruses without prior adaption of virus, but on the other hand they are poor models for viral pathogenesis (Belser *et al.*, 2011) and use of non-human primates and pigs are limited due to ethics, size and costs regarding housing (Barnard, 2009;Belser *et al.*, 2011).

The use of ferrets as a model organism for the transmission of IAV was first described in 1933 (Smith *et al.*, 1933) and subsequently, the ferret has been widely used as a small animal model for the study of IAV pathobiology. Ferrets have been found to be naturally susceptible to infection with IAVs without requirement of prior adaption (Belser *et al.*, 2011;Shope, 1934;Smith *et al.*, 1933). Furthermore, clinical symptoms are similar to those observed for humans and IAV in ferrets also follows a similar disease pattern. The cause of disease is acute and clinical signs typically include sneezing, nasal discharge, elevated body-temperature and weight loss (Maher and DeStefano, 2004;Reuman *et al.*, 1989). The ferret has been used in several studies for the analysis of transmission of both swine and human IAVs (Duan *et al.*, 2010;Pascua *et al.*, 2012;Pearce *et al.*, 2012;Yen *et al.*, 2011), due to its receptor distribution in the respiratory tract resembling that of humans (van Riel *et al.*, 2007).

Two drawbacks of the ferret model is; 1) the problem of obtaining IAV sero-negative animals for experiments and 2) the lack of knowledge of immune function. The missing knowledge of immune function is due to the limited availability of ferret-specific reagents as well as incomplete ferret genome sequencing and therefore enhanced efforts for mapping of the ferret immune response and closing of

this gap, will also help to provide an improved understanding of the human immune response to IAV (Belser *et al.*, 2011;Teijaro *et al.*, 2014).

Reverse genetics

Reverse genetics is a critically important system for the study of IAVs since the system provides the possibility of studying the exchange of segments and/or mutations at specific residues involved in host restriction, pathogenesis and virulence as well as the potential development of new vaccine candidates.

The establishment of a reverse genetics system requires several steps involving extraction and amplification of vRNA, sequence analysis of amplified vRNA, cloning of the eight segments into individual plasmids, transformation of plasmids into bacteria, selection of bacterial clones possessing the correct insert and then transfection of several plasmids into cells for the formation of live virus particles. Subsequent to transfection of the eight IAV segments into cells, it is necessary that the polymerase proteins, as well as the NP protein, are co-expressed since infectious virions otherwise cannot be formed. Traditionally, an eight plus four system has been used, in which four plasmids expressing the necessary viral proteins, have been co-transfected with the eight plasmids containing the vRNA inserts (Fig. 13) (Fodor *et al.*, 1999;Neumann *et al.*, 1999).
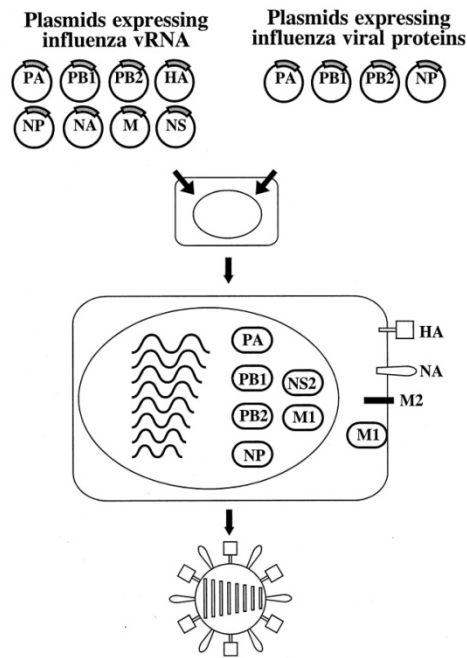
Figure 13 Schematic representation of the eight plus four system. The eight plasmids expressing vRNA are transfected into cells together with the plasmids expressing the viral proteins. Modified from (Neumann *et al.*, 1999).

For an improved efficiency of the cloning process and to reduce the number of plasmids used for transfection and to enhance transfection efficiency, a bidirectional plasmid was constructed (Hoffmann *et al.*, 2000b;Hoffmann *et al.*, 2000a;Hoffmann and Webster, 2000). This plasmid (pHW2000) (Fig. 14) was constructed so that it both contained the human DNA-dependent RNA polymerase I (Pol I) promoter, controlling the expression of vRNA and the Pol II promoter in the opposite direction flanked by a polyadenylation signal, controlling the transcription of mRNA. The backbone of the plasmid was derived from the cloning vector pcDNA3 (Hoffmann *et al.*, 2000b;Hoffmann *et al.*, 2000a). Hence, it was now possible to transfect cells with only eight plasmids and still obtain the protein expression necessary for the formation of infectious virions (Hoffmann *et al.*, 2000a). Later on, this system was further refined to remove the dependency of restriction enzymes, which was done by the insertion of the conserved influenza gene termini. Between the conserved termini, the negative selection marker *ccd*B was added and by this, it was now possible to obtain plasmids containing any of the eight segments by target-primed plasmid amplification (Stech *et al.*, 2008).
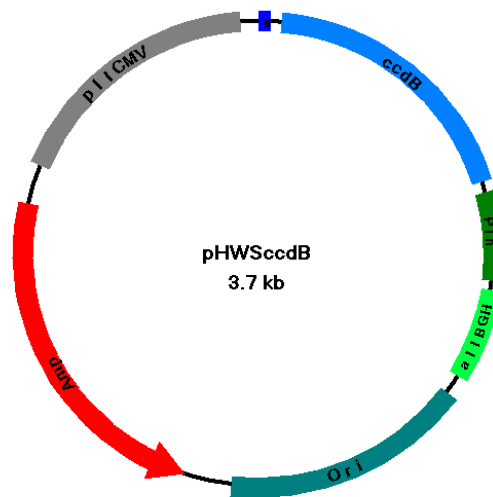
Figure 14 Schematic representation of the pHWS*ccdB* plasmid. The plasmid contains the Pol I promoter (P_{Ih}), followed by a murine terminator (t_I) sequence. The Pol I promoter and the terminator sequence are separated by the conserved influenza gene termini and between these the negative selection marker *ccdB* was inserted. Flanking the Pol I promoter and the murine terminator sequences are the pol II promoter ($p_{II}CMV$, derived from human cytomegalovirus) and a polyadenylation signal ($a_{II}BGH$, from the gene encoding bovine growth hormone). The backbone of the plasmid was derived from the cloning vector pcDNA3.

MANUSCRIPT I

Genetic and antigenic characterization of influenza A virus isolated from Danish swine in the period 2003-2012

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(Ready for submission to Journal of Clinical Microbiology)

ABSTRACT

Influenza A virus is a highly contagious disease in pigs and has been endemic in Danish pigs for more than 30 years, with H1N1 and H1N2 being the dominating subtypes. In Denmark, the import of live pigs is very limited and the country can therefore be considered as a closed population, providing the perfect opportunity for studying the evolution of swine influenza viruses. The purpose of this study was to investigate the genetic and antigenic evolution of the avian-like H1N1 and H1N2 subtypes found in Danish pigs during the last 10 years. Viruses from a total of 78 samples were isolated and the hemagglutinin and neuraminidase genes full length sequenced. Genetic analysis of H1 revealed an overall genetic identity of 89-100 %, but no chronological drift was revealed. For N1, the overall genetic identity was 89-99% and phylogeny resembled that of H1. Phylogenetic analysis of the N2 gene revealed an overall genetic identity of 88-100 % with two separate co-circulating clades. Surprisingly, estimation of most recent common ancestor of HA suggested the time of emergence for H1 to be around 1913-1919. Estimated selection pressures indicated that more purifying and less diversifying selection controlled the H1 evolution. For antigenic characterization, 58 of the viruses were tested in hemagglutination inhibition tests (HI) against a panel of representative reference sera. Analysis by antigenic cartography showed that most H1s clustered together, regardless of the subtype and year of isolation, with few outliers. These outliers were mainly within the H1N2 subtype and many of these were found to possess mutations at the receptor binding site 225. In conclusion, this study provides an important contribution to the complex epidemiology of circulating swine influenza viruses.

INTRODUCTION

Swine influenza is an acute contagious respiratory disease caused by the influenza A virus (IAV). In swine, IAV is an important respiratory disease and today swine influenza virus (SIV) is endemic in the global pig population (Brown, 2012). IAV is able to escape herd immunity by the accumulation of point mutations in the surface glycoproteins hemagglutnin (HA) and neuraminidase (NA), known as antigenic drift, or by reassortment of segments from different viruses during co-infection, a process known as antigenic shift (Schild *et al.*, 1974).

The HA protein is exposed at the surface of the virus particle and is involved in viral fusion with host cell membranes upon infection, whereas the NA protein is involved in the release of newly formed viral particles from the cell surface. Due to the exposure of the HA and NA glycoproteins at the viral surface, both proteins are easily recognized by host immune systems (Baigent and McCauley, 2003).

Mutations in the antigenic sites, as well as mutations at key residues involved in receptor binding and the deletion or addition of glycosylation sites of the HA and NA glycoproteins are all factors that have been reported to influence the recognition of the IAV by the host immune system (Sun *et al.*, 2011). Antibodies targets specific sites on the globular head domain of the HA termed Sa, Sb, Ca1, Ca2 and Cb, and antigenic drift is usually caused by accumulation of amino acid substitutions in these sites (Caton *et al.*, 1982). Recently, however, one amino acid alone, located at residue 145 in site Ca2, was found to be responsible for antigenic change of human H1N1 (Koel *et al.*, 2013).

Presently, three major SIV subtypes are co-circulating in the Danish swine population. The first subtype isolated from Danish swine was the classical swine H1N1 subtype which was initially detected in 1981(Kuntz-Simon and Madec, 2009;Nardelli *et al.*, 1978;Sorensen *et al.*, 1981). In 1979 outbreaks of influenza among swine in the Netherlands and Germany was found to be caused by a different H1N1 subtype (Pensaert *et al.*, 1981). This new subtype was the result of a transmission event from birds to swine and created a new and stable lineage, afterwards referred to as the European avian-like swine H1N1. The European avian-like swine H1N1 spread throughout Europe and replaced the classical swine H1N1 in Europe (Abusugra *et al.*, 1987;Schultz *et al.*, 1991).

Shortly after the human IAV pandemic in 1968, the pandemic H3N2 virus was isolated from pigs in Asia, became adapted to swine and started circulating at low levels in Europe (Ottis *et al.*, 1982;Tumova *et al.*, 1980). In 1984, this human-like swine H3N2 reassorted with the avian-like swine H1N1 and established the European reassortant human-like swine H3N2, with internal genes originating from the avian-like swine H1N1 and the HA and NA from the human-like swine H3N2 subtype (Castrucci *et al.*, 1993). This European reassortant human-like swine H3N2 gradually replaced the original human-like swine H3N2 in Europe and during the 1990-ies it was found in several European countries (Castro *et al.*, 1988;Yus *et al.*, 1992;Zhang *et al.*, 1989) including Denmark (A. Bøtner, personal communication).

In 1994, an H1N2 virus was isolated from pigs in the UK. The HA of this virus was found to originate from a human-like H1N1 that had been circulating in pigs (Brown *et al.*, 1993), and NA and internal segments all originated from the European reassortant human-like swine H3N2 and was therefore named reassortant human-like swine H1N2. This virus quickly spread to the western continental Europe (Kuntz-Simon and Madec, 2009), but has never been detected in Denmark (Bøtner, 1994;Trebbien *et al.*, 2013). In 2003 a different H1N2 was, however, detected in Denmark, containing an avian-like swine H1 and a reassortant human-like swine N2 (Trebbien *et al.*, 2013). This subtype became established in Denmark and constitutes now approximately 20% of the IAVs found in Danish pigs (unpublished data). This H1N2 has subsequently also been detected in Sweden, Germany and Italy (Balint *et al.*, 2009;Harder *et al.*, 2013;Metreveli *et al.*, 2011;Moreno *et al.*, 2012). The emergence of the pandemic H1N1 (H1N1pdm09) virus in 2009 in humans followed by reverse zoonotic events, introduced yet another H1N1 virus in pigs in many countries (Pereda *et al.*, 2010;Vijaykrishna *et al.*, 2010). This virus was identified for the first time in Denmark in 2010 (Breum *et al.*, 2012).

According to results of a passive surveillance program, the dominating subtypes in Danish pigs are, the avian-like swine H1N1, the avian-like swine H1N2 and H1N1pdm09, whereas H3N2 was not detected in 2011 and 2012 (unpublished data). Until now, none of these subtypes have been characterized in detail, creating a gap in the knowledge of the level of genetic and antigenic drift. Thus, the aim of the present study was to characterize the genetic and antigenic evolution of the two dominating SIV subtypes H1N1 and H1N2 by analyzing the genetic and antigenic changes of HA and NA of 78 H1 subtype viruses collected in Denmark during the past ten years.

METHODS

Virus isolation

A total of 78 viruses were isolated from samples submitted for diagnostic purposes from swine with a history of respiratory disease. Briefly, Madin-Darby canine kidney (MDCK) cells were grown to 60-70% confluency in MEM (Gibco, Carlsbad, CA, USA) containing 5% FBS, 2 mM L-glutamine, $1\times$ Non-essential amino acids (NEAA) and $1\times$ penicilin-streptomycin. Lung tissue was homogenized on TissueLyser (Qiagen, GmbH, Germany) in 1.5 mL $1\times$ MEM supplemented with $1\times$penicillin-streptomycin and sterile filtered. Cells were inoculated with 500 µL lung tissue homogenate for 30 min at 37°C, 5% CO_2. Following incubation, 10 mL MEM containing $1\times$penicillin-streptomycin, $1\times$NEAA, 2 mM L-glutamine and 2 µg/mL TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. The cells were incubated for 72 hours and daily monitored for CPE. The supernatant was harvested and centrifuged at 2500 rpm for 30 minutes to clarify cell debris and then stored at -80°C until further analysis.

RNA purification and real-time RT-PCR screening

Viral RNA was purified from cultured viruses by RNeasy Mini Kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. Cell culture supernatant was prepared by mixing 200 µl isolate with 400 µl RLT-buffer containing β–mercaptoethanol. Total RNA was eluted in 60 µl RNase-free water and stored at -80°C.

The presence of IAV was confirmed by real-time RT-PCR using an in-house modified assay for detection of the matrix gene (De *et al.*, 2009).

Full length sequencing of HA and NA genes

Nucleic acid amplification was performed by one-step RT-PCR using in-house primers modified from Hoffmann *et al.*, (Hoffmann *et al.*, 2001) and Superscript III One-Step RT-PCR kit with Platinum *Taq* High Fidelity (Invitrogen, Carlsbad, CA, USA). The full length amplification of HA required 600 nM of the pQE-HA-F 5′-**CGGATAACAATTTCACACAG**AGCAAAAGCAGGGG-3′ and of the pQE-HA-R 5′ -**GTTCTGAGGTCATTACTGG**AGTAGAAACAAGGGTGTTTT-3′ primers.

PCR cycling conditions for HA was: 30 min at 55°C, 2 min at 94°C, 4 cycles of 94°C for 30 sec, 55°C for 30 sec and 68°C for 180sec, followed by 40 cycles of 94°C for 30 sec and 68°C for 210 sec, then 68°C for 10min. For the full length amplification of NA 600 nM of each of the primers: pQE-NA-F 5′-**CGGATAACAATTTCACACAG** AGCAAAAGCAGGAGT-3′ and pQE-NA-R 5′-**GTTCTGAGGTCATTACTGG** AGTAGAAACAAGGAGTTTTTT-3′ were used. PCR conditions were the same as for HA, except that RT temperature was at 54°C and annealing temperature at 58°C.

The PCR products were visualized by gel electrophoresis using E-Gel 0.8% agarose gels (Invitrogen, Carlsbad, CA, USA) and purified with High Pure PCR Product Purification Kit (Roche Diagnostics, GmbH, Germany). Purified PCR products were send for Sanger sequencing at LGC Genomics (GmbH, Germany).

Sequence alignment and phylogenetic analysis

Assembly and proofreading of sequences were performed using CLC DNA Workbench 6.0.2 (CLC bio A/S, Aarhus, Denmark). The full length sequences were aligned using MAFFT, followed by an alignment using RevTrans 1.4 with standard settings. The substitution model was determined using jModelTest version 2.1.1 (Posada, 2008). Based on the Akaike information criterion (AIC), a general time reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites was used for inferring Maximum Likelihood (ML) trees.

Phylogenetic trees from the aligned sequences were inferred using the Bayesian Markov-Chain Monte Carlo (MCMC) method integrated in Mr.Bayes 3.1 (Ronquist and Huelsenbeck, 2003). The MCMC was run for 20 million iterations for H1 and 2.5 million iterations for N1 and N2 with 25% burn-in using a sampling frequency of 2500. The phylogenetic trees were rooted with appropriate outgroups and visualized using FigTree, version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Selection pressures

The mean number of substitutions per site (ω = dN/dS) (Miyata and Yasunaga, 1980) were applied to determine the degree of natural selection acting on the HA and NA genes. The dN/dS ratio was estimated using the Codeml program implemented in the PAML package (Yang, 2007). Inferred ML trees from the DNA substitution model test in PAUP with the GTR+I+G substitution model was used.

Nucleotide substitution rate and time of the most recent common ancestor

Overall rates of evolutionary change (nucleotide substitutions per site per year) and time of the most recent common ancestor (tMRCA) were estimated using the BEAST program version 1.7.5 (http://beast.bio.ed.ac.uk/; (Drummond and Rambaut, 2007). For H1 analysis, the lognormal relaxed molecular clock and a tree prior with coalescent constant were used with the GTR+I+G model. For N1 and N2 analyses, the lognormal relaxed molecular clock and a tree prior population with birth/death rate were used, also with the GTR+I+G nucleotide substitution model.

Two independent Bayesian MCMC analyses were performed for each gene for 20 million generations with sampling every 2000 generations. Convergences and effective sample sizes of the estimates were checked using Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) and a burn-in of 20% was used. Uncertainty in parameter estimates is reported as values of the 95% high posterior density (HPD).

Virus antigenic characterization

A hemagglutination (HA) assay was performed to determine the HA titer for each virus. An equal volume of 0.5% turkey red blood cell suspension was added to a serial dilution of the virus. Virus with HA titers above four were used for hemagglutination inhibition (HI) assays.

HI assays against reference swine antisera were performed to compare the antigenic properties of the SIVs. Eight representative swine antisera collected between 1982 and 2010, representing the HAs of avian-like swine H1N1, avian-like swine H1N2, human-like swine H1N1, human-like swine H1N2 and H1N1pdm09 were used as reference sera. Sera were treated with receptor destroying enzyme (RDE), followed by heat inactivation at 56°C for 30 min to remove nonspecific inhibitors of hemagglutination. Four HA units of virus were mixed with a 10-fold serial dilution of each reference serum, followed by the addition of 0.5% turkey red blood cells. The reciprocal of the highest dilution of serum to completely prevent agglutination of turkey red blood cells by virus was considered the HI titer for that virus.

Antigenic cartography

The quantitative analysis of the antigenic properties of the Danish H1 viruses were performed using antigenic cartography, as previously described for human (H3) and swine (H1) IAVs (Lorusso *et al.*, 2011;Smith *et al.*, 2004). In this study, the antigenic map was generated based on HI data from 58 H1 SIVs (divided between 35 H1N1 and 23 H1N2 isolates) versus eight reference swine antisera. The eight reference swine antisera were: A/swine/Côtes d'Armor/0070/2010 (rH1N1), A/swine/Finistere/2889/1982 (H1N1), A/swine/Côtes d'Armor/0388/2009 (H1N1), A/swine/Denmark/19126/1993 (H1N1), A/swine/Scotland/410440/1994 (H1N2), A/swine/Côtes d'Armor /0113/2006 (H1N2), A/swine/Côtes d'Armor/0186/2010 (rH1N2) and A/swine/Sarthe/0155/2010 (H1N1).

Detection of possible N-linked glycosylation sites

N-linked glycosylation of HA and NA was estimated using the program NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc), to which aligned files of HA and NA were submitted. Potential glycosylation sites that did not exceed the threshold were excluded. Only N-glycosylation sites with high specificity, i.e. sites exceeding the 0.75 threshold and with Jury agreement were selected.

Modeling of 3D structure

Model prediction of the 3D structure of N1 was performed using CPHmodels ver. 3.2 (https://www.cbs.dtu.dk/services/CPHmodels/). This program predicted an expected structure based on a consensus sequence and the best matching PDB structure, in this case 4b7q. The structure was further modified using PYMOL ver. 1.6.0.0.

RESULTS

Phylogenetic analysis

Full length HA gene sequences were determined for a total of 78 SIVs collected between 2003 and 2012 (Table 1). Comparisons of the H1 sequences revealed 89-100% identity at the nucleotide level and 87-100% identity at the amino acid level. The identity within the clades of the H1 tree ranged from

93-100% at the amino acid level. The level of nucleotide identities among the viruses isolated within each year decreased from 94-97 % in 2003 to 89-94% in 2012 (Table 1).

The phylogenetic analysis of the H1 genes from Danish SIV sequences revealed a high degree of polytomy and the presence of several clades, mostly comprised of H1s originating from both the H1N2 and H1N1 subtypes and no chronologic drift was observed, considering the replacement of older strains by newer ones (Fig 1A). For the N1 gene, the level of nucleotide similarity was 89-99%, whereas the identity at the amino acid level ranged from 89-100%. Similar to the H1 tree, the phylogenetic tree of N1 also revealed a polytomic tree with few clades (Fig 1B). The identity within the clades of the N1 tree ranged from 93-100% at the amino acid level.

Phylogenetic analysis of the N2 gene revealed two different and clearly distinct lineages, which are most illustratively shown in a radial tree (Fig 2). Recently, German swine N2 subtype viruses were divided into three different clades that were designated N2 group 1 through 3 (Fig 2) (Starick *et al.*, 2012). Most of the Danish N2 sequences were found to form one large clade within group 1, sharing 93-100% identity at the nucleotide level (92-100% at the amino acid level). The second and much smaller clade showed 91-99% identity at the nucleotide level (90-99% identity at the amino acid level) and was found to belong to the clade designated group 2. The overall similarity of the Danish SIV N2 gene sequences were 88-100% at the nucleotide level and 86-100% at the amino acid level. Three of the group 2 strains, A/swine/Denmark/1053-1/2006(H1N2), A/swine/Denmark/16525-1/2008(H1N2) and A/swine/Denmark/101404-1/2011(H1N2), were 97-99% identical at both amino acid and nucleotide levels. In contrast, the last group 2 strain (A/swine/Denmark/12687/2003/H1N2), shared only 91-92% similarity at the nucleotide level (90-91% similarity at the amino acid level) with the other three group 2 strains.

In contrast to the finding for the H1 genes, no specific differences were observed in the percentage of nucleotide identities of N1 and for N2, more sequences would be necessary in order to evaluate a potential difference (Table 1).

Selection pressures on Danish swine influenza viruses

Global ratios of dN/dS were estimated to be 0.18 in the entire HA protein and 0.21 in the HA1 coding region. Estimation of dN/dS for N1 gave an estimate of 0.23 and for N2 an estimate of 0.21. All dN/dS

values for all datasets were below 1.0, indicating lack of positive selection on the genes as a whole. Analysis of selection pressure at specific sites revealed that no positively selected sites were present in the H1 and N2 genes, but positive selection was detected at specific amino acids in the N1 gene. The sites predicted to be under positive selection in the N1 gene were 53V, 331M and 339Y which all were predicted to be located at the surface of the N1 protein (Fig 3). Position 53V is not shown in the figure since the 3D structure of this part of the protein has yet to be determined.

Evolutionary rates and times of most recent common ancestor (tMRCA)

The mean evolutionary rate described by the number of substitutions per site per year and tMRCA with 95% HPD range for H1, N1 and N2 are summarized in table 2. Estimation of tMRCA for the H1 in H1N1 and H1N2 was calculated individually, to analyze if the H1 had divided into two different lineages after emergence of the H1N2 strain. The tMRCA for H1 in H1N1 was estimated to be 1919 (95% HPD: 1900-1938) and the H1 in H1N2 tMRCA was estimated to be 1913 (95% HPD: 1896-1930). The most recent common ancestor of the European (avian-like) swine N1 was dated back to 1980 (95% HPD: 1974-1984). For the N2 gene, the tMRCA was dated to 1977 (95% HPD: 1972-1979).

Antigenic cartography

A total of 23 H1N2 and 35 H1N1 Danish isolates were included in the antigenic cartography analysis. The isolates shown in the in three-dimensional (3D) antigenic map (Fig 4A) were colored according to every two years of isolation. It is evident from this map that none of the Danish H1 viruses clustered according to the year of isolation and H1s from the ten years of sampling were interspersed among one another, as seen also in the phylogenetic analysis of the genes. Antigenic outliers were defined as isolates distant from its counterparts by more than three antigenic units (corresponding to an eight fold difference in HI assay). Using A/swine/Denmark/102586-1/2007(H1N1) which was situated in the center of the map, as reference strain, a total of 17 isolates could be defined as antigenic outliers. Of these antigenic outliers, six were H1s from H1N1 and 11 were H1's from H1N2 and five outliers were from 2011/12. To analyze if viruses grouped according to subtypes, H1N1 and H1N2 strains were colored separately (Fig 4B), which showed that HA from H1N1 and H1N2 were co-located on the antigenic map.

Substitutions associated with cluster transitions

Previously it has been shown that certain amino acids have been associated with cluster transitions, in antigenic cartography, in both human and swine H3N2 (Koel *et al.*, 2013;Lewis *et al.*, 2014). These amino acid substitutions were identified at residues 145, 155, 156, 158, 159 and 189 (throughout this study H3 numbering for identification of amino acid residues was used). Analysis of these residues in all of the Danish SIV sequences revealed that residue 145 was for most sequences N, 155 most often V, 156 was highly conserved among all sequences as K, 158 was often K, but showed more variation. Residue 159 was conserved in most cases as N and residue 189 was conserved in most sequences as S (Table 3). Sequences from the 17 antigenic outliers only showed high level of conservation at residue 156, whereas the remaining amino acids showed a higher degree of variability (Table 3).

Changes in antigenic sites

Analysis of the five antigenic sites, previously identified in SIVs (Caton *et al.*, 1982;Gerhard *et al.*, 1981;Luoh *et al.*, 1992), in all Danish H1 SIV sequences showed that site Ca2 was found to be most prone to mutations (100% changes, meaning that mutations from consensus was observed at all residues in this site). Site Ca1 followed site Ca2 (with 91% changes), whereas site Sb (58% changes) was found to be the most conserved antigenic site in the Danish viruses. In comparison to these results, analysis of the 17 antigenic outliers showed that site Cb was the least conserved antigenic site (67% amino acids changes compared to consensus sequence), followed by site Ca1 (64%) and the most conserved site was site Sb (33%) (Fig 5).

Genetic changes in receptor binding sites

Sequences of all the Danish H1 viruses versus the sequences of the 17 antigenic outliers were analyzed for identification of potential mutations in receptor binding sites either specific for the Danish SIVs or mutations potentially involved in changes in antigenic properties of the viruses.

Amino acids at positions 133, 138, 192, 193, 226 and 228 has previously been shown to be involved in receptor binding (Matrosovich *et al.*, 2000). Residues 190D, 192Q, 226Q and 228G were highly conserved among all Danish SIV sequences. Residue 222K was also very well conserved, whereas residues 133, 138, 155, 193 and 225 revealed more diversity (Table 3). Residue 155 has been shown to be important for receptor affinity (Matrosovich *et al.*, 2000;Suzuki *et al.*, 1997) and six of the antigenic

outliers showed 155T and one showed 155I, whereas the remaining isolates showed 155V (Fig 5). Furthermore, residue 222, known to interact with α2,6-receptors (Stevens *et al.*, 2006), was found to be highly conserved as K and only two Danish SIV sequences (both antigenic outliers) possessed different amino acids at this residue in that A/swine/Denmark/101713-1/2010(H1N1) had acquired an E and A/swine/Denmark/101958-1/2011(H1N2) had acquired an R (Fig 5).

Analysis of residue 225, involved in receptor binding properties of the virus as well as adaption from birds to humans and swine (Matrosovich *et al.*, 2000), showed some variability, since most sequences possessed the 225E substitution, but also Q/N/D/T/K was observed.

Genetic analysis of important residues in the neuraminidase proteins

The neuraminidase proteins of all N1 and N2 Danish SIV sequences maintained the 15 conserved charged amino acids that have been found to constitute the active site of the enzyme (Colman *et al.*, 1983).

The substitutions H274Y and N294S known to induce drug resistance in N1 viruses (Le *et al.*, 2005) were not detected in any of the Danish SIVs. Further, no stalk deletion within the Danish N2 viruses was observed, which has been associated with replication efficiency *in vivo*, increased host range and reduced NA enzymatic activity (Castrucci and Kawaoka, 1993).

Glycosylation

The degree of conservation of glycosylation sites in H1N1 HA and H1N2 HA are illustrated in table 4A. The analysis revealed that three potential glycosylation sites at positions 15, 26 and 542 in the stalk region were strictly conserved for both H1N1 and H1N2 HA (Table 4A). In contrast, the glycosylation site at position 58, located at the globular head region, was shared only by 22 of the isolates. Further, some of the less well conserved glycosylation sites were found in or nearby antigenic sites. This was found for the glycosylation site at position 157 which was located in antigenic site Sa. The glycosylation site at position 218 was located close to site Ca2 and position 186/187 was found to be located right outside the antigenic site Sb. Several more glycosylation sites were estimated, but none of those were conserved.

Inspection of glycosylation sites of the antigenic outliers revealed that most H1 sequences had the same potential glycosylation sites as the rest of the Danish SIV sequences and only the isolates A/swine/Denmark/101958-1/2011/H1N2 and A/swine/Denmark/101107-1/2009/H1N2 appeared to have an extra glycosylation site at positions 186 and 58, respectively. For N1 a high degree of conservation was observed for all the putative glycosylation sites at positions 63, 88, 146 and 235, where position 63 and 88 are located in the stalk of N1 and 146 and 235 are located in a conserved part of the protein (Table 4B). Furthermore, position 44 was also shared by some of the isolates. N2 sequences was found to share three highly conserved glycosylation sites at positions 61, 146 and 234, where positions 146 and 234 are located in the conserved part of the protein, and one less conserved site at position 402 (Table 4B).

DISCUSSION

In this study, the genetic and antigenic diversity and evolution of the two most prevalent subtypes, the avian-like H1N1 and H1N2, were analyzed. The analysis of SIV isolates collected from diseased pigs over a period of more than 10 years revealed several interesting features and greatly enhance the understanding of the evolution of IAV in commercial pig holdings.

The level of diversity of the H1 gene was found to increase from 3-7% in 2003 to 6-11% in 2012, indicating that the selective pressure on the virus has increased over the years. This may be due to the increased herd sizes, even though there did not seem to be a very high level of selection on the HA gene as a whole. This gradual increase in diversity with time has also been observed for the HA gene of H1N1pdm09 circulating in humans (Li *et al.*, 2013).

Phylogenetic analysis of the N2 gene revealed two clearly distinct lineages, as previously observed (Starick *et al.*, 2012;Trebbien *et al.*, 2013). This study has confirmed the existence of these two lineages in Denmark and further showed that both lineages continuously co-circulates in the Danish swine herds, though dominated by NA clade 1. Phylogenetic analysis revealed that the Danish lineage is clearly different from the German lineage within clade 2, which is interesting since a large proportion of living pigs (10 million per year) are exported from Denmark to Germany. In clade 2, A/swine/Denmark/12687/2003(H1N2) was found to differ from the remaining Danish N2 sequences and as the oldest sequence in this clade, this could indicate an adaption to Danish pigs.

Analysis of the selection pressure in H1 genes revealed a higher dN/dS value for HA1 compared to the full HA sequence, indicating a higher degree of immunological pressure on HA1 in accordance with this region being part of the extracellular region of the protein. The dN/dS value estimated for the entire HA sequence is considered the most correct of the two, since immunological changes in the extracellular region can affect the intracellular region and vice versa. Previous values for avian-like H1s has been estimated to 0.208 (Moreno *et al.*, 2013) and 0.17 (Lam *et al.*, 2008;Li *et al.*, 2011) for the full HA sequence and both values are in agreement with the calculated level of selection pressure on Danish H1sequences. Comparison with values for human H1N1 HA reveals a similar level of selection (Bragstad *et al.*, 2008;Wolf *et al.*, 2006).

The selection pressure on the N1 gene was higher, but still comparable with previous estimates for European avian-like swine viruses (Fourment *et al.*, 2010;Li *et al.*, 2011;Xu *et al.*, 2012). In comparison, the ratio for the Danish N2 gene was found to differ from previous estimates for European human-like H1N2 viruses (Lam *et al.*, 2008), but it appears that there in general is some differences between dN/dS values for N2 genes, depending on both host and subtype from which the N2 originates (Lam *et al.*, 2008;Westgeest *et al.*, 2012;Xu *et al.*, 2012).

The observed lack of positive selection on the Danish HA and NA genes as a whole was in accordance with previous results on SIV, as well as for human H1N1 viruses (Bragstad *et al.*, 2008;Li *et al.*, 2011;Moreno *et al.*, 2013;Wolf *et al.*, 2006).

Results from the estimation of selection pressures on the N1 gene revealed three amino acids under positive selection and a closer analysis of these sites revealed that positions 53 and 331 were both found to be located in the T-cell antigenic regions, while position 339 was found to be located in the B-cell antigenic regions and mutations at these sites might provide the neuraminidase with a selective advantage by allowing escape from the host immune-system. Position 339 is furthermore associated with host adaption after introduction of the virus from birds to humans (Tamuri *et al.*, 2009). The findings are in accordance with a previous study that also identified positions 53 and 339 in North American SIVs as being under positive selection (Li *et al.*, 2011), indicating the overall importance of these sites in the N1 gene evolution.

Further analysis of the selection pressure and evolution of the Danish HA and NA genes involved the determination of their evolutionary rates. This analysis revealed that the rate of Danish H1 and N2 was in accordance with previously estimated values from a study of H1N2 viruses in Italian swine (Moreno *et al.*, 2013). For the Danish N1 gene the evolutionary rate was found to be higher than previously estimated substitution rates for Eurasian avian-like swine viruses and also higher than both classical swine H1N1 viruses and North American triple reassortant (TRIG) swine viruses (Dunham *et al.*, 2009;Fourment *et al.*, 2010;Xu *et al.*, 2012).

Recently, however, a study has shown that the Eurasian avian-like HA and NA genes generally has a higher evolutionary rate compared to classical and TRIG swine viruses (Bhatt *et al.*, 2013), which correlates well with the estimated evolutionary rate for the Danish N1 gene. This higher evolutionary rate for avian-like viruses has been proposed to be related to the longer circulation of the classical swine virus (approximately 80 years), compared to the Eurasian avian-like virus, which was not introduced until approximately 30 years ago. Hence, the classical swine virus could have become adapted to its swine host, whereas the Eurasian avian-like viruses could still be adapting to swine (Bhatt *et al.*, 2013). The differences may also be explained by different management practices that put different pressure on viruses in one production system compared to others.

Time of most recent common ancestor has previously been determined for H1 viruses and in this study it was of particular interest due to the co-circulation of two different subtypes in terms of determining if the two H1s could be in the process of separating into two different lineages. It was evident from both the antigenic cartography and the phylogeny that this was not the case. Interestingly the estimated tMRCA for the Danish avian-like H1 gene revealed a tMRCA to be around 1913-1919, a prediction that does not correlate with the established view on the evolution of European avian-like H1N1 viruses. The introduction of the avian-like H1 in 1979 has been regarded as an introduction of a circulating virus directly from an avian host, since identical H1 genes were identified in contemporary samples from birds (Pensaert *et al.*, 1981). The estimated tMRCA in this study fits quite well with the presumed introduction of the H1 of the classical H1N1 swine virus which originated from the human H1N1 responsible for the "Spanish" influenza pandemic in 1918 (Brown, 2000). Yet the narrow range of the 95% HPD and the independent calculations for H1 originating from H1N1 and H1 from H1N2 are good indicators of sound estimates and the results are definitely worth a closer investigation.

The origin of the surface genes of the European reassortant human-like swine H3N2 virus is believed to originate from the 1968 pandemic virus, but only few studies have been performed on the origin of the N2 gene. The tMRCA estimated in this study correlated well with the time of the first introductions of human H3N2 into European swine herds in the early and mid-1970'ies (Bikour *et al.*, 1995;Haesebrouck and Pensaert, 1988;Tumova *et al.*, 1980). Another study estimated the time of MRCA of swine H3N2 to be around 1956 for N2 (Xu *et al.*, 2012), which correlates well with the time of introduction of this lineage from birds into humans during the H2N2 pandemic in 1957. This early estimate could be due to the inclusion of sequences from the H2N2 lineage as well as from H3N2 and H1N2, whereas the present study only included H1N2 sequences and a few H3N2 sequences. The tMRCA for N1 was estimated to be 1980, which is one year later than the first isolation of this lineage in swine in Belgium (Pensaert *et al.*, 1981), and fits well with a previous study where the tMRCA for the Eurasian avian-like swine N1 was estimated to be 1978 (Xu *et al.*, 2012).

In terms of obtaining a more profound picture of the evolution of the Danish SIVs during the past ten years, the antigenic relationship among the viruses was also investigated, which revealed grouping of both H1N1 HAs and H1N2 HAs into one large group with some outliers. This further supported the co-circulation of the H1N1 and H1N2 strains, as also seen in the phylogeny, in the herds with no apparent replacement of one strain by the other. The antigenic outliers determined in the cartography should be subject to caution, as these could be early warnings of antigenic escape from vaccines and/or herd immunity (Lewis *et al.*, 2011;Smith *et al.*, 2004). Some of the outliers were more recent strains from 2011 and 2012 and in general many of the recent strains seemed to move away from the reference strain (A/swine/Denmark/19126/1993 (H1N1)), which could be an early evidence of drift that may lead to generation of escape mutants. In this study, 58 strains were used to create the antigenic cartography map and 17 of these strains could be determined as antigenic outliers, suggesting that addition of more strains, potentially would reveal more than just one cluster. Most outliers were found to be of H1N2 strains and it could be speculated if the interplay between the H1 and N2 genes could affect fitness and antigenicity of the virus.

Amino acid sequences of the antigenic outliers were further investigated to determine if certain residue changes could be specific for the outliers. The swine adapted H1N1 viruses carries certain characteristic amino acids and one of these, residue 155V, is usually connected with increased binding

affinity for the Neu5Gc receptor (Matrosovich *et al.*, 2000), a receptor present on the respiratory cells of pigs (Sriwilaijaroen *et al.*, 2011). In the Danish SIVs, the V/I155T mutation was found to be present in a total of nine sequences, of these, six were found in sequences of antigenic outliers, two did not show HA activity and could therefore not be included in the antigenic map. The remaining strains were situated in the lower part of the cluster of the Danish SIVs in the antigenic map. These findings suggested that mutations at this position could potentially play a role in antigenic escape from the original H1s. Residue 155 has also previously been associated as being a key amino acid in cluster transitions in H3N2 viruses (Koel *et al.*, 2013;Lewis *et al.*, 2014), but the findings also indicated that a V to T mutation alone is not enough to place the strains as antigenic outliers. The cluster transitions in H3N2 viruses was found to involve at least six key amino acids at residues 145, 155, 156, 158, 159 and 189 and were common for both human and swine IAVs (Koel *et al.*, 2013;Lewis *et al.*, 2014). For human H1 viruses, one corresponding residue was also identified as being responsible for cluster transitions. This amino acid was identified at residue 145 (140 in H1 numbering) (Koel *et al.*, 2013) and the Danish antigenic outliers did show variation at this residue, but the N145K substitution was observed in several other sequences than those of the antigenic outliers, demonstrating that this substitution alone cannot explain the outliers. Interestingly, two of the outliers showed the N145S variation as the only mutation and the possibility that their positions as antigenic outliers could be ascribed to this substitution is more likely. A closer investigation of the remaining residues associated with cluster transitions in H3N2 viruses, showed that all of the Danish SIV antigenic outliers revealed atypical amino acids at least at one of these positions and it was previously shown that only a single amino acid substitution could lead to a given strain becoming outlier (Lewis *et al.*, 2014).

Only one cluster was formed in the antigenic map, which may be because too few strains were included, but there is also the possibility that the constant supply of naïve pigs, as well as the lack of widespread vaccination, leads to a generally low immunological pressure on the virus and thereby less antigenic variability. In Denmark, the vaccine used for vaccinating sows against SIV was for decades a heterologous commercial bivalent vaccine containing the strains A/New Jersey/8/1976/H1N1 and A/Port Chalmers /1/1973/H3N2. Both strains were of human origin and it has previously been shown that swine vaccinated with the commercial bivalent vaccine and infected with a Eurasian avian-like swine H1N1 did not greatly lead to immune selection (Murcia *et al.*, 2012). In 2010, an updated

trivalent vaccine against SIV was introduced. This contained the lineages A/swine/Bakum/IDT1769/2003/H3N2, A/swine/Haselünne/IDT2617/2003/H1N1 and A/swine/Bakum/1832/2000/H1N2. This trivalent vaccine now contains the European SIV strains that are currently circulating in swine, which could enhance the immunological pressure on SIVs and thereby also enhance antigenic drift of the viruses.

European avian-like swine strains have previously been shown to have significant variability in the receptor binding residue 138, unlike classical strains that maintains the avian consensus (A) and it was furthermore observed that some European avian-like swine strains showed A138S changes (Dunham *et al.*, 2009). Most of the Danish SIV sequences possessed V138, few had I138, two possessed the original avian A and only one sequence possessed the S138 change. Most of these amino acid changes would probably not change the receptor binding properties significantly, I is often interchangeable with V and A is, like V, also a hydrophobic amino acid. Only S, being hydrophilic, could potentially have an impact on receptor binding properties, but it is questionable if this change is beneficial since this substitution was detected in only 1 out of 78 sequences.

Further analysis of differences in sequences at residues important for receptor binding and host specificity, revealed that the amino acid at residue 222, one of the amino acids critical for interaction with long α-2,6-receptors (Stevens *et al.*, 2006), was found to be highly conserved in the Danish SIV sequences. Only two sequences possessed substitutions at this position, A/swine/Denmark/101958-1/2011(H1N2) with K222R and A/swine/Denmark/101713-1/2010(H1N1) with the K222E mutation. Since the original amino acid, K, and the mutated R are both positively charged amino acids, only the K222E substitution, with the negatively charged E, would potentially create a major difference in RBS structure.

Other amino acid residues known to be critical for receptor binding and host specificity, are positions 190 and 225, where 190E and 225G preferentially binds to α-2,3-receptors in avian species, 190D and 225G favors both α-2,3- and α-2,6-receptor bindings in pigs, and 190D and 225D effectively binds to α-2,6-receptors in humans (Shen *et al.*, 2009). Residue 225 has previously been found to be variable in European avian-like swine strains and included the avian 225G, but also G225E and G225K (Das *et al.*, 2010;Dunham *et al.*, 2009). A closer inspection of residue 225 in the sequences of the antigenic outliers

revealed a rather high variation at this position. Indeed both 225E and 225K was observed, with 225E being the most common amino acid in the Danish SIV sequences. For the antigenic outliers 225T/D/N/Q were also observed. The observed variable amino acids at this residue in sequences of antigenic outliers indicate an importance of this site for antigenic variability and eventual escape from herd immunity.

Analysis of the neuraminidase genes revealed no stalk deletion in the Danish N2 viruses. It has previously been shown that the length of the stalk region correlates with the efficacy of virus replication (Castrucci and Kawaoka, 1993), thus indicating a potentially high replication efficacy for the N2 viruses, which could explain the rather fast establishment of this lineage in the Danish swine herds during a relatively short period of time.

Investigation of the level of glycosylation in the Danish SIV H1 sequences revealed three highly conserved glycosylation sites, which all were located in the stem region of the virus. Conservation of these sites are thought to be involved in proper association with glycan binding-endoplasmic reticulum chaperones that facilitate the folding and assembly of HA (Hebert *et al.*, 1996). For human H1 strains, HA have been found to contain five potential glycosylation sites at positions 14, 15, 26, 289 and 483 (Das *et al.*, 2010;Sun *et al.*, 2011). Human seasonal H1N1s are more heavily glycosylated compared to swine viruses and least glycosylated are the avian H1s (Sun *et al.*, 2011). These observations correlate very well with our results and are also in agreement with the lower immunological pressure exerted by the swine host.

The potential glycosylation site at position 542 has been found to be strictly conserved in our study which is in accordance with previous studies (Das *et al.*, 2010;Sun *et al.*, 2011), but it has been speculated that this site might not be glycosylated since it is located at the intracellular region of HA (Sun *et al.*, 2011). Observation of glycosylation sites located in or near antigenic sites were in also accordance with previous studies showing that oligosaccharides have the ability to sterically block antibody binding to HA antigenic sites and thereby shields the virus from immune recognition (Abe *et al.*, 2004). Only two of the antigenic outliers were found to contain extra glycosylation sites so it is unlikely that alternate glycosylation sites are predictive for antigenic outliers which is in agreement with previous observations that found that viruses belonging to the same antigenic cluster often had different glycosylation states (Blackburne *et al.*, 2008). Estimation of glycosylation sites for N1 and N2

revealed that site 146 was highly conserved in both subtypes and it has previously been found that the glycosylation of this site is conserved in all NA subtypes. Furthermore, it has been shown that the N-glycan at this glycosylation site affects NA enzymatic activity by causing a 20-fold decrease in activity (Chen *et al.*, 2012;Li *et al.*, 1993).

In general, variation of the glycosylation sites is an important way for the IAVs to escape host immunity, but for Danish SIV this ability does not appear to be very highly prioritized, due to the lower extent of glycosylation compared to human IAVs. This observation is in agreement with the lower immunological pressure exerted by the swine host.

Denmark is a country renowned for its large export of living swine to other countries rendering it important to map both evolution of enzootic IAVs, as well as characterization of novel reassortants, but also to gain an increased understanding of the population dynamics. This study has provided a more detailed picture of the two most common enzootic viruses circulating in Danish pig herds. We have shown that the evolution of Danish avian-like H1 viruses are not chronological and that there is a lack of concordance between H1s and NA subtypes, which is probably due to frequent reassortment events. Antigenic cartography revealed that most of the Danish H1s are relatively antigenic alike, but outliers were also identified. Of these antigenic outliers, five were from 2011/2012 which could indicate an increasing drift away from the predominant herd immunity.

ACKNOWLEDGEMENTS

REFERENCES

Abe,Y., Takashita,E., Sugawara,K., Matsuzaki,Y., Muraki,Y., and Hongo,S. (2004). Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. J. Virol. *78*, 9605-9611.

Abusugra,I.A., Linne,T., and Klingeborn,B. (1987). The provenance of the Swedish swine influenza H1N1 virus of 1983. Zentralbl. Veterinarmed. B *34*, 566-572.

Baigent,S.J. and McCauley,J.W. (2003). Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays *25*, 657-671.

Balint,A. *et al.* (2009). The first Swedish H1N2 swine influenza virus isolate represents an uncommon reassortant. Virol. J. *6*, 180.

Bhatt,S. *et al.* (2013). The evolutionary dynamics of influenza A virus adaptation to mammalian hosts. Philos. Trans. R. Soc. Lond B Biol. Sci. *368*, 20120382.

Bikour,M.H., Frost,E.H., Deslandes,S., Talbot,B., Weber,J.M., and Elazhary,Y. (1995). Recent H3N2 swine influenza virus with haemagglutinin and nucleoprotein genes similar to 1975 human strains. J. Gen. Virol. *76 (Pt 3)*, 697-703.

Blackburne,B.P., Hay,A.J., and Goldstein,R.A. (2008). Changing selective pressure during antigenic changes in human influenza H3. PLoS. Pathog. *4*, e1000058.

Bøtner,A. (1994). Svineinfluenza. Veterinær Information *6*, 24-27.

Bragstad,K., Nielsen,L.P., and Fomsgaard,A. (2008). The evolution of human influenza A viruses from 1999 to 2006: a complete genome study. Virol. J. *5*, 40.

Breum,S.O., Hjulsager,C.K., and Larsen,L.E. New influenza A reassortments have been found in Danish swine in 2011. Influenza 2012, Oxford, UK. 2012.
Abstract

Brown,I.H. (2012). History and Epidemiology of Swine Influenza in Europe. Curr. Top. Microbiol. Immunol.

Brown,I.H. (2000). The epidemiology and evolution of influenza viruses in pigs. Vet. Microbiol. *74*, 29-46.

Brown,I.H., Done,S.H., Spencer,Y.I., Cooley,W.A., Harris,P.A., and Alexander,D.J. (1993). Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet. Rec. *132*, 598-602.

Castro,J.M., del,P.M., and Simarro,I. (1988). Identification of H3N2 influenza virus isolated from pigs with respiratory problems in Spain. Vet. Rec. *122*, 418-419.

Castrucci,M.R., Donatelli,I., Sidoli,L., Barigazzi,G., Kawaoka,Y., and Webster,R.G. (1993). Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology *193*, 503-506.

Castrucci,M.R. and Kawaoka,Y. (1993). Biologic importance of neuraminidase stalk length in influenza A virus. J. Virol. *67*, 759-764.

Caton,A.J., Brownlee,G.G., Yewdell,J.W., and Gerhard,W. (1982). The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell *31*, 417-427.

Chen,W., Zhong,Y., Qin,Y., Sun,S., and Li,Z. (2012). The evolutionary pattern of glycosylation sites in influenza virus (H5N1) hemagglutinin and neuraminidase. PLoS. One. *7*, e49224.

Colman,P.M., Varghese,J.N., and Laver,W.G. (1983). Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature *303*, 41-44.

Das,S.R., Puigbo,P., Hensley,S.E., Hurt,D.E., Bennink,J.R., and Yewdell,J.W. (2010). Glycosylation focuses sequence variation in the influenza A virus H1 hemagglutinin globular domain. PLoS. Pathog. *6*, e1001211.

De,V.A., Atanasova,K., Van,B.S., van den,B.T., Rasmussen,T.B., Uttenthal,A., and Van,R.K. (2009). Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs. PLoS. One. *4*, e6662.

Drummond,A.J. and Rambaut,A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. BMC. Evol. Biol. *7*, 214.

Dunham,E.J., Dugan,V.G., Kaser,E.K., Perkins,S.E., Brown,I.H., Holmes,E.C., and Taubenberger,J.K. (2009). Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses. J. Virol. *83*, 5485-5494.

Fourment,M., Wood,J.T., Gibbs,A.J., and Gibbs,M.J. (2010). Evolutionary dynamics of the N1 neuraminidases of the main lineages of influenza A viruses. Mol. Phylogenet. Evol. *56*, 526-535.

Gerhard,W., Yewdell,J., Frankel,M.E., and Webster,R. (1981). Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature *290*, 713-717.

Haesebrouck,F. and Pensaert,M. (1988). Influenza in swine in Belgium (1969-1986): epizootiologic aspects. Comp Immunol. Microbiol. Infect. Dis. *11*, 215-222.

Harder,T.C., Grosse,B.E., Lange,E., Meiners,C., Dohring,S., Pesch,S., Noe,T., Grund,C., Beer,M., and Starick,E. (2013). Expanded cocirculation of stable subtypes, emerging lineages, and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany. J. Virol. *87*, 10460-10476.

Hebert,D.N., Foellmer,B., and Helenius,A. (1996). Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. EMBO J. *15*, 2961-2968.

Hoffmann,E., Stech,J., Guan,Y., Webster,R.G., and Perez,D.R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. *146*, 2275-2289.

Koel,B.F. *et al.* (2013). Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science *342*, 976-979.

Kuntz-Simon,G. and Madec,F. (2009). Genetic and antigenic evolution of swine influenza viruses in Europe and evaluation of their zoonotic potential. Zoonoses. Public Health *56*, 310-325.

Lam,T.Y., Hon,C.C., Wang,Z., Hui,R.K., Zeng,F., and Leung,F.C. (2008). Evolutionary analyses of European H1N2 swine influenza A virus by placing timestamps on the multiple reassortment events. Virus Res. *131*, 271-278.

Le,Q.M. *et al.* (2005). Avian flu: isolation of drug-resistant H5N1 virus. Nature *437*, 1108.

Lewis,N.S., Anderson,T.K., Kitikoon,P., Skepner,E., Burke,D.F., and Vincent,A.L. (2014). Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J. Virol. *88*, 4752-4763.

Lewis,N.S. *et al.* (2011). Antigenic and genetic evolution of equine influenza A (H3N8) virus from 1968 to 2007. J. Virol. *85*, 12742-12749.

Li,S., Schulman,J., Itamura,S., and Palese,P. (1993). Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. J. Virol. *67*, 6667-6673.

Li,W., Shi,W., Qiao,H., Ho,S.Y., Luo,A., Zhang,Y., and Zhu,C. (2011). Positive selection on hemagglutinin and neuraminidase genes of H1N1 influenza viruses. Virol. J. *8*, 183.

Li,X., Kong,M., Chen,J., Su,X., Zou,M., Guo,L., Li,L., and Ding,J. (2013). Epidemiology and full genome sequence analysis of H1N1pdm09 from Northeast China. Virus Genes *47*, 199-209.

Lorusso,A. *et al.* (2011). Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. J. Gen. Virol. *92*, 919-930.

Luoh,S.M., McGregor,M.W., and Hinshaw,V.S. (1992). Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses. J. Virol. *66*, 1066-1073.

Matrosovich,M., Tuzikov,A., Bovin,N., Gambaryan,A., Klimov,A., Castrucci,M.R., Donatelli,I., and Kawaoka,Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. *74*, 8502-8512.

Metreveli,G. *et al.* (2011). Comparison of two H1N2 swine influenza A viruses from disease outbreaks in pigs in Sweden during 2009 and 2010. Virus Genes *42*, 236-244.

Miyata,T. and Yasunaga,T. (1980). Molecular evolution of mRNA: a method for estimating evolutionary rates of synonymous and amino acid substitutions from homologous nucleotide sequences and its application. J. Mol. Evol. *16*, 23-36.

Moreno,A., Chiapponi,C., Boniotti,M.B., Sozzi,E., Foni,E., Barbieri,I., Zanoni,M.G., Faccini,S., Lelli,D., and Cordioli,P. (2012). Genomic characterization of H1N2 swine influenza viruses in Italy. Vet. Microbiol. *156*, 265-276.

Moreno,A., Gabanelli,E., Sozzi,E., Lelli,D., Chiapponi,C., Ciccozzi,M., Zehender,G., and Cordioli,P. (2013). Different evolutionary trends of swine H1N2 influenza viruses in Italy compared to European viruses. Vet. Res. *44*, 112.

Murcia,P.R. *et al.* (2012). Evolution of an Eurasian avian-like influenza virus in naive and vaccinated pigs. PLoS. Pathog. *8*, e1002730.

Nardelli,L., Pascucci,S., Gualandi,G.L., and Loda,P. (1978). Outbreaks of classical swine influenza in Italy in 1976. Zentralbl. Veterinarmed. B *25*, 853-857.

Ottis,K., Sidoli,L., Bachmann,P.A., Webster,R.G., and Kaplan,M.M. (1982). Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. Arch. Virol. *73*, 103-108.

Pensaert,M., Ottis,K., Vandeputte,J., Kaplan,M.M., and Bachmann,P.A. (1981). Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man. Bull. World Health Organ *59*, 75-78.

Pereda,A. *et al.* (2010). Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg. Infect. Dis. *16*, 304-307.

Posada,D. (2008). jModelTest: phylogenetic model averaging. Mol. Biol. Evol. *25*, 1253-1256.

Ronquist,F. and Huelsenbeck,J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. *19*, 1572-1574.

Schild,G.C., Oxford,J.S., Dowdle,W.R., Coleman,M., Pereira,M.S., and Chakraverty,P. (1974). Antigenic variation in current influenza A viruses: evidence for a high frequency of antigenic 'drift' for the Hong Kong virus. Bull. World Health Organ *51*, 1-11.

Schultz,U., Fitch,W.M., Ludwig,S., Mandler,J., and Scholtissek,C. (1991). Evolution of pig influenza viruses. Virology *183*, 61-73.

Shen,J., Ma,J., and Wang,Q. (2009). Evolutionary trends of A(H1N1) influenza virus hemagglutinin since 1918. PLoS. One. *4*, e7789.

Smith,D.J., Lapedes,A.S., de Jong,J.C., Bestebroer,T.M., Rimmelzwaan,G.F., Osterhaus,A.D., and Fouchier,R.A. (2004). Mapping the antigenic and genetic evolution of influenza virus. Science *305*, 371-376.

Sorensen,K.J., Have,P.D., Ronsholt,L., Hage,J.M., Westergaard, and Pedersen,K.B. (1981). Influenza hos svin i Danmark. Dan. Vet. Tidsskr. *64*, 826-829.

Sriwilaijaroen,N., Kondo,S., Yagi,H., Takemae,N., Saito,T., Hiramatsu,H., Kato,K., and Suzuki,Y. (2011). N-glycans from porcine trachea and lung: predominant NeuAcalpha2-6Gal could be a selective pressure for influenza variants in favor of human-type receptor. PLoS. One. *6*, e16302.

Starick,E., Lange,E., Grund,C., Grosse,B.E., Dohring,S., Maas,A., Noe,T., Beer,M., and Harder,T.C. (2012). Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. J. Gen. Virol. *93*, 1658-1663.

Stevens,J., Blixt,O., Glaser,L., Taubenberger,J.K., Palese,P., Paulson,J.C., and Wilson,I.A. (2006). Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J. Mol. Biol. *355*, 1143-1155.

Sun,S., Wang,Q., Zhao,F., Chen,W., and Li,Z. (2011). Glycosylation site alteration in the evolution of influenza A (H1N1) viruses. PLoS. One. *6*, e22844.

Suzuki,T. *et al.* (1997). Swine influenza virus strains recognize sialylsugar chains containing the molecular species of sialic acid predominantly present in the swine tracheal epithelium. FEBS Lett. *404*, 192-196.

Tamuri,A.U., Dos,R.M., Hay,A.J., and Goldstein,R.A. (2009). Identifying changes in selective constraints: host shifts in influenza. PLoS. Comput. Biol. *5*, e1000564.

Trebbien,R., Bragstad,K., Larsen,L.E., Nielsen,J., Botner,A., Heegaard,P.M., Fomsgaard,A., Viuff,B., and Hjulsager,C.K. (2013). Genetic and biological characterisation of an avian-like H1N2 swine influenza virus generated by reassortment of circulating avian-like H1N1 and H3N2 subtypes in Denmark. Virol. J. *10*, 290.

Tumova,B., Veznikova,D., Mensik,J., and Stumpa,A. (1980). Surveillance of influenza in pig herds in Czechoslovakia in 1974--1979. 1. Introduction of influenza epidemic A (H3N2) viruses into pig herds. Zentralbl. Veterinarmed. B *27*, 517-523.

Vijaykrishna,D., Poon,L.L., Zhu,H.C., Ma,S.K., Li,O.T., Cheung,C.L., Smith,G.J., Peiris,J.S., and Guan,Y. (2010). Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science *328*, 1529.

Westgeest,K.B. *et al.* (2012). Genetic evolution of the neuraminidase of influenza A (H3N2) viruses from 1968 to 2009 and its correspondence to haemagglutinin evolution. J. Gen. Virol. *93*, 1996-2007.

Wolf,Y.I., Viboud,C., Holmes,E.C., Koonin,E.V., and Lipman,D.J. (2006). Long intervals of stasis punctuated by bursts of positive selection in the seasonal evolution of influenza A virus. Biol. Direct. *1*, 34.

Xu,J., Davis,C.T., Christman,M.C., Rivailler,P., Zhong,H., Donis,R.O., and Lu,G. (2012). Evolutionary history and phylodynamics of influenza A and B neuraminidase (NA) genes inferred from large-scale sequence analyses. PLoS. One. *7*, e38665.

Yang,Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. *24*, 1586-1591.

Yus,E., Sanjuan,M.L., Garcia,F., Castro,J.M., and Simarro,I. (1992). Influenza A viruses: epidemiologic study in fatteners in Spain (1987-89). Zentralbl. Veterinarmed. B *39*, 113-118.

Zhang,X.M., Herbst,W., Lange-Herbst,H., and Schliesser,T. (1989). Seroprevalence of porcine and human influenza A virus antibodies in pigs between 1986 and 1988 in Hassia. Zentralbl. Veterinarmed. B *36*, 765-770.

FIG 1A Phylogenetic tree of H1 sequences was inferred using the Bayesian MCMC method integrated in Mr.Bayes 3.1 under the GTR+I+G model by running 20 million MCMCs with 25% burn-in and a sampling frequency of 500. Scale bar indicates number of nucleotide substitutions per site. Danish swine H1N2 sequences are colored pink, Danish swine H1N1 sequences included in this study are light blue, European reference swine H1N1 and H1N2 sequences are black, avian sequences are green, human and human-like sequences are dark blue and pandemic sequences are red.
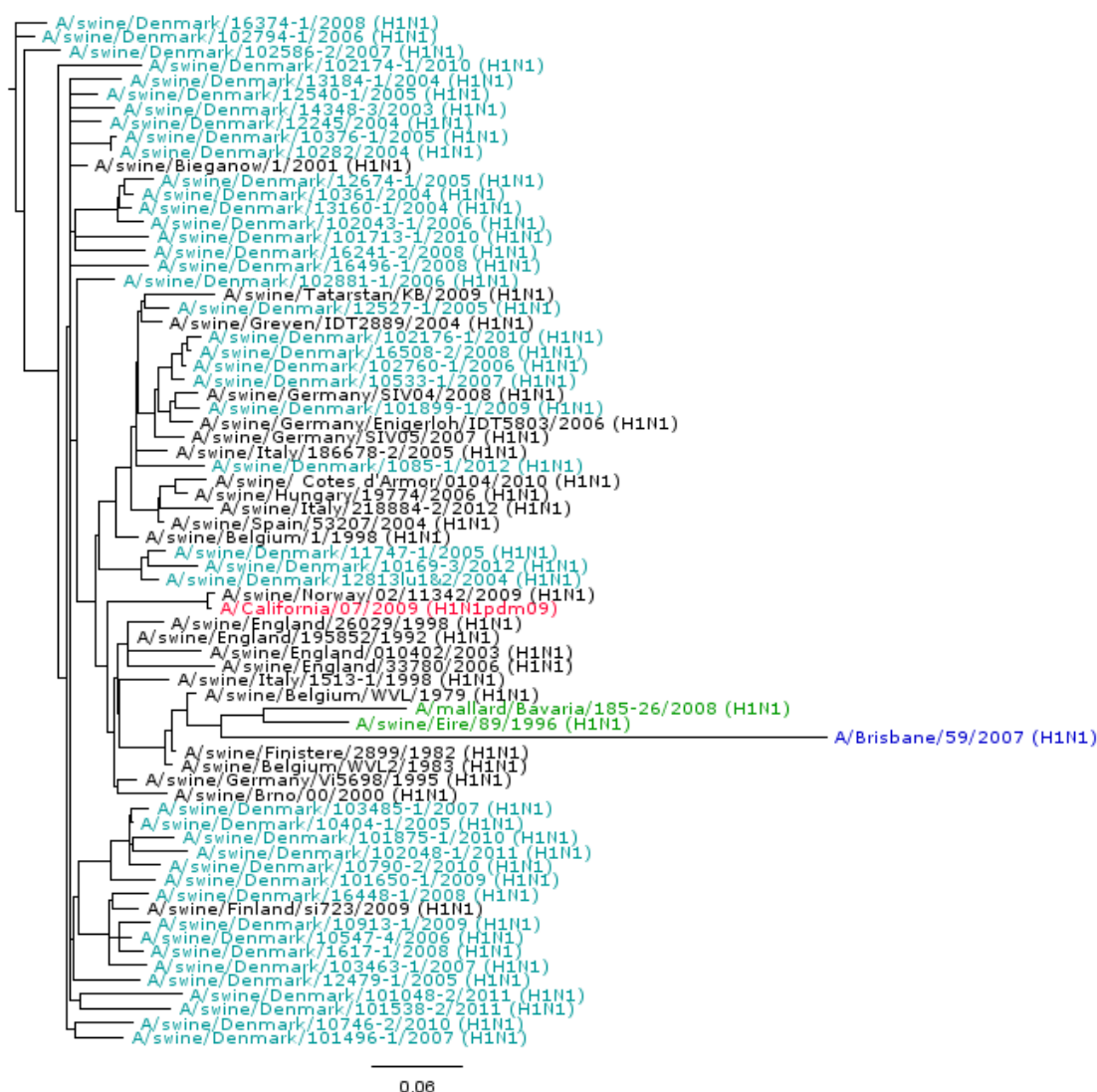
FIG 1B Phylogenetic tree of N1 sequences was inferred using the Bayesian MCMC method integrated in Mr.Bayes 3.1 under the GTR+I+G model by running 2.5 million MCMCs with 25% burn-in and a sampling frequency of 2500. Scale bar indicates number of nucleotide substitutions per site. Danish swine H1N1 sequences are light blue, reference swine H1N1 sequences are black, avian sequences are green, human and human-like sequences are dark blue and pandemic sequences are red.
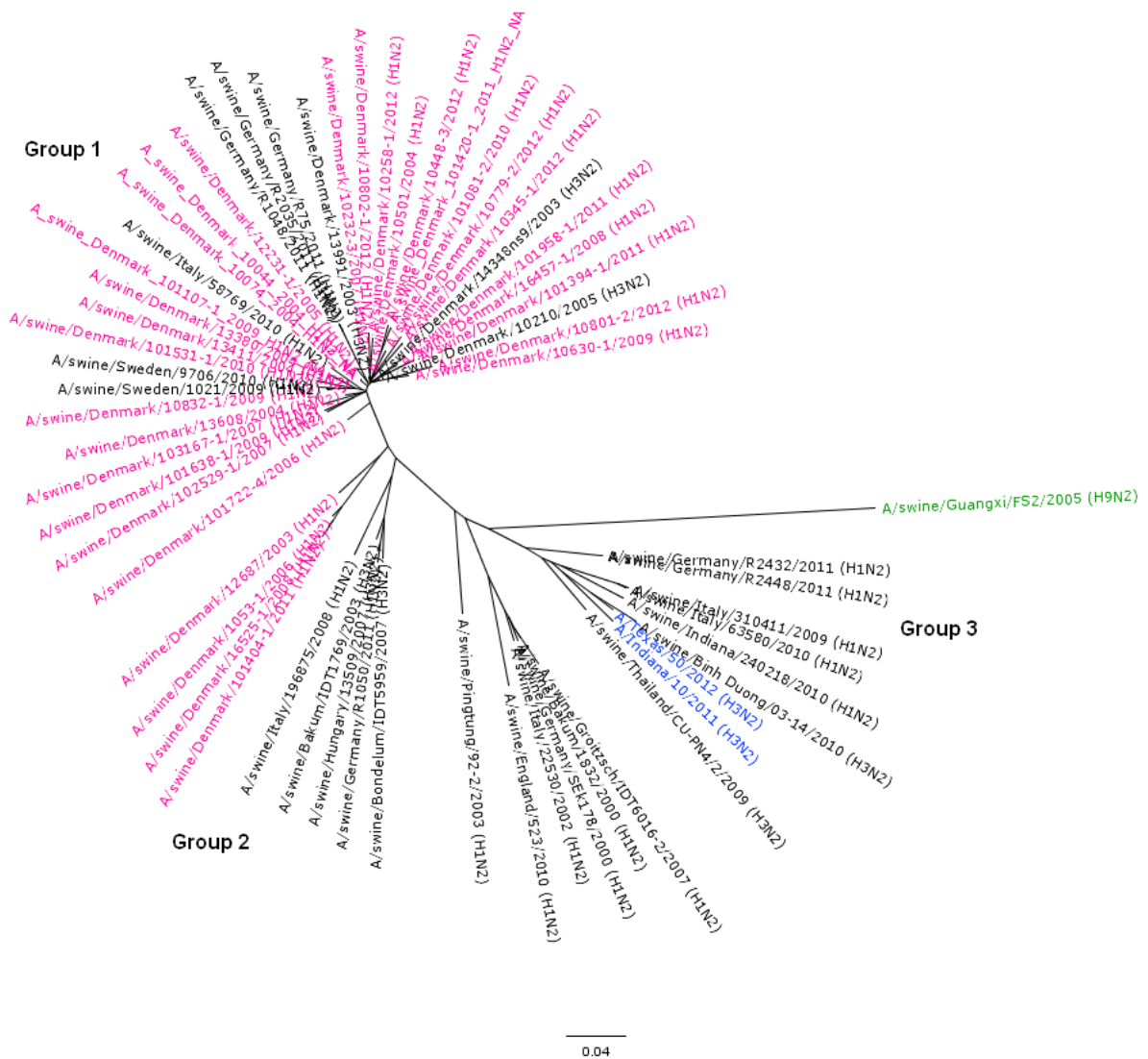
FIG 2 Phylogenetic tree of N2 sequences was inferred using the Bayesian MCMC method integrated in Mr.Bayes 3.1 under the GTR+I+G model by running 2.5 million MCMCs with 25% burn-in and a sampling frequency of 2500. Scale bar indicates number of nucleotide substitutions per site. Danish swine H1N2 sequences are colored pink, reference swine H3N2 and H1N2 sequences are black, avian virus isolated from a pig is green and human sequences are dark blue.
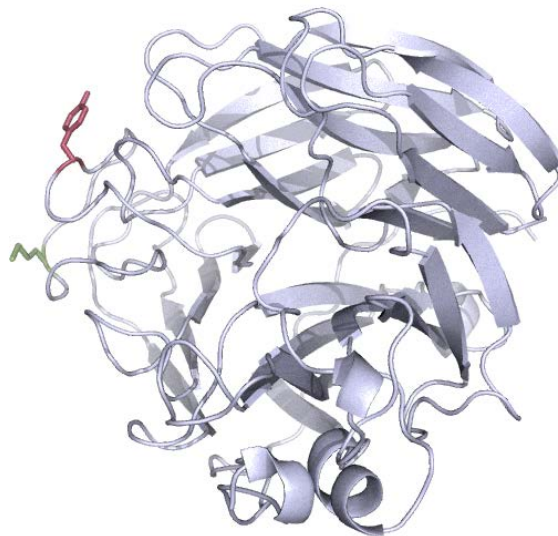
FIG 3 Location of the positively selected sites 331M and 339Y in N1. Red is 339Y and green is 331M. The model was based on prediction of the N1 structure using the program CPH models and for this the PDB entry 4b7q was used.
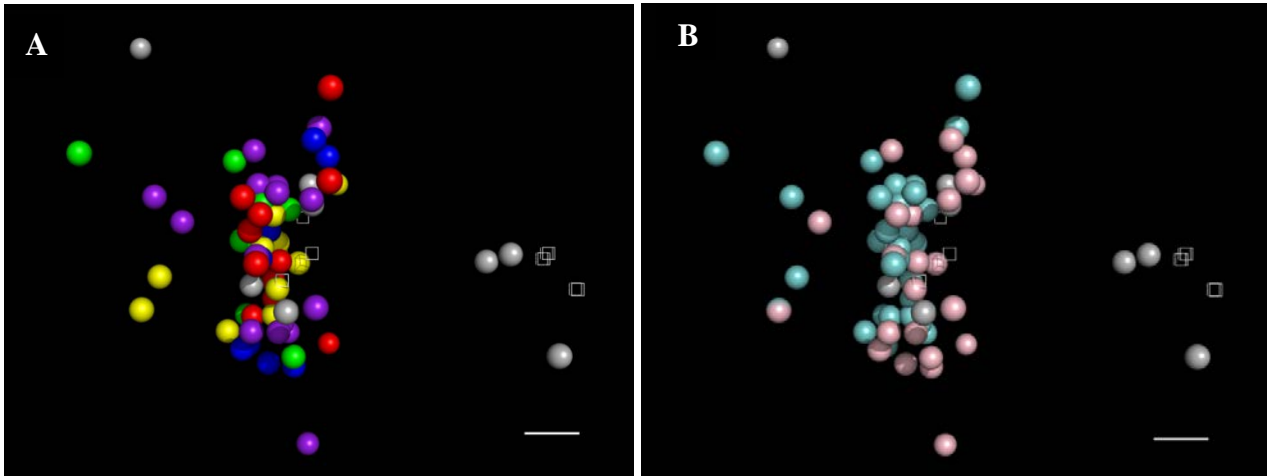
FIG 4 3D Antigenic cartography maps of the Danish H1 viruses, subtype H1 from 2003 to 2012. The relative positions of isolates (colored dots) and swine hyperimmune antisera (open squares) were computed such that the distances between isolates and antisera in the map with the least error represents the corresponding HI measurements (Smith *et al.*, 2004). (A) The isolates are colored according to their year of isolation, 2003/04 are blue, 2005/06 are green, 2007/08 yellow, 2009/10 are red and 2011/12 are purple, reference strains are colored grey. (B) The isolates are colored according to their subtype, H1N1 are blue, H1N2 are pink and reference strains are colored grey. The scale bar on the maps represents 1 unit of antigenic distance, corresponding to a two-fold difference in the HI assay.

FIG 5 Comparison of amino acid sequences from outliers in the antigenic cartography. Antigenic sites are shown in colored boxes, Cb is shown in dark blue, Sa in red, Ca1 in light green, Ca2 in dark green and Sb in light blue. The 130 loop, 190 helix and 220 loop are indicated in the blankets under the sequences. The host specific amino acid residue 155 is shown in a black box.

TABLE 1 Percentage of nucleotide similarity for H1, N1 and N2 sequences per year

Year of isolation

		2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Nucleotide similarity (%)	H1	95-97 (6)	93-100 (12)	93-95 (7)	92-94 (7)	91-93 (8)	91-95 (8)	89-95 (7)	89-96 (8)	90-93 (7)	89-94 (8)
	N1	- (1)	93-99 (6)	92-96 (7)	92-95 (5)	91-99 (5)	91-96 (6)	90-93 (3)	90-96 (6)	90-91 (3)	91 (2)
	N2	- (1)	98-100 (6)	- (1)	91 (2)	95 (3)	89 (2)	94-97 (4)	95 (2)	88-95 (4)	93-95 (6)

Numbers in parentheses represents the number of sequences used for the calculation

TABLE 2 Mean rates of nucleotide substitutions and time of most common recent ancestor of HA and NA

Segment	Mean rate of nucleotide substitution ($\times 10^{-3}$ substitutions per site per year)			Time of MRCA		
	Mean	**95% HPD***		**Mean**	**95% HPD**	
		Lower	**Upper**		**Lower**	**Upper**
H1	4.65	4.09	5.17	1913-1919	1896-1900	1930-1938
N1	4.12	3.39	4.91	1980	1974	1984
N2	3.66	3.15	4.17	1977	1972	1979

* HPD = Highest probability density

TABLE 3 Selected amino acids at residues important for host specificity, receptor binding and species specificity, as well as residues associated with potential cluster transitions in antigenic cartography in Danish SIV sequences

Function	Position	Amino acid in A/swine/Denmark/19126/1993 and A/swine/Denmark/WVL3/1993	Ratio of substitutions in all sequences	Ratio of substitutions in antigenic outliers
130-loop	133	T	46K/25R/3-*/2G/1Q/1N	11K/3-/1R/1Q/1G
	138	A	65A/13S	11A/6S
	145	R/S	52N/24K/2S	9N/6K/2S
Species specific	155	V	63V/6I/9T	10V/1I/6T
	156	K	78K	17K
	158	G	52G/14E/5R/3-*/2N/1D/1K	8G/6E/1R/1D/1K
	159	N	70N/4S/3D/1K	16N/1K
	189	S	68S/6N/2D/1R/1G/1I	12S/2N/1D/1G/1I
	190	D	78D	17D
190-helix	192	Q	78Q	17Q
	193	T/P	60T/18A	8T/9A
				15K/1R/1E
220-loop	222	K	76K/1R/1E	
	225	G	65E/1Q/4N/1D/5T/2K	8E/1Q/4N/1D/2T/1K
	226	Q	78Q	17Q
	228	G	78G	17G

*- deletion

TABLE 4A Glycosylation sites in H1N1 HA and H1N2 HA from the present study

Glycosylation site	% conserved H1N1	% conserved H1N2
14NNST	2	0
15NSTD	100	100
26NVTV	100	100
58NCSV	12	10
123NRTS/NATS	0	7
133NVTV	2	0
157NNSY	2	0
186NYSD	0	7
187NYSD	2	0
218NRTK	2	0
260NKSS	2	7
276NHTY	0	3
487NYSK	2	0
541NGSL	2	0
542NGSL	96	93

TABLE 4B Glycosylation sites in N1 and N2

Glycosylation site N1	% conserved	Glycosylation site N2	% conserved
44NQTE	20	-	-
50NQSV	7	-	-
58NKTW	2	-	-
63NQTY	100	61NMTK/NVTK/NITE	88
68NISN	9	70NTTI	6
87NSSL	2	86NWTK	3
88NSSL	75		
146NGTI	100	146NDTI	100
234NGSC	2	234NGTC	91
235NGSC	82	-	-
341NGTN	2	-	-
-	-	402NRSG	18

MANUSCRIPT II

Zoonotic potential of four European swine influenza viruses in the ferret model

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(Submitted for Journal of Virology)

ABSTRACT

The reverse zoonotic events that introduced the 2009 pandemic influenza virus into pigs have drastically increased the diversity of swine influenza viruses in Europe. The pandemic potential of these novel reassortments is still unclear, necessitating enhanced surveillance of European pigs with additional focus on risk assessment of these new viruses. In this study, four European swine influenza viruses were assessed for their zoonotic potential. Two of the four viruses were enzootic viruses of subtype H1N2 (with avian-like H1) and H3N2 and two were new reassortants, one with avian-like H1 and human-like N2 and one with 2009 pandemic H1 and swine-like N2. All viruses replicated to high titers in nasal wash- and nasal turbinate samples from inoculated ferrets and transmitted efficiently by direct contact. Only the H3N2 virus transmitted to naïve ferrets via the airborne route. Growth kinetics using a differentiated human bronchial epithelial cell line showed that all four viruses were able to replicate to high titers. Further, the viruses revealed preferential binding to the α2,6-silalylated glycans and investigation of the antiviral susceptibility of the viruses revealed that all were sensitive to neuraminidase inhibitors. These findings suggest that these viruses have the potential to infect humans and further underline the need for continued surveillance as well as biological characterization of new influenza A viruses.

INTRODUCTION

Influenza A virus causes disease in humans, birds and some domestic animals, including swine. Swine influenza viruses (SIV) are enzootic in pigs worldwide and infection with the virus causes substantial economic loss for farmers due to secondary infections and reduced weight gain in affected pigs. Further, the presence of influenza A virus in swine also pose a potential health risk to humans due to development of new reassortant viruses with zoonotic potential as recently seen with the 2009 H1N1 pandemic virus (H1N1pdm09) (2011a).

Within the last few years, several new reassortant SIV have been detected throughout Europe, Asia and the US (Breum *et al.*, 2013;Moreno *et al.*, 2011;Pascua *et al.*, 2013;Starick *et al.*, 2011;Starick *et al.*, 2012;Tremblay *et al.*, 2011), with many carrying segments from enzootic SIVs and the H1N1pdm09 virus. In the US, recent reassortment events have led to a novel variant of the triple-reassortant H3N2, now containing the matrix gene from the H1N1pdm09 (2011a;2011b;2011c). This variant H3N2 (H3N2v) has proven efficient in transmission from swine to humans, as observed at several agricultural fairs in the US during 2012-2013 (http://www.cdc.gov/flu/weekly/pastreports.htm).

In Denmark, at least four SIV subtypes are enzootic in pigs, the avian-like H1N1 (Abusugra *et al*., 1987;Schultz *et al*., 1991), the European reassortant human-like swine H3N2 (Bøtner, 1990;Castrucci *et al.*, 1993) and the H1N2 with an avian-like swine H1 and N2 from the reassortant human-like swine H3N2 (Trebbien *et al*., 2013). In 2010, a reverse zoonotic event led to the introduction of the H1N1pdm09 into Danish swine. The virus spread and rapidly accounted a prevalence of 15-20% of SIV's isolated in the country (unpublished data). During the national passive surveillance program in 2012, at least two new reassortants were detected in Danish pigs, one with an avian-like H1N1 backbone and an N2 gene most closely related to that of a human seasonal H3N2 virus that was circulating in the mid 1990-ies (H1avN2hu) (Breum *et al*., 2013) and one with a pandemic backbone and a N2 gene from the reassortant human-like swine H3N2 (H1pdmN2sw) (unpublished data), also detected in German pig populations (Starick *et al.*, 2012).

The increasing rate of new reassortments and the lack of knowledge regarding their zoonotic potential is a worrying aspect and it is therefore of significant importance to genetically and phenotypically characterize reassortants.

Influenza A virus infection in ferrets closely resembles that of humans considering clinical signs and pathogenesis (Maher and DeStefano, 2004) and therefore ferrets have been widely used as a model for assessment of the pandemic potential of influenza viruses. Additionally, receptor expression patterns in the respiratory tract of ferrets are similar to humans (Baum and Paulson, 1990;van Reeth *et al*., 2007).

In this study, the pathogenicity and transmissibility of two new reassortant SIV's, the H1avN2hu and H1pdmN2sw, were compared to H1N2 and H3N2 enzootic European SIV strains in the ferret model. This study is the first to assess the zoonotic potential of not just new reassortant viruses, but also contemporary virus isolates of the European H1N2 with avian-like H1 and the European reassortant human-like swine H3N2 virus.

METHODS

Viruses

Four influenza A viruses, A/swine/Denmark/10302-2/2012(H1N2), A/swine/Denmark/10845-1/2012(H1N2), A/swine/Denmark/101394-1/2011(H1N2) and A/swine/Denmark/101501-1/2010(H3N2) (hereafter referred to as H1avN2hu, H1pdmN2sw, H1N2 and H3N2, respectively) were isolated from lung samples submitted for diagnostic purposes from swine with a history of respiratory disease. Viruses were grown and titrated in Madin-Darby canine kidney (MDCK) cells. Before inoculation, influenza viruses were passaged in the allantoic cavity of 10-day-old embryonated chicken eggs (Marshall Durbin, Birmingham, AL) at 35°C for 72 h. All isolates underwent a maximum of two passages in eggs and/or cells.

Cell cultures

MDCK cells were grown in Minimum Essential Medium Eagle (MEM) (Gibco, Carlsbad, CA, USA) containing 5% fetal calf serum (FCS), 2 mM L-glutamine, Non-essential amino acids (NEAA) and penicillin-streptomycin. Normal human bronchial epithelial cells (NHBE) in individual inserts were obtained from MatTek Corporation (Ashland, MA, USA). The cells were grown in AIR-100-ASY (MatTek Corporation Ashland, MA, USA) serum free media containing growth factors. The apical surface was washed to remove mucus and media was changed every other day.

Primary swine respiratory epithelial cells (pSREC) were seeded into type VI collagen (Sigma-Aldrich, St. Louis, MO, USA)-coated tissue culture flasks and grown in Bronchial Epithelial Cell Growth

medium (BEGM, Lonza, Walkersville, MD, USA) with SingleQuots™ Kit containing growth factors, and cytokines. Medium was further supplemented with 5% FCS and 1% penicillin/streptomycin/amphotericin (Sigma, St. Louis, MO, US) and passaged up to five times prior to infection. All cells were grown at 37°C in a 5% CO_2 atmosphere.

Infection and replication kinetics

For preparation of viral stocks, lung tissue was homogenized on TissueLyser (Qiagen, GmbH, Germany) in 1.5 mL MEM supplemented with penicillin-streptomycin and sterile filtered. MDCK cells were inoculated with 500 µL lung tissue homogenate for 30 min at 37°C, 5% CO_2. Following incubation, 10 mL MEM containing penicillin-streptomycin, NEAA, 2 mM L-glutamine and 2 µg/mL tosylsulfonyl-phenylalanyl-chloromethyl-ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. The cells were incubated for 72 h and daily monitored for cytopathic effects (CPE). The supernatant was harvested and centrifuged at 2500 rpm for 30 minutes to clarify cell debris and then stored at -80°C until further use.

The 50% tissue culture infectious dose ($TCID_{50}$) and plaque-forming units (pfu) were determined by incubating serial dilutions of virus in MDCK cells at 37°C for 72 h. A hemagglutination assay was performed to determine the end point of infection and $TCID_{50}$ was calculated using the method of Reed and Muench (Reed and Muench, 1938).

Replication kinetics in MDCK cells were determined at a multiplicity of infection (MOI) of 0.01 pfu per cell. After 1 h of incubation the MDCK cells were washed and overlaid with infection medium (MEM containing 2% BSA, pen-strep, amino acids and TPCK-treated trypsin). Supernatants were collected and stored at -80°C for virus titration. Replication kinetics in differentiated NHBE cells were determined at a MOI of 0.01 pfu per cell. Cells were washed with phosphate buffered saline (PBS) to remove mucus before infection and were inoculated via the apical side with 200 µl diluted virus in the absence of trypsin. After 1 h of incubation at 37°C, viral inoculum was removed and cells were washed. Cells were then pre-incubated with 200 µl fresh medium at 37°C for 30 min prior to sample collection at specified time points. Samples were stored at -80°C. Replication kinetic in pSRECs was determined at an MOI of 0.01 pfu per cell. Cells were washed and infection medium (BEGM, Lonza, Walkersville, MD, USA) containing 0.5% BSA was added, supernatants were collected at specified time points and stored at -80°C for virus titration.

RNA purification and real time RT-PCR screening

Viral RNA was purified from cultured viruses by RNeasy Mini Kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. Cell culture supernatant was prepared by mixing 200 µl sample with 400 µl RLT-buffer containing β–mercaptoethanol. Total RNA was eluted in 60 µl RNase-free water and stored at -80°C.

The presence of influenza A virus was confirmed by real-time RT-PCR using an in-house modified assay for detection of the matrix gene (De Vleeschauwer *et al*., 2009).

Full genome sequencing and RT-PCR

Nucleic acid amplification was performed by one-step RT-PCR using primers modified from Hoffmann *et al*., (Hoffmann *et al*., 2001) and the Superscript III One-Step RT-PCR kit with Platinum *Taq* High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions for HA was as follows: 30 min at 55°C, 2 min at 94°C, four cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 180 sec, followed by 40 cycles of 94°C for 30 sec and 68°C for 210 sec and then 68°C for 10 min. For NA the same conditions were used, except that reverse transcriptase temperature was 54°C and annealing temperature 58°C.

The PCR products were visualized by gel electrophoresis using E-Gel 0.8% agarose gels (Invitrogen, Carlsbad, CA, USA) and purified with High Pure PCR Product Purification Kit (Roche Diagnostics, GmbH, Germany). Purified PCR products were sent for single sample sequencing at LGC Genomics (GmbH, Germany).

Full genome sequencing was performed on two cell culture propagated influenza positive samples by full length amplification of all 8 gene segments with in-house designed primers (primers available upon request) using SuperScript III OneStep RT-PCR System with Platinum Taq High Fidelity and PCR conditions similar to that described for HA full length amplification. Purified PCR products for all gene segments were pooled in equimolar quantity to a final amount of 1 µg and used for next generation sequencing (NGS) on the Ion Torrent PGM™ sequencer (Life Technologies, Carlsbad, CA, USA). NGS including library preparation was carried out at the Multi-Assay Core facility located at the Technical University of Denmark.

Sequence analysis

Sequences obtained by NGS were assembled using the *de novo* and reference assembly tools of CLC Genomics Workbench 4.6.1 (CLC bio A/S, Århus, Denmark). Sequences obtained by Sanger sequencing was analyzed using CLC Main Workbench Version 6.9 (CLC bio A/S).

Animals

All animal experiments were performed in Animal Biosafety Level 2 facilities at St. Jude Children Research Hospital (Memphis, TN, USA), in compliance with the policies of the National Institutes of Health and the Animal Welfare Act and with the approval of the St. Jude Children's Research Hospital Animal Care and Use Committee. In total, 36 four to six month old ferrets (Triple F farms, Sayre, PA, US), weighing 0.8-1.6 kg, that had been tested negative to current circulating human influenza subtypes by hemagglutination inhibition (HI) assay were used.

Transmission and pathogenicity studies

Before inoculation of the donor ferrets, baseline body temperatures and weights were documented for all ferrets. To test viral pathogenicity and transmission, two donor ferrets were housed in the upper cages of the isolator and two donor ferrets were housed in the lower cages of the isolator. Donor ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 TCID$_{50}$ influenza virus in 1 ml PBS with antibiotics and antimycotic (Sigma, St. Louis, MO; 100 U/ml penicillin, 100 mg streptomycin, and 0.25 mg amphomycin per ml) with 500 µl in each nostril. At day 1 post infection (dpi) two of the donor ferrets were moved to separate cages each containing one naïve ferret, for direct contact (DC) transmission assay. For the airborne transmission (AT) assay two naïve ferrets were housed adjacent to the donor ferrets, separated by a double-layered grill in order to allow unobstructed airflow and prevent direct contact.

After inoculation, temperature, weight and clinical signs were recorded every other day for 11 days.

Nasal washes

Nasal washes were collected at 1 dpi for donor ferrets and at 3, 5, 7, 9 and 11 dpi for all ferrets. Ferrets were anesthetized intramuscularly with 20-50 mg/kg of Ketamine, nostrils were flushed with 1 ml of PBS containing antibiotics and antimycotic (Sigma, St. Louis, MO, USA; 100 U/ml penicillin, 100 mg streptomycin, and 0.25 mg amphomycin per ml) with 500 µl in each nostril and collected into cups.

Nasal washes were spun down and stored at -80°C until further analysis. $TCID_{50}$ were determined in MDCK cells and expressed as $TCID_{50}$/ml.

Ferret organ collection and virus titration

At 5 dpi two inoculated ferrets from each group were sacrificed for pathological examination. The remaining ferrets were sacrificed at 21 dpi and the following tissues were collected from all animals; nasal turbinates, trachea, Right/left caudal and cranial lung lobes, lymph nodes, intestine, liver and spleen. Tissues were weighed and homogenized in MEM with antibiotics. Virus titers were determined in MDCK cells as described above and expressed as $TCID_{50}$/g tissue.

Serological tests

Serum samples collected at 0 and 1 dpi and at 19 and 21 dpi were tested for antibodies. The serum samples were tested in a blocking ELISA using the commercially available influenza A antibody test kit, detecting antibodies against the NP gene (IDEXX Laboratories A/S, Switzerland), according to manufacturer's instructions. The ELISA antibody values were calculated as optical density (OD) from each sample and presented as percent transmittance.

Histopathology

Samples of trachea and the left/right cranial and dorsal lung lobes were collected from two control ferrets and all inoculated ferrets at 5 dpi, and the remaining ferrets at 21 dpi. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and slides were processed by routine methods for histology, stained with hematoxylin and eosin and examined in a blinded fashion.

Receptor assay

The four viruses were tested for their HA binding activity to the following glycans; α2,3-SL (Neu5Acα2-3Galβ1-4Glcβ-PAA-Biotin), α2,6-SL (Neu5Acα2-6Galβ1-4Glcβ-PAA-Biotin) and α2,6-SLN (Neu5Acα2-6Galβ1-4GlcNAcβ-PAA-Biotin) (Glycotech Corp., Gaithersburg, MD, US) as previously described (Matrosovich and Gambaryan, 2012). Briefly, 32 hemagglutinating units (HAU) of each virus were coated to wells of enzyme-linked immunosorbent assay (ELISA) plates previously coated with fetuin and incubated overnight at 4°C. Plates were washed with ice-cold PBS and biotinylated glycans were prepared in reaction buffer (PBST and 0.1% BSA) and added to the plates, which were incubated at 4°C for 2 h. Plates were washed and incubated with tetramethylbenzidine

substrate (product) solution for 10 min. The reaction was stopped by adding 50 mM HCl and the absorbance (450 nM) was read in a Synergy microplate reader (Biotek Instruments Inc., Winooski, VT, USA).

NA kinetics and antiviral susceptibility to neuraminidase inhibitors

NA kinetics and antiviral susceptibility to NA inhibitors (NAI) were based on the method of Potier et al. (Potier *et al.*, 1979) using methylumbelliferone *N*-acetylneuraminic acid (MUNANA; Sigma-Aldrich, St Louis, MO) substrate, as described by (Jones *et al.*, 2014). Substrate cleavage by the NA enzyme releases the fluorescent product 4-methylumbelliferone (4-MU) (Sigma-Aldrich, St Louis, MO), after which fluorescence was monitored using excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Enzyme kinetics data were fitted by non-linear regression to the Michaelis-Menten equation using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) to determine the Michaelis Menten constant and maximum velocity (V_{max}) of substrate conversion.

Sensitivity of NA to oseltamivir carboxylate (oseltamivir) from Hoffmann-La Roche (Basel, Switzerland), zanamivir from Glaxo-SmithKline (Research Triangle Park, NC, USA), and peramivir from BioCryst Pharmaceuticals (Birmingham, AL, USA) was tested by using dilutions of inhibitors ranging from 5×10^{-7} µM to 50 µM. The drug concentration that inhibited 50% of the NA enzymatic activity (IC_{50}) was determined from the dose-response curve with GraphPad Software version 5. Results are expressed as the average of two independent tests.

Statistical analysis

Two-way ANOVA with Bonferoni's posttest was performed using GraphPad Prism version 5.

RESULTS

Clinical signs and virus shedding

Ferrets were inoculated with 10^6 TCID$_{50}$/ml of each virus, a dose previously shown to initiate an infection (Lednicky *et al.*, 2010;Stark *et al.*, 2013). None of the ferrets developed fever, had nasal discharges or showed signs of lethargy during the study. The ferrets showed variable weight losses from mild to moderate (3-10%).

All four viruses replicated in inoculated ferrets and were also found to be transmitted to naïve DC ferrets (Fig 1) as assessed by isolation of virus from nasal washes. Infectious virus was detected in nasal washes until approximately 7 dpi for donor ferrets and 9 dpi for ferrets inoculated with H3N2. The day of observed peak titers for the four viruses was found to vary (Fig 1). Ferrets inoculated with H1avN2hu showed a mean viral peak titer in nasal washes at 4.6 log$_{10}$ TCID$_{50}$/ml at day 1 p.i. and DC ferrets in this group had a mean viral peak titer at 3.5 log$_{10}$ TCID$_{50}$/ml at 7 dpi For ferrets inoculated with H1pdmN2sw a mean peak titer at 6.5 log$_{10}$ TCID$_{50}$/ml was observed at 1 dpi and DC ferrets showed a mean peak titer at 5.2 log$_{10}$ TCID$_{50}$/ml at 9 dpi. Infection with the H1N2 virus resulted in a mean peak viral titer at 5.1 log$_{10}$ TCID$_{50}$/ml 5 dpi and a mean peak viral titer at 5.5 log$_{10}$ TCID$_{50}$/ml 7 dpi for DC ferrets. Finally, infection with H3N2 gave a mean peak viral titer at 5.9 log$_{10}$ TCID$_{50}$/ml 3 dpi for inoculated ferrets and a mean peak viral titer at 5.4 log$_{10}$ TCID$_{50}$/ml 5 dpi for DC ferrets.

Airborne transmission was observed in ferrets inoculated with H3N2 with a mean peak viral titer at 3.8 log$_{10}$ TCID$_{50}$/ml 7 dpi (Fig 1). One of the AT ferrets died at 11 dpi although it was concluded that the cause of death was not related to the infection. By the end of the experiment IAV antibodies were detected in serum from all inoculated and DC ferrets, as well as the AT ferret belonging to the H3N2 group (Fig 2). The ELISA assay was chosen for antibody detection, due to a too high background response in the HI assay. At 19 and 21 dpi, inoculated and DC ferrets had all seroconverted.

Virus detection by real time RT-PCR

Nasal wash samples from all days post infection were tested by real time RT-PCR to determine if virus could be replicating at levels too low to be detected by the TCID$_{50}$ assay (Table 1). In general, viral RNA was detected in all samples from inoculated ferrets at 1 dpi, viral load was found to peak at 3 or 5 dpi with a subsequent decline in viral RNA at 7-11 dpi. For DC ferrets the viral load for H1avN2hu and

H1pdmN2sw peaked between 7 and 9 dpi with a rapid decline already 2 days after viral RNA had peaked. For H1N2 the day of the highest viral load could not be determined due to lack of material from one of the ferrets at 7 dpi. The H3N2 virus was found to peak at 7 dpi, but viral load remained high even after the last nasal wash sampling at 11 dpi. Results from AT ferrets showed no ct values for H1avN2hu and H1N2, whereas low viral load was detected in one of the AT ferrets in the H1pdmN2sw group at 3 and 5 dpi. In the H3N2 group, AT ferrets showed viral RNA for one ferret at 5 dpi and viral load in both AT ferrets peaked at 11 dpi.

Viral load in respiratory organs

From each group, two inoculated ferrets were euthanized at 5 dpi for determination of viral titers in nasal turbinates, trachea, caudal and cranial lung lobe, lymph nodes, intestine, liver and spleen. No viral replication was detected in liver, spleen, intestines or lymph nodes.

For all viruses, infectious virus particles were recovered from nasal turbinates, trachea and lung lobe 1 and 2 (Fig 3). Highest viral titers were observed in nasal turbinates with mean titers ranging from 6.1 $\log_{10}$ TCID$_{50}$/g to 7.9 $\log_{10}$ TCID$_{50}$/g with the highest level of virus particles observed in ferrets inoculated with H1avN2hu and H1N2. From the trachea, viral titers ranged from 3.82 TCID$_{50}$/g to 5.45 TCID$_{50}$/g, with highest titers observed in ferrets inoculated with H1avN2hu and H3N2. Viral replication in caudal and cranial lung lobes lung lobes 1 and 2 showed mean titers ranging from 1.5 $\log_{10}$ TCID$_{50}$/g to 3.6 $\log_{10}$ TCID$_{50}$/g with the highest level of virus particles observed in ferrets inoculated with H1pdmN2sw and H3N2.

Histopathology

No significant lesions were seen in trachea or lung tissue of the control group (Fig 4A). All four viruses caused hyperemia, edema and hemorrhage into alveoli as well as varying degrees of non-suppurative interstitial pneumonia, dysplasia of the bronchiolar epithelium and hyperplasia of the bronchus associated lymphoid tissue (BALT). Apart from these common characteristics, the severity of the lesions differed among the groups depending on the virus used for inoculation.

Findings in trachea were alike in the four virus groups, ranging from no lesions to mild dysplasia of the epithelium, moderate suppurative tracheitis and single cell necrosis.

The viruses H3N2 and H1pdmN2sw caused the most severe lung lesions (Fig 4B and C) consisting of suppurative bronchiolitis and bronchitis, as well as epithelial necrosis of serous glands and perivascular accumulation of mononuclear cells. The inoculated ferrets (euthanized 5 dpi) furthermore had necrosis of bronchiolar epithelium, and in one of the H3N2 inoculated ferrets mixed bronchopneumonia was seen.

The H1N2 and H1avN2hu viruses caused infiltration of a few neutrophilic granulocytes and mononuclear cells in the alveoli (Fig 4D and E). Both groups of inoculated ferrets (euthanized 5 dpi) had necrosis of the bronchiolar epithelium. Inoculation with H1avN2hu also induced perivascular accumulation of mononuclear cells, desquamation of bronchiolar epithelium, suppurative bronchiolitis and focal epithelial necrosis of serous glands.

In vitro growth kinetics

The *in vitro* replication capacity of the four European SIV strains was assessed and compared in a human cell line (NHBE), a swine cell line (pSRECs) and MDCK cells. Cells were inoculated with a low MOI (0.01).

All four swine viruses were able to infect and replicate to high titers in NHBE cells. The H1avN2hu and H1pdmN2sw viruses progressively increased in titers until 48 hours post-inoculation (hpi) with mean peak titers at 8.0 $\log_{10}$ TCID$_{50}$/ml and 8.9 $\log_{10}$ TCID$_{50}$/ml, respectively. The H1N2 and H3N2 titers increased until 60 hpi and showed mean peak titers at 9.2 $\log_{10}$ TCID$_{50}$/ml and 9.4 $\log_{10}$ TCID$_{50}$/ml, respectively (Fig 5A).

In pSREC an increase in titers were observed until 36 hpi for H1avN2hu and H1N2 with mean peak titers at 6.8 $\log_{10}$ TCID$_{50}$/ml and 7.2 $\log_{10}$ TCID$_{50}$/ml, respectively. Mean peak titers for H1pdmN2sw and H3N2 were 6.6 $\log_{10}$ TCID$_{50}$/ml at 48 hpi and 6.75 $\log_{10}$ TCID$_{50}$/ml at 60 hpi, respectively (Fig 5B).

In MDCK cells, the virus titers progressively increased during the first 36 hpi with highest mean peak titers in the range from 6.3 $\log_{10}$ TCID$_{50}$/ml to 7.9 $\log_{10}$ TCID$_{50}$/ml and the highest mean peak titer was observed for H1N2 (Fig 5C).

Receptor binding

Binding of the viral HA to host receptors is known be important for the determination of transmissibility efficiency and host range restriction. Hence glycan binding properties of the four viruses were investigated by testing for their ability to bind biotinylated sialylglycopolymers in a dose-dependent fashion.

None of the viruses exhibited strong binding preference towards the avian $\alpha2,3$ sialylglycopolymer ($\alpha2,3$-SL) and binding to this glycan barely exceeded the threshold. The European swine viruses preferentially bound to the human/swine $\alpha2,6$ sialylglycopolymer, with H3N2 and H1pdmN2sw showing the highest affinity for the human/swine $\alpha2,6$ sialylglycopolymer ($\alpha2,6$-SLN). H1avN2hu and H3N2 were found to also bind the short version of the $\alpha2,6$ sialylglycopolymer ($\alpha2,6$-SL). For H1avN2hu and H3N2, it appeared that as the concentration of sialyglycopolymers were decreased the receptor preference shifted from the long to the short version of the $\alpha2,6$ sialylglycopolymer. Results are summarized in figure 6.

Sequencing

For examination of molecular determinants involved in receptor binding of the European SIVs, full length HA sequences were obtained from all four viruses using nasal washes from the day of their highest mean peak viral titers as templates. Amino acids previously shown to be involved in receptor binding are located at the distal tip of the HA monomer in positions 111 to 265 and is formed by three secondary structures, termed the 130-loop, the 190-helix and the 220-loop (Gamblin *et al.*, 2004) (Table 2).

The analysis of the H1 viruses, showed that variable amino acids were found at the receptor binding residue 138 (Dunham *et al.*, 2009), where H1N2 possessed 138V, H1avN2hu 138I and H1pdmN2sw 138A.

In the 190-helix, 190D was found for H1N2 and H1pdmN2sw, whereas H1avN2hu possessed 190S and also residue 193 was found be variable among the European H1 viruses. At this residue, H1N2 possessed 193T, H1avN2hu possessed 193A and H1pdmN2sw possessed 193S.

In the 220-loop, an E225K mutation in H1N2 was observed during the study, with the 225E found in inoculum, an inoculated ferret and a DC ferret. The 225K variation was found in an inoculated ferret

and a DC ferret. The ferrets possessing the E225K mutation had not been cohoused. H1avN2hu was found to possess 225E and H1pdmN2sw 225D.

Further, all H1 viruses possessed a K at position 222, which is one of the amino acids critical for interaction with long α2,6-receptors (Stevens *et al.*, 2006).

For pandemic H1 viruses, two amino acid positions, 200 and 227, have been found to play an important role in terms of binding preferences of pandemic H1 versus swine H1 to sialic acid receptors (de Vries *et al.*, 2011). Amino acids at these positions were found for H1pdmN2sw to be 200A and 227E and for comparison, H1avN2hu possessed 200T and 227E and at the same positions, H1N2 possessed 200T and 227A.

Position 155 have previously been shown to be a residue important for host specificity (Matrosovich *et al.*, 2000) and at this position, all three H1 viruses possessed 155V.

Investigation of the receptor binding properties of the H3N2 virus showed that the European H3N2 SIV possessed 155Y and 158G. These two residues have previously been shown to play a critical role in receptor recognition (Takahashi *et al.*, 2009). Further, amino acids at residue 226 and 228 have also previously been shown to play a role in receptor binding (Matrosovich *et al.*, 2000). The European SIV H3N2 was found to possess 226L and 228S.

NA kinetics

To compare the NA enzyme kinetics of the four European SIVs, the sialidase activity of the neuraminidases were determined using the MUNANA fluorogenic substrate.

Previously it has been suggested that NA activity may facilitate the transmissibility of IAVs (Campbell *et al.*, 2014) and hence NA enzyme kinetics of the four European SIVs were determined, using the MUNANA fluorogenic substrate. The Michaelis-Menten constant (K_M) is an estimate of the dissociation equilibrium for substrate binding to enzyme and thereby reflects the enzyme affinity for the substrate, where V_{max} reflect the enzyme's catalytic activity. All four European SIVs showed high Km values, ranging from 281 µM for H3N2 to 492 µM for H1N2. Vmax values were found to be in the range from 842 U/sec for H3N2 to 1845 U/sec for H1pdmN2sw (Table 3).

Antiviral susceptibility

The antiviral susceptibility of the four European SIVs to three of the most commonly used neuraminidase inhibitors (Oseltamivir carboxylase, Zanamivir and Peramivir) was tested. All of the European swine influenza viruses were found to be sensitive to all of the neuraminidase inhibitors tested (Table 3). H3N2 was found to be the most sensitive (IC_{50} of 0.08 nM) to Oseltamivir Carboxylate and H1N2 to be the least sensitive (IC_{50} of 0.47 nM). For Zanamivir, H3N2 was found to be the most sensitive (IC_{50} of 0.14nM) and H1pdmN2sw to be the least sensitive (IC_{50} of 1.59 nM) and for Peramivir, the results showed that also H3N2 was the most sensitive (IC_{50} of 0.10 nM) and H1N2 was the least sensitive (IC_{50} of 0.66 nM).

DISCUSSION

The genetic background of the H1N1pdm09 virus, the recent incidents with infection of humans with swine derived H3N2v in the US, as well as the increased prevalence of new SIV reassortants detected globally (Ducatez *et al.*, 2011;Harder *et al.*, 2013;Mukherjee *et al.*, 2012;Pascua *et al.*, 2013) have highlighted the need to investigate the zoonotic potential of SIVs. In this study, four European SIVs were characterized. Two of these viruses were recently discovered reassortants and two were enzootic viruses having circulated in swine herds for decades.

The ferret model is a widely used model for investigating potential pandemic and zoonotic threats of both swine and avian influenza viruses due to its disease course resembling that of humans (Maher and DeStefano, 2004). This trait also makes ferrets a suitable model for assessment of the zoonotic potential of the four SIVs investigated in this study. All four viruses were found to be able to infect ferrets and were also able to be transmitted directly to naïve cohoused ferrets. In comparison, a previous study showed that A/sw/Italy/3169-7/1994(H1N1), an avian-like H1N1, transmitted poorly via direct contact and was not able to transmit to AT ferrets. The same study also found that North American triple reassortant swine (TRS) viruses were readily transmissible via direct contact and that TRS viruses carrying swine-like HA and human-like NA versus human-like HA and human-like NA were moderate and efficient, respectively, in airborne transmission (Barman *et al.*, 2012). In agreement with these observations, we found that the H1avN2hu/H1pdmN2sw viruses carrying human-origin NA and human-origin HA, respectively, were less efficient in transmission compared to the H3N2 virus carrying both human-origin HA and NA.

H1avN2hu and H1pdmN2sw showed the highest virus titers in nasal turbinates, whereas in the nasal washes, H1avN2hu and H1N2 showed the lowest mean peak viral titers. These findings indicate that H1avN2hu replicates very efficiently in tissues, but suggests that release of new viral particles, are not very efficient. An observation indicating that the balance between the HA and NA of this virus, is not optimal for efficient cleavage of viral particles.

H3N2 and H1pdmN2sw showed the highest virus titers in lungs. The ability of H3N2 and H1pdmN2sw to replicate in the lungs of ferrets, were also consistent with the observation that these viruses seemed to induce the most severe lung lesions compared to the other two viruses. We therefore propose that the human-like HA (H3N2 and H1pdmN2sw) plays a more significant role in transmission than the human-like NA (H1avN2hu). Further supporting this theory was the detection of viral RNA in AT ferrets from both the H3N2 and the H1pdmN2sw virus groups. Even though the AT ferret in the H1pdmN2sw group showed a high ct value, this finding still suggests that H1pdmN2sw could also be able to transmit via airborne contact, but considerably less efficiently than the H3N2 virus.

The European H3N2 showed a mean peak viral titer already at day 5 pi in DC ferrets, which indicated that this virus was the most efficient in transmitting to naïve DC ferrets of the four viruses. H3N2 was further found to be the only virus able to transmit and initiate an effective viral replication via airborne transmission. These results, combined with the efficient transmission to DC ferrets and the high viral titers in both nasal turbinates and trachea, showed that this virus was the most effective, of the four European SIVs, for replication and transmission in the ferret model.

During this study, one of the AT ferrets in the group inoculated with H3N2 was found dead at 11 dpi. Pathological examinations of the ferret revealed that the death was not related to the infection. Since both AT ferrets in the group inoculated with H3N2 shed virus and virus was detected in nasal wash from the deceased ferret at 11 dpi, the results of the airborne transmission were not compromised by its death.

Previously, several studies have investigated the pathogenesis of SIV in the ferret model, but most of these studies have investigated infections with different TRS viruses and reassortments hereof (Barman *et al.*, 2012;Pascua *et al.*, 2012;Pearce *et al.*, 2012), as well as infection studies using the H1N1pdm09 virus (Guarnaccia *et al.*, 2013;Maines *et al.*, 2009;Yen *et al.*, 2011). In 2012, a study of the pathogenesis of Korean North American-like TRS H1N2 and H3N2 in ferrets, also showed high viral

titers from nasal washes of inoculated ferrets, but in contrast to the European SIVs, the A/Sw/Korea/1204/2009(H1N2) also caused severe disease in all inoculated ferrets. Further, the TRS viruses were shown to transmit via direct contact and A/Sw/Korea/1204/2009(H1N2) was found also to replicate in the lungs of ferrets, though to lower titer values than the European H3N2 and H1pdmN2sw (Pascua *et al.*, 2012).

The H1N1pdm09 has also previously been shown to transmit via direct contact between ferrets (Yen *et al.*, 2011), but did not transmit efficiently via airborne contact. The study by Yen *et al*., also detected H1N1pdm09 in the lower respiratory tract at high titers and in comparison to this, the European H1pdmN2sw lung titers were found to be considerably lower.

For further assessment of the zoonotic potential of the European SIVs, they were all tested for their ability to replicate in a human airway cell line and compared to their replication efficiency in a swine airway cell line and MDCK cells, the cell line primarily used for isolation of IAVs.

A classification criteria has previously been established based on the observation that the *in vitro* replication of IAVs from various species between 8 and 24 hpi correlates with infection in humans (Ilyushina *et al.*, 2012). This system classified viruses into three groups on the basis of replication kinetics at 24 hpi: rapid/high (≥ 4 Log_{10} $TCID_{50}$/ml), delayed/intermediate (<4 Log_{10}, but surpassed this level at subsequent time points), and low (<4 Log_{10} throughout the study) (Jones *et al.*, 2014). Applying this classification to the European SIVs showed that they at 24 hpi in NHBE cells all belonged to the rapid/high replication kinetics and only in pSREC cells could the H3N2 be classified as replicating at delayed/intermediate level. In conclusion, the high level of replication in NHBE cells of all four SIVs, suggested that these viruses have the ability to efficiently infect cells in the human respiratory airway.

The potential ability of the European SIVs to replicate efficiently in humans was also analyzed in terms of their receptor binding properties, important amino acids involved in receptor binding and host specificities. Human IAVs bind preferentially to α2,6-SL, and α2,6-SLN and to a lesser extent α2,3-SL as representatives of α2,6- and α2,3-linked Neu5Ac receptors (Stevens *et al.*, 2006). This is consistent with the high efficiency of infection and transmission of the European H3N2 virus, since it was found to bind both α2,6-SL and α2,6-SLN, but does not explain the less efficient infection and transmission of the H1avN2hu, that was also shown to bind these two receptors.

The observed preferences of the European SIVs fit well with the high viral replication in the upper respiratory tracts, where these receptor types mainly are present. The predominant binding to the α2,6 receptors also correlates well with amino acids in the receptor binding area that are characteristic for binding to this receptor in positions 190, 225, 226 and 228 that are typically observed in human adapted virus strains (Matrosovich *et al.*, 2000).

The 190D was present in H1N2 and H1pdmN2sw, whereas H1avN2hu possessed 190S. This amino acid has been shown to play an important role in binding of SIVs and human IAVs to the α-2,6 receptor in concert with the amino acid at position 225 (Matrosovich *et al.*, 2000). It could be speculated that the D to S mutation potentially affects binding of the H1avN2hu to the α2,6 receptor. Concerning the amino acid at residue 225, this was found to vary between all four European SIVs, with the H1pdmN2sw showing the "avian" 225D and H1avN2hu a 225E variation. Most interesting was H1N2 that showed both 225E and 225K. The 225E variant was found in inoculum, whereas the 225K variation was found in an inoculated ferret and a DC ferret, but since these ferrets were not cohoused, this mutation may have been random. The receptor binding domain at residue 225 has previously been found to be variable in European avian-like swine strains and has been shown to include the avian 225G, but also 225E and 225K (Dunham *et al.*, 2009), thus demonstrating that the E225K mutation observed in H1N2 is not uncommon.

In H1 HA, "avian" residues 226Q and 228G has been found to be present in human viruses (Glaser *et al.*, 2005;Matrosovich *et al.*, 2000) and these "avian" residues were also present in the European swine influenza H1 viruses. The European H3N2 virus possessed 226L and 228S at these respective residues. These amino acids have previously been shown, for the human H3 subtype, to reduce the affinity for α2,3 receptor binding, as well as increase the affinity for α2,6 receptors (Matrosovich *et al.*, 2000;Nobusawa *et al.*, 2000;Rogers *et al.*, 1983). Taken together, these findings are in accordance with the observed ability of the European H3N2 to infect both human and swine cell lines, as well as infecting and transmitting between ferrets, considering the α2,6 specificity required for infection of the above mentioned systems and also strongly supports its observed preference for binding of α2,6 sialylglycopolymers.

Earlier studies of RBS in H1 subtypes has shown that the European avian-like swine strains have significant variability in the receptor binding residue 138, unlike classical strains that maintains the

avian consensus (138A) (Dunham *et al.*, 2009). This observation is in agreement with the findings in this study where the two avian-like H1's showed both 138I/V and the H1pdm09, where the HA originates from a North American TRS lineage, which yet again originates from the classical H1N1 swine lineage (Smith *et al.*, 2009), showed the 138A variation.

The European H3N2 was shown in this study to possess amino acids 155Y and 158G, mutations found to be present in human H3's and both mutations have previously been shown to play a critical role in recognition of two major molecular species of sialic acids, namely 5-*N*-acetylneuraminic acid (Neu5Ac) and 5-*N*-glycolylneuraminic acid (Neu5Gc), where Neu5GC is an analog of sialic acid, expressed in many animal tissues, but absent from humans (Chou *et al.*, 1998;Matrosovich *et al.*, 2000;Takahashi *et al.*, 2009). Previously, T155Y and E158G substitutions was found to preferably bind to α2,6 receptors and have also been proposed to be mainly responsible for the wide binding pattern of H3 viruses that potentially are able to infect a variety of animals (Takahashi *et al.*, 2009). All the European H1 SIVs were found to possess 155V, which have previously been shown to increase the affinity of SIVs for Neu5Gc (Bateman *et al.*, 2010;Chou *et al.*, 1998;Sriwilaijaroen *et al.*, 2011). In human H1 viruses, 155T has been found to be conserved at this position (Matrosovich *et al.*, 2000), which could explain the observed enhanced growth of especially H1N2 and H1avN2hu in pSRECs compared to that of H3N2.

Specifically for the H1pdmN2sw, it has previously been shown that T200A and E227A mutations in pandemic H1 were very important for strong fetuin binding (a blood-glycoprotein containing α2,3 and α2,6 sialic acids in a 2:1 ratio) of HA (de Vries *et al.*, 2011). This suggests that since H1pdmN2sw only possesses one of these two mutations this could possibly affect the binding of this virus to the α2,6 receptor, but also suggests that the virus could be in the process of adapting to swine.

It has previously been found that the level of NA activity of particular influenza strains affects transmission efficiency in both guinea pigs and ferrets (Campbell *et al.*, 2014;Govorkova, 2013). Further it was found that PR8 modified by reverse genetics to contain a pandemic matrix (M) gene enhanced NA activity (Campbell *et al.*, 2014). Of the European SIVs, only H1pdmN2sw contained a pandemic M gene and interestingly, the H1pdmN2sw showed higher enzyme activity (a higher V_{max}) than the H3N2 virus, despite comparable K_M values. These findings suggest that NA activity is not sufficient to confer airborne contact transmission of the H3N2 virus.

It would be interesting though, to test if NA activity and transmission efficiency of the European H3N2 virus would increase further if the original M gene was replaced with a pandemic M gene.

Ferrets used for transmission studies are usually sero-negative to circulating human IAVs and is hence expected not to have acquired any previous immunity. For this reason, infection of the ferrets does not quite mirror the case in the human population, since vaccination as well as previously acquired immunity plays a role in a potential infection. With the transmission efficiency and ability to replicate in human respiratory cells observed in this study for the European SIV H3N2, there is a possibility that this virus could potentially transmit to humans. For the past 25 years, the European H3N2 virus has been adapting to swine and the degree of antigenic relation between this virus and the human seasonal H3N2 virus is likely to have increased dramatically. A recent study did show that contemporary human seasonal H3N2 viruses had obtained substantial antigenic distance from swine H3N2 viruses, even though the lineages shared a common ancestor. Hence, the authors speculated that this increasing distance could pose a risk for the youngest of the human population, since they could become increasingly susceptible to infections with swine H3N2 due to the lack of cross-reacting immunity (Lewis *et al.*, 2014).

Here we have shown the ability of four European SIVs to transmit efficiently in the ferret model via direct contact. Furthermore we showed that one of these viruses, H3N2, also had the ability to efficiently transmit via the airborne route. These findings stress the need for a continued and systematic surveillance of the European SIVs in order to detect new reassortants as well as monitoring the evolution of both reassortants and enzootic strains and further shows the importance of assessing their zoonotic potentials.

AKNOWLEDGEMENTS

REFERENCES

(2011b). Limited human-to-human transmission of novel influenza A (H3N2) virus--Iowa, November 2011. MMWR Morb. Mortal. Wkly. Rep. *60*, 1615-1617.

(2011c). Swine-origin influenza A (H3N2) virus infection in two children--Indiana and Pennsylvania, July-August 2011. MMWR Morb. Mortal. Wkly. Rep. *60*, 1213-1215.

(2011a). Update: influenza activity--United States, 2010-11 season, and composition of the 2011-12 influenza vaccine. MMWR Morb. Mortal. Wkly. Rep. *60*, 705-712.

Abusugra,I.A., Linne,T., and Klingeborn,B. (1987). The provenance of the Swedish swine influenza H1N1 virus of 1983. Zentralbl. Veterinarmed. B *34*, 566-572.

Barman,S. *et al.* (2012). Pathogenicity and transmissibility of North American triple reassortant swine influenza A viruses in ferrets. PLoS. Pathog. *8*, e1002791.

Bateman,A.C., Karamanska,R., Busch,M.G., Dell,A., Olsen,C.W., and Haslam,S.M. (2010). Glycan analysis and influenza A virus infection of primary swine respiratory epithelial cells: the importance of NeuAc{alpha}2-6 glycans. J. Biol. Chem. *285*, 34016-34026.

Baum,L.G. and Paulson,J.C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem. Suppl *40*, 35-38.

Bøtner,A. (1990).
Personal Communication

Breum,S.O., Hjulsager,C.K., Trebbien,R., and Larsen,L.E. (2013). Influenza a virus with a human-like n2 gene is circulating in pigs. Genome Announc. *1*.

Campbell,P.J., Danzy,S., Kyriakis,C.S., Deymier,M.J., Lowen,A.C., and Steel,J. (2014). The M segment of the 2009 pandemic influenza virus confers increased neuraminidase activity, filamentous morphology, and efficient contact transmissibility to A/Puerto Rico/8/1934-based reassortant viruses. J. Virol. *88*, 3802-3814.

Castrucci,M.R., Donatelli,I., Sidoli,L., Barigazzi,G., Kawaoka,Y., and Webster,R.G. (1993). Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology *193*, 503-506.

Chou,H.H., Takematsu,H., Diaz,S., Iber,J., Nickerson,E., Wright,K.L., Muchmore,E.A., Nelson,D.L., Warren,S.T., and Varki,A. (1998). A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. Proc. Natl. Acad. Sci. U. S. A *95*, 11751-11756.

de Vries,R.P., de,V.E., Moore,K.S., Rigter,A., Rottier,P.J., and de Haan,C.A. (2011). Only two residues are responsible for the dramatic difference in receptor binding between swine and new pandemic H1 hemagglutinin. J. Biol. Chem. *286*, 5868-5875.

De Vleeschauwer,A., Atanasova,K., Van,B.S., van den,B.T., Rasmussen,T.B., Uttenthal,A., and Van,R.K. (2009). Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs. PLoS. One. *4*, e6662.

Ducatez,M.F. *et al.* (2011). Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerg. Infect. Dis. *17*, 1624-1629.

Dunham,E.J., Dugan,V.G., Kaser,E.K., Perkins,S.E., Brown,I.H., Holmes,E.C., and Taubenberger,J.K. (2009). Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses. J. Virol. *83*, 5485-5494.

Gamblin,S.J. *et al.* (2004). The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science *303*, 1838-1842.

Glaser,L., Stevens,J., Zamarin,D., Wilson,I.A., Garcia-Sastre,A., Tumpey,T.M., Basler,C.F., Taubenberger,J.K., and Palese,P. (2005). A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J. Virol. *79*, 11533-11536.

Govorkova,E.A. (2013). Consequences of resistance: in vitro fitness, in vivo infectivity, and transmissibility of oseltamivir-resistant influenza A viruses. Influenza. Other Respir. Viruses. *7 Suppl 1*, 50-57.

Guarnaccia,T. *et al.* (2013). Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. PLoS. Pathog. *9*, e1003354.

Harder,T.C., Grosse,B.E., Lange,E., Meiners,C., Dohring,S., Pesch,S., Noe,T., Grund,C., Beer,M., and Starick,E. (2013). Expanded cocirculation of stable subtypes, emerging lineages, and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany. J. Virol. *87*, 10460-10476.

Hoffmann,E., Stech,J., Guan,Y., Webster,R.G., and Perez,D.R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. *146*, 2275-2289.

Ilyushina,N.A., Ikizler,M.R., Kawaoka,Y., Rudenko,L.G., Treanor,J.J., Subbarao,K., and Wright,P.F. (2012). Comparative study of influenza virus replication in MDCK cells and in primary cells derived from adenoids and airway epithelium. J. Virol. *86*, 11725-11734.

Jones,J.C., Baranovich,T., Marathe,B.M., Danner,A.F., Seiler,J.P., Franks,J., Govorkova,E.A., Krauss,S., and Webster,R.G. (2014). Risk assessment of H2N2 influenza viruses from the avian reservoir. J. Virol. *88*, 1175-1188.

Lednicky,J.A., Croutch,C.R., Lawrence,S.J., Hamilton,S.B., Daniels,D.E., and Astroff,B. (2010). A nonlethal young domesticated ferret (Mustela putorius furo) model for studying pandemic influenza virus A/California/04/2009 (H1N1). Comp Med. *60*, 364-368.

Lewis,N.S., Anderson,T.K., Kitikoon,P., Skepner,E., Burke,D.F., and Vincent,A.L. (2014). Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J. Virol. *88*, 4752-4763.

Maher,J.A. and DeStefano,J. (2004). The ferret: an animal model to study influenza virus. Lab Anim (NY) *33*, 50-53.

Maines,T.R. *et al.* (2009). Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science *325*, 484-487.

Matrosovich,M., Tuzikov,A., Bovin,N., Gambaryan,A., Klimov,A., Castrucci,M.R., Donatelli,I., and Kawaoka,Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. *74*, 8502-8512.

Matrosovich,M.N. and Gambaryan,A.S. (2012). Solid-phase assays of receptor-binding specificity. Methods Mol. Biol. *865*, 71-94.

Moreno,A. *et al.* (2011). Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. Vet. Microbiol. *149*, 472-477.

Mukherjee,T.R., Agrawal,A.S., Chakrabarti,S., and Chawla-Sarkar,M. (2012). Full genomic analysis of an influenza A (H1N2) virus identified during 2009 pandemic in Eastern India: evidence of reassortment event between co-circulating A(H1N1)pdm09 and A/Brisbane/10/2007-like H3N2 strains. Virol. J. *9*, 233.

Nobusawa,E., Ishihara,H., Morishita,T., Sato,K., and Nakajima,K. (2000). Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. Virology *278*, 587-596.

Pascua,P.N., Lim,G.J., Kwon,H.I., Park,S.J., Kim,E.H., Song,M.S., Kim,C.J., and Choi,Y.K. (2013). Emergence of H3N2pM-like and novel reassortant H3N1 swine viruses possessing segments derived from the A (H1N1)pdm09 influenza virus, Korea. Influenza. Other Respir. Viruses. *7*, 1283-1291.

Pascua,P.N. *et al.* (2012). Virulence and transmissibility of H1N2 influenza virus in ferrets imply the continuing threat of triple-reassortant swine viruses. Proc. Natl. Acad. Sci. U. S. A *109*, 15900-15905.

Pearce,M.B. *et al.* (2012). Pathogenesis and transmission of swine origin A(H3N2)v influenza viruses in ferrets. Proc. Natl. Acad. Sci. U. S. A *109*, 3944-3949.

Potier,M., Mameli,L., Belisle,M., Dallaire,L., and Melancon,S.B. (1979). Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. Anal. Biochem. *94*, 287-296.

Reed,L.J. and Muench,H. (1938).A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS. American Journal of Epidemiology 27[3], 493-497.

Rogers,G.N., Paulson,J.C., Daniels,R.S., Skehel,J.J., Wilson,I.A., and Wiley,D.C. (1983). Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature *304*, 76-78.

Schultz,U., Fitch,W.M., Ludwig,S., Mandler,J., and Scholtissek,C. (1991). Evolution of pig influenza viruses. Virology *183*, 61-73.

Smith,G.J. *et al.* (2009). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature *459*, 1122-1125.

Sriwilaijaroen,N., Kondo,S., Yagi,H., Takemae,N., Saito,T., Hiramatsu,H., Kato,K., and Suzuki,Y. (2011). N-glycans from porcine trachea and lung: predominant NeuAcalpha2-6Gal could be a selective pressure for influenza variants in favor of human-type receptor. PLoS. One. *6*, e16302.

Starick,E. *et al.* (2011). Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. J. Gen. Virol. *92*, 1184-1188.

Starick,E., Lange,E., Grund,C., Grosse,B.E., Dohring,S., Maas,A., Noe,T., Beer,M., and Harder,T.C. (2012). Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. J. Gen. Virol. *93*, 1658-1663.

Stark,G.V., Long,J.P., Ortiz,D.I., Gainey,M., Carper,B.A., Feng,J., Miller,S.M., Bigger,J.E., and Vela,E.M. (2013). Clinical profiles associated with influenza disease in the ferret model. PLoS. One. *8*, e58337.

Stevens,J., Blixt,O., Glaser,L., Taubenberger,J.K., Palese,P., Paulson,J.C., and Wilson,I.A. (2006). Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J. Mol. Biol. *355*, 1143-1155.

Takahashi,T., Hashimoto,A., Maruyama,M., Ishida,H., Kiso,M., Kawaoka,Y., Suzuki,Y., and Suzuki,T. (2009). Identification of amino acid residues of influenza A virus H3 HA contributing to the recognition of molecular species of sialic acid. FEBS Lett. *583*, 3171-3174.

Trebbien,R., Bragstad,K., Larsen,L.E., Nielsen,J., Botner,A., Heegaard,P.M., Fomsgaard,A., Viuff,B., and Hjulsager,C.K. (2013). Genetic and biological characterisation of an avian-like H1N2 swine influenza virus generated by reassortment of circulating avian-like H1N1 and H3N2 subtypes in Denmark. Virol. J. *10*, 290.

Tremblay,D., Allard,V., Doyon,J.F., Bellehumeur,C., Spearman,J.G., Harel,J., and Gagnon,C.A. (2011). Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine. J. Clin. Microbiol. *49*, 4386-4390.

Van Reeth,D., Munster,V.J., de,W.E., Rimmelzwaan,G.F., Fouchier,R.A., Osterhaus,A.D., and Kuiken,T. (2007). Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am. J. Pathol. *171*, 1215-1223.

Yen,H.L. *et al.* (2011). Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proc. Natl. Acad. Sci. U. S. A *108*, 14264-14269.
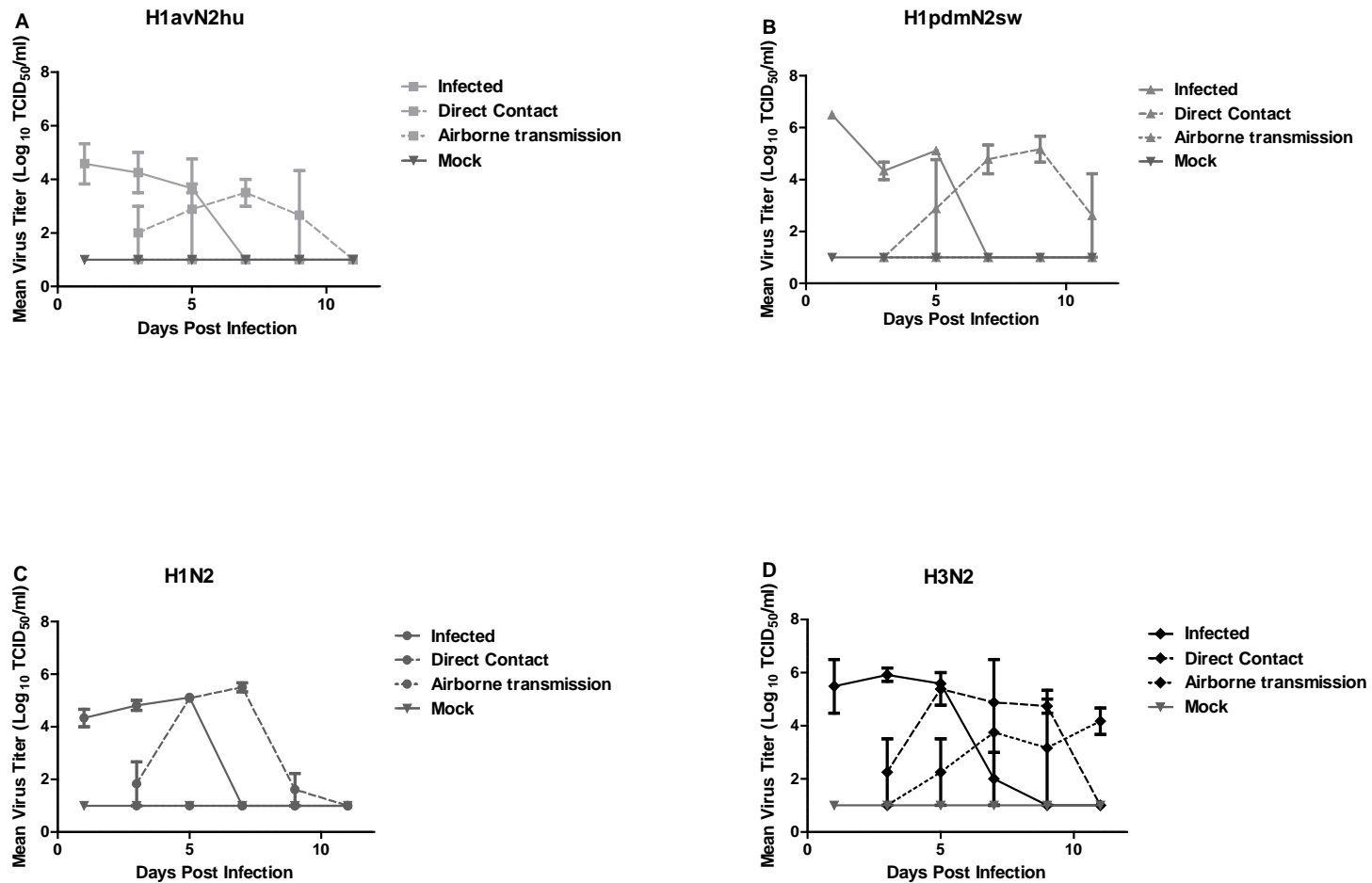
FIG 1 Replication kinetics in the upper respiratory tract of the four European swine influenza viruses. Groups of four ferrets were intranasally inoculated with 1 ml of 10^6 TCID$_{50}$ (0.5 ml per nostril) of the respective viruses. Each virus group contained eight ferrets, four donor ferrets (until 5 dpi), two direct contacts and two airborne transmission contacts. (A) H1avN2hu (B) H1N2 (C) H3N2 (D) H1pdmN2sw. Nasal washes were collected on days 1, 3, 5, 7, 9 and 11 after infection. Data are presented as mean virus titer ± SEM (log$_{10}$ TCID$_{50}$/ml) on the indicated day. Limit of detection was 10^1 TCID$_{50}$/ml.
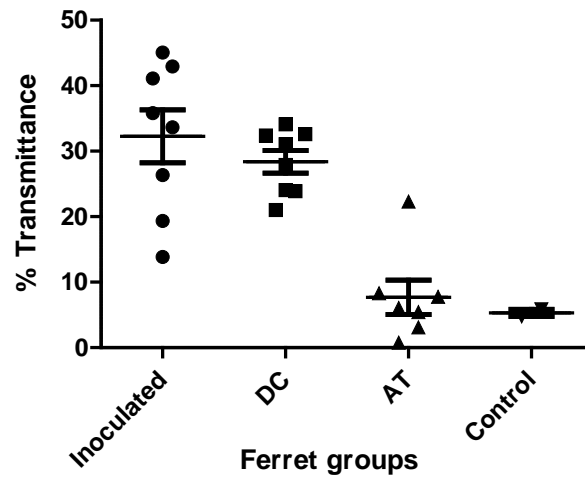
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FIG 2 Seroconversion of ferrets. Ferrets were tested for seroconversion at 19 and 21 dpi, using a blocking-ELISA, detecting antibodies against the NP gene. Serum samples were tested in duplicate and mean values are presented as percent transmittance ± SEM
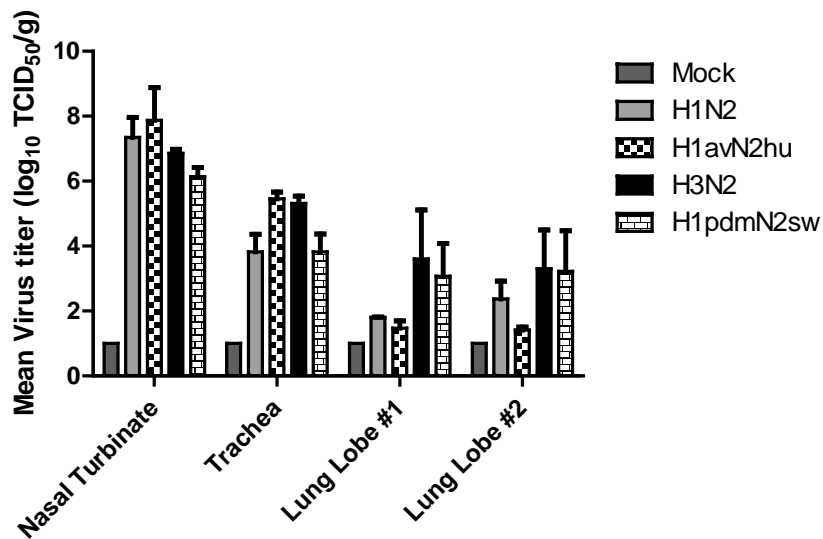
FIG 3 Comparison of European swine influenza virus titers recovered from ferret tissues. Ferrets were inoculated intranasally with 1 ml of 10^6 TCID$_{50}$ (0.5 ml per nostril) with H1N2, H1avN2hu, H3N2 or H1pdmN2sw, and tissues were collected at day 5 p.i. Titers are expressed as $\log_{10}$ TCID$_{50}$ per gram of tissue. Each bar represents two ferrets. Data are presented as mean virus titer ± SEM ($\log_{10}$ TCID$_{50}$/g) from the indicated tissue. Limit of detection was 10^1 TCID$_{50}$/g.

FIG 4 Lung pathology in ferrets inoculated with one of the four European swine influenza viruses A) Normal lung tissue from control ferret; B) Ferret inoculated with H3N2, euthanized 5 days post infection (dpi). Suppurative bronchiolitis, dys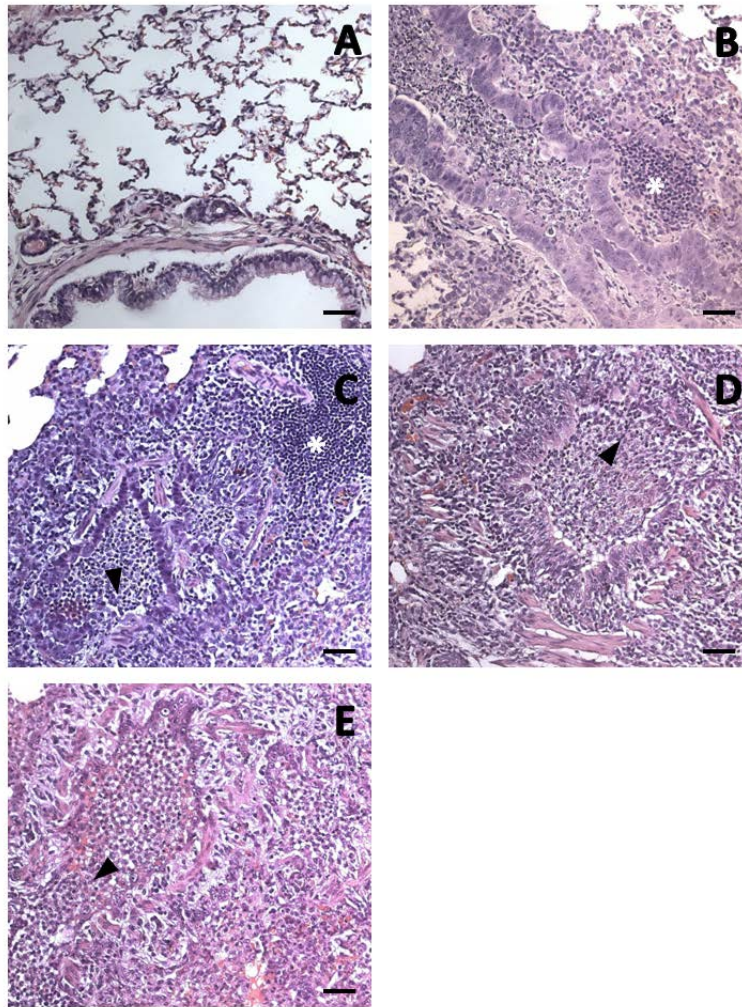plasia of bronchiolar epithelium and hyperplasia of the bronchus associated lymphoid tissue (BALT) (asterisks); C) Ferret inoculated with H1pdmN2sw, euthanized 5 dpi. Suppurative bronchiolitis, dysplasia and desquamation (arrowhead) of bronchiolar epithelium and BALT hyperplasia (asterisks); D) Ferret inoculated with H1N2, euthanized 5 dpi. Suppurative bronchiolitis and peribronchiolar infiltration of mononuclear cells. Dysplasia, necrosis and desquamation (arrowhead) of bronchiolar epithelium; E) Ferret inoculated with H1avN2hu, euthanized 5 dpi. Suppurative bronchiolitis, and dysplasia, necrosis and desquamation (arrowhead) of bronchiolar epithelium. Hematoxylin and eosin, scale bar 100 µm.
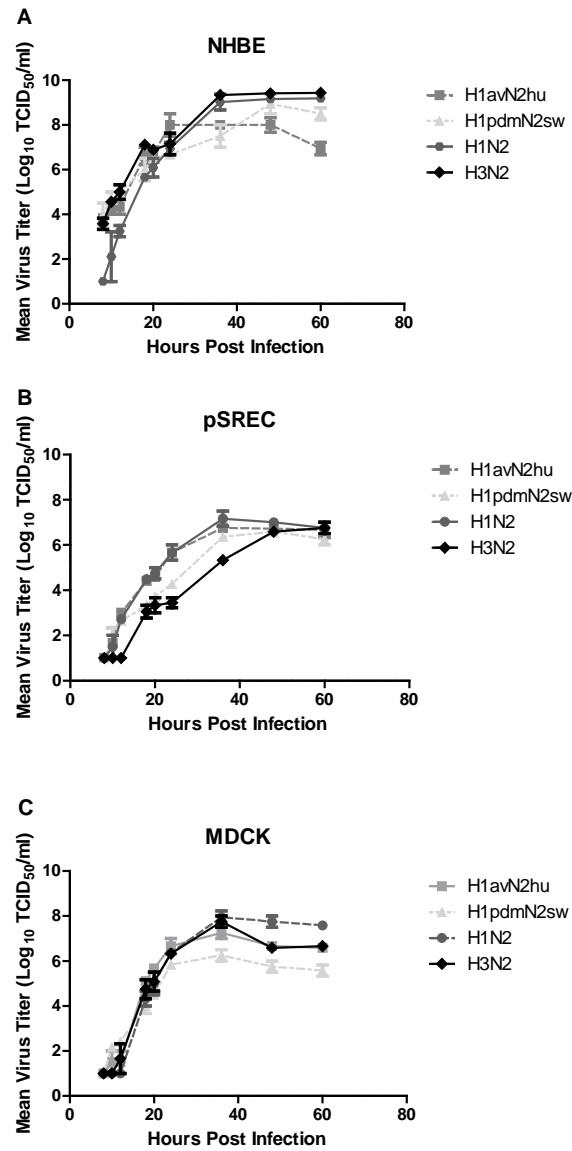
FIG 5 Replication kinetics of European swine influenza viruses in normal human bronchial epithelial (NHBE) cells, primary swine respiratory epithelial (pSREC) cells and MDCK cells. Growth curves were obtained by inoculating cells with an MOI of 0.01 PFU/cell with H1N2, H1avN2hu, H1pdmN2sw or H3N2, respectively. Supernatant was harvested and titrated in MDCK cells at 8, 10, 12, 18, 20, 24, 36, 48 and 60 hours post infection. Data are expressed as mean $\log_{10}$ $TCID_{50} \pm SEM$ from three independent experiments performed in duplicate.
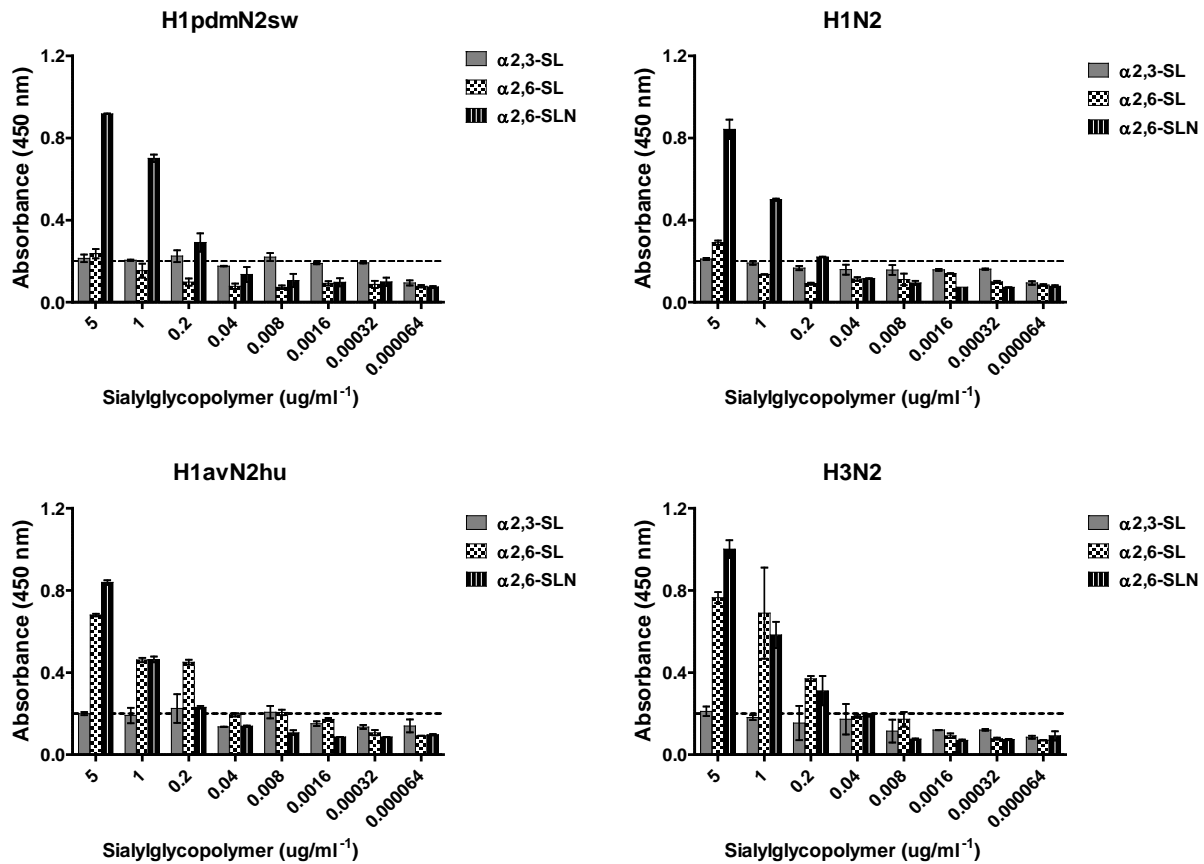
FIG 6 Receptor specificity of four European swine influenza viruses. analysis of receptor-binding specificity against sialyl glycans. The receptor-binding specificity of the four European swine influenza viruses H1N2, H1avN2hu, H1pdmN2sw and H3N2 were tested in a dose dependent glycan array assay against the sialyl glycans α2,6-SL and α2,6-SLN (representing the human/swine receptors) and α2,3SL (representing the avian receptor).

Table 1 Virus detection in nasal wash samples by real time RT-PCR

Virus/ group		Day post infection					
		1	3	5	7	9	11
H1avN2hu	Inoculated	++/++	+++/+++	+++/+++	++/+	+/+	+/+
	DC	n.a.	-/+	-/+++	++/+++	++/++	++/+
	AT	n.a.	-/-	-/-	-/-	-/-	-/-
H1pdmN2sw	Inoculated	+++/+++	+++/++	+++/+++	++/+	+/+	+/-
	DC	n.a.	+/+	+/+++	+/+++	+++/+++	++/+
	AT	n.a.	+/-	+/-	-/-	-/-	-/-
H1N2	Inoculated	++/++	+++/+++	+++/+++	++/++	++/+	+/+
	DC	n.a.	+/+	+++/+++	+++/*	++/++	+/+
	AT	n.a.	-/-	-/-	-/-	-/-	-/-
H3N2	Inoculated	+++/++	+++/+++	+++/+++	+/++	++/++	+/+
	DC	n.a.	++/+	+++/++	+++/+++	+++/+++	+++/+++
	AT	n.a.	-/-	+/-	+++/-	+++/-	+++/+++
Controls		-/-	-/-	-/-	-/-	-/-	-/-

+++: ct < 25, ++: ct = 25-30, +: ct < 30, (+): ct > 40, - :no ct, n.a.: Not applied, *: Not enough sample

Table 2 Sequence analysis of receptor binding residues and host specific amino acids in the four European swine influenza viruses

	Amino acid position													
	130 loop					190 helix				220 loop				
Subtype	133	137	138	155	158	190	193	194	200	222	225	226	227	228
H3N2	-	N	-	Y	G	E	N	L	-	-	G	L	-	S
H1N2	T	T	V	V	-	D	T	L	T	K	E/K	Q	A	G
H1avN2hu	T	T	I	V	-	S	A	L	T	K	E	Q	E	G
H1pdmN2sw	N	T	A	V	-	D	S	L	A	K	D	Q	E	G

TABLE 3 NA enzyme kinetics and antiviral susceptibility of European swine influenza viruses using MUNANA substrate

Virus	Vmax [mean (95% CI)]	Km (µM) [mean (95% CI)]	IC_{50} (nM) [mean (95% CI)]		
			Oseltamivir	Zanamivir	Peramivir
H1avN2hu	1364 (1257-1472)	376.5 (272.6-480.4)	0.10 (0.07-0.14)	0.43 (0.25-0.75)	0.14 (0.08-0.26)
H1pdmN2sw	1845 (1655-2036)	487 (320.5-653.6)	0.29 (0.19-0.44)	1.59 (0.85-2.98)	0.48 (0.30-0.77)
H1N2	1495 (1219-1772)	492 (191.7-792.4)	0.47 (0.31-0.72)	1.48 (0.86-2.56)	0.66 (0.43-1.01)
H3N2	842.4 (743.9-940.9)	281.1 (158.7-403.5)	0.08 (0.05-0.12)	0.14 (0.05-0.35)	0.10 (0.07-0.14)

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) of substrate conversion were fitted to the Michaelis-Menten kinetics by non-linear regression. CI, confidence interval

MANUSCRIPT III

Establishment of a reverse genetics system for functional analysis of swine influenza viruses

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(Work in progress)

INTRODUCTION

Influenza A virus (IAV) is an important pathogen affecting many different species. The natural reservoir of influenza A virus are waterfowls, from which 16 different hemagglutinins (HA) and 9 neuraminidases (NA), have been isolated (Fouchier *et al.*, 2005;Webster *et al.*, 1992). HA and NA are both surface glycoproteins and the subtypes of IAV is defined by these proteins. IAVs are able to circumvent host immunity either by subtle changes at antigenic sites in the surface glycoproteins, a process termed antigenic drift or by the exchange of segments between different subtypes thus creating new reassortants, a process termed antigenic shift (Schild *et al.*, 1974;Webster *et al.*, 1992).

Many different species, including humans and pigs, serves as hosts for IAV. Seasonal epidemics in the human population yearly claims between 250,000 and 500,000 live worldwide (http://www.who.int/mediacentre/factsheets/fs211/en/). During the last century, four different IAV subtypes have caused pandemics in the human population. In 1918, H1N1 caused "the Spanish flu" that was responsible for the death of more than 50 million people worldwide (Johnson and Mueller, 2002). In 1957, a pandemic was caused by an H2N2 subtype, followed by the pandemic in 1968 caused by H3N2 and the latest pandemic in 2009 caused by a novel H1N1 virus (Neumann and Kawaoka, 2011). This virus was termed H1N1pdm09 to distinguish it from the previous seasonal H1N1. At present, H3N2 and H1N1pdm09, which have largely replaced the seasonal H1N1, continue to co-circulate in the human population (Neumann and Kawaoka, 2011). Until 2009, subtypes found to circulate in the pig population included H3N2, H1N1 and H1N2, though a great variability exists regarding the gene constellation and origin, depending on the continent and region in which the different subtypes circulates (Lorusso *et al.*, 2012;Zell *et al.*, 2012;Zhu H *et al.*, 2012). In 2009, human-to-swine transmissions introduced the H1N1pdm09 in the pig population (Forgie *et al.*, 2011;Howden *et al.*, 2009), and was subsequently detected throughout the pig population worldwide (Moreno *et al.*, 2010;Pereda *et al.*, 2010;Song *et al.*, 2010;Sreta *et al.*, 2010;Welsh *et al.*, 2010). It appears that the introduction of the H1N1pdm09 to the pig population has increased the number of reassortants between this virus and endemic SIVs in the recent years (Kitikoon *et al.*, 2011;Moreno *et al.*, 2011;Nelson *et al.*, 2012;Pereda *et al.*, 2011;Starick *et al.*, 2011;Zhu *et al.*, 2011). Recent events in the US where a new reassortant of the US SIV H3N2 containing the matrix protein from the H1N1pdm09 and termed H3N2v, were found to be transmitted to humans on several occasions. Further, this reassortant virus

was found capable of human-to-human transmission in few cases (2011b;2011a;2012). These recent incidents indicates an important role of the pandemic matrix gene for effective transmission and further stress the importance of surveillance in pig populations as well as studies aimed at elucidating how the acquisition of genes derived from the H1N1pdm09 affects transmissibility, replication and viral fitness in human and pig *in vitro* and *in vivo* models.

Reverse genetics systems for IAV offer the ability to generate recombinant viruses, which then can be used for investigation of interactions between segments from different subtypes as well as replication and viral fitness. A reverse genetics system established by Stech et al, has the advantage of omitting the use of restriction enzymes and instead relies on the incorporation of the segment of interest to be by target-primed plasmid amplification (Stech *et al.*, 2008). This feature provides a fast and efficient way of cloning the eight segments needed for the generation of the desired IAV.

In this study, the reverse genetics system developed by Stech et al. (Stech *et al.*, 2008) was established with a Danish SIV of the H1N2 subtype used as backbone. The effects of exchanging the original M gene with an M gene originating from the H1N1pdm09 was sought elucidated in terms of assessing the infectious potential in cell types of both human and swine origin of the recombinant virus compared to the wild type virus.

METHODS

Cells and viruses

A/swine/Denmark/10845-1/2012(H1pdmN2sw) and A/swine/Denmark/101394-1/2011(H1N2) were isolated from samples submitted for diagnostic purposes from swine with a history of respiratory disease. Briefly, Madin-Darby canine kidney (MDCK) cells were grown to 60-70% confluency in MEM (Gibco, Carlsbad, CA, USA) containing 5% FBS, 2 mM L-glutamine, $1\times$ Non-essential amino acids (NEAA) and $1\times$ penicillin-streptomycin. Lung tissue was homogenized on TissueLyser (Qiagen, GmbH, Germany) in 1.5 mL $1\times$ MEM supplemented with $1\times$penicillin-streptomycin and sterile filtered. Cells were inoculated with 500 µL lung tissue homogenate for 30 min at 37°C, 5% CO_2. Following incubation, 10 mL MEM containing $1\times$penicillin-streptomycin, $1\times$NEAA, 2 mM L-glutamine and 2 µg/mL tosylsulfonyl-phenylalanyl-chloromethyl-ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. The cells were incubated for 72 hours and daily monitored for CPE. The supernatant was harvested and centrifuged at 2500 Rpm for 30 minutes to clarify cell debris and then stored at -80°C until further use.

RNA purification and real time RT-PCR screening

Viral RNA was purified from cultured viruses by RNeasy Mini Kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. Cell culture supernatant was prepared by mixing 200 µl sample with 400 µl RLT-buffer containing β–mercaptoethanol. Total RNA was eluted in 60 µl RNase-free water and stored at -80°C. The presence of influenza A virus was confirmed by real-time RT-PCR using an in-house modified assay for detection of the matrix gene (De Vleeschauwer *et al.*, 2009).

Full genome sequencing and RT-PCR

Nucleic acid amplification for sequencing of specific influenza A virus genes, were performed by one-step RT-PCR using primers modified from Hoffmann et al., (Hoffmann *et al.*, 2001) and the Superscript III One-Step RT-PCR kit with Platinum *Taq* High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions for NA was as follows: 30 min at 58°C, 2 min at 94°C, four cycles of 94°C for 30 sec, 54°C for 30 sec, 68°C for 180 sec, followed by 40 cycles of 94°C for 30 sec and 68°C for 210 sec and then 68°C for 10 min. PCR cycling conditions for M was as follows: 30 min at 50°C, 2

min at 94°C, 40 cycles of 94°C for 30 sec, 56°C for 30 sec and 68°C for 180 sec and then 68°C for 10 min.

For the amplification of nucleic acid for target-primed plasmid amplification, primers modified from Stech *et al.*, were used (Table1) and amplification was performed using the same RT-PCR kit as for the amplification of specific influenza A virus genes. PCR cycling conditions for PB1, PB2, PA, NP and HA were similar to those used for amplification of NA, whereas conditions for NS were similar to those used for amplification of M. Specific temperatures used for the reverse transcription and annealing steps are listed in table 1.

The PCR products were visualized by gel electrophoresis using E-Gel 0.8% agarose gels (Invitrogen, Carlsbad, CA, USA) and purified with High Pure PCR Product Purification Kit (Roche Diagnostics, GmbH, Germany). Purified PCR products of specific influenza A virus genes were send for single sample sequencing at LGC Genomics (GmbH, Germany).

Full genome sequencing was performed on the A/swine/Denmark/101394-1/2011(H1N2) cell culture propagated virus by full length amplification of all 8 gene segments with in-house designed primers (primers available upon request) using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity and PCR conditions similar to those described for NA and M full length amplification. Purified PCR products for all gene segments were pooled in equimolar quantity to a final amount of 1 µg and used for next generation sequencing (NGS) on the Ion Torrent PGM™ sequencer (Life Technologies, Carlsbad, CA, USA). NGS including library preparation was carried out at the Multi-Assay Core facility located at the Technical University of Denmark.

Sequences obtained by NGS were assembled using the features *de novo* and reference assembly of CLC Genomics Workbench 4.6.1 (CLC bio A/S, Århus, Denmark). Sequences obtained by Sanger sequencing was analyzed using CLC Main Workbench Version 6.9 (CLC bio A/S, Århus, Denmark).

Phylogenetic tree

Phylogenetic trees from aligned sequences were inferred using the Bayesian Markov-Chain Monte Carlo (MCMC) method integrated in Mr.Bayes 3.1 (Ronquist and Huelsenbeck, 2003). The MCMC was run for 2 million iterations under the GTR+I+G model, and using a 25% burn-in and a sampling frequency of 2000. The phylogenetic tree was visualized using FigTree, version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) and midpoint rooted for clarity.

Target-primed plasmid amplification and transformation

For the target primed plasmid amplification, 100 ng of pHWS*ccd*B plasmid (kindly provided by Dr. Jürgen Stech) and PCR product of varying concentration dependent of the gene as previously described (Stech *et al.*, 2008). For each gene, the plasmid and the gene specific PCR product was mixed with 2U Phusion High-Fidelity DNA Polymerase according to manufacturer's instructions (Thermo Fischer Scientific, Copenhagen, Denmark). PCR cycling conditions were as follows: 30 sec at 98°C, followed by 35 cycles of 98°C for 10 sec and 48°C for 60 sec and 72°C for 5.30 min. Following target-primed plasmid amplification, 5 or 10 µl of the reaction mixture was transformed into One-shot TOP10 competent cells (Life technologies, Carlsbad, CA, USA), according to manufacturer's instructions and streaked on LB plates containing 100 µg/mL ampicillin.

Verification of inserts

For PCR screening, four to five colonies were selected and streaked on fresh LB plates with ampicillin and were subsequently screened using Qiagen OneStep RT-PCR kit (Qiagen, GmbH, Germany). Primers targeting the area surrounding the insert in the plasmids were used for amplification (primers available upon request). For PCR, the following conditions were used: 30 sec at 98°C, followed by 35 cycles of 98°C for 10 sec and 55°C for 60 sec and 72°C for 5.30 min.

Plasmids containing inserts of correct size were purified using Pure-Link Quick Plasmid DNA Mini-prep kit (Life technologies, Carlsbad, CA, USA) according to manufacturer's instructions and send for single sample sequencing at LGC Genomics (GmbH, Germany). Sequences were verified using CLC Main Workbench Version 6.9 (CLC bio A/S, Århus, Denmark). Plasmids from correct clones were purified and stored at -80°C until further use.

RESULTS AND DISCUSSION

The work plan of this study was to amplify each segment from a Danish H1N2 SIV and a pandemic M segment, clone the obtained PCR products into plasmids and transform the plasmids into competent E. coli bacteria. Following selection and verification of correct clones, each of the cloned genes are tested by co-transfection with seven plasmids encoding the genes of the A/Puerto Rico/8/1934(H1N1) (PR8) strain into a co-culture of 293T and MDCK cells. After the test, all eight genes to be used for the final recombinant IAVs are transfected into a 293T/MDCK co-culture (Fig. 1). This will result in two recombinant IAVs; one virus will be wild type and contain the original M segment, whereas the second virus will contain the pandemic M segment. Finally, growth kinetics of the recombinant IAVs will be tested and compared *in vitro* in the human epithelial cell line A549, MDCK cells and a primary swine kidney cell culture. Furthermore, receptor binding preferences and NA kinetics of the recombinant IAVs will also be tested.

For the establishment of the reverse genetics backbone, it was chosen to use a strain of H1N2 subtype, since this subtype is one of the two most prevalent subtypes circulating in Danish pigs and also due to its high success in becoming an established subtype in Denmark (unpublished data), since its first detection in 2003 (Trebbien *et al.*, 2013). The specific strain name of the virus to be used as backbone was A/swine/Denmark/101394-1/2011(H1N2). Furthermore, HA and NA sequences of this subtype were analyzed and H1 was found not to possess any substitutions that distinguished this virus from the main part of the Danish swine H1N2s. The H1 of this virus was shown to be antigenically closely related to the remaining part of the Danish H1 viruses and phylogeny of the N2 gene showed that the virus belonged to clade 1 together with the main part of Danish N2 viruses (Fobian *et al.*, 2014).

Selection of the pandemic M gene was based on phylogeny (Fig. 2). Due to the previous cases of the North American H3N2 viruses containing the pandemic M segment, it was decided that the Danish M segment to be used for the recombinant virus, had to be as closely related to the North American pandemic M segment as possible, in order to more closely mimic the suggested role of this segment in transmission as well as its apparent impact on NA activity (Campbell *et al.*, 2014;Lakdawala *et al.*, 2011).

At present, five of the eight segments of the H1N2 backbone, as well as the pandemic M segment, have been successfully amplified and verified (Table 2). PB1, PA and HA have all been amplified but specific sequencing primers for verification of these segments remains to be designed.

In recent studies, the reverse genetics systems have been used to analyze various aspects related to virulence and host range restrictions. One study showed that mutations in a triple reassortant H1N2 virus that was acquired in HA and NA through a single passage in ferrets, substantially altered virulence and transmissibility of the virus (Pascua *et al.*, 2012). Another study showed that the introduction of the pandemic NA into a triple reassortant H1N2 increased airborne transmission in ferrets (Yen *et al.*, 2011).

Therefore the establishment of this reverse genetics system will be invaluable in the assessment of the zoonotic potential of new reassortants as well as in the analysis of host range restrictions in Danish SIVs.

ACKNOWLEDGEMENTS

REFERENCES

(2011b). Swine-origin influenza A (H3N2) virus infection in two children--Indiana and Pennsylvania, July-August 2011. MMWR Morb. Mortal. Wkly. Rep. *60*, 1213-1215.

(Li *et al.*, 2009). Evaluation of rapid influenza diagnostic tests for influenza A (H3N2)v virus and updated case count--United States, 2012. MMWR Morb. Mortal. Wkly. Rep. *61*, 619-621.

(2011a). Limited human-to-human transmission of novel influenza A (H3N2) virus--Iowa, November 2011. MMWR Morb. Mortal. Wkly. Rep. *60*, 1615-1617.

Campbell,P.J., Danzy,S., Kyriakis,C.S., Deymier,M.J., Lowen,A.C., and Steel,J. (Steain *et al.*, 2009). The M segment of the 2009 pandemic influenza virus confers increased neuraminidase activity, filamentous morphology, and efficient contact transmissibility to A/Puerto Rico/8/1934-based reassortant viruses. J. Virol. *88*, 3802-3814.

De Vleeschauwer,A., Atanasova,K., Van,B.S., van den,B.T., Rasmussen,T.B., Uttenthal,A., and Van,R.K. (Goodwin *et al.*, 2009). Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs. PLoS. One. *4*, e6662.

Fobian,K., Larsen,L.E., Lewis,A., Kirk,I.K., Hjulsager,C.K., and Breum,S.O. (Steain *et al.*, 2009). Genetic and antigenic characterization of influenza A virus isolated from Danish swine in the period 2003-2012. Ready for submission.

Forgie,S.E. *et al.* (Sebastian *et al.*, 2009). Swine outbreak of pandemic influenza A virus on a Canadian research farm supports human-to-swine transmission. Clin. Infect. Dis. *52*, 10-18.

Fouchier,R.A., Munster,V., Wallensten,A., Bestebroer,T.M., Herfst,S., Smith,D., Rimmelzwaan,G.F., Olsen,B., and Osterhaus,A.D. (Tashiro *et al.*, 2009). Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. *79*, 2814-2822.

Hoffmann,E., Stech,J., Guan,Y., Webster,R.G., and Perez,D.R. (Jiang *et al.*, 2010). Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. *146*, 2275-2289.

Howden,K.J. *et al.* (Goodwin *et al.*, 2009). An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can. Vet. J. *50*, 1153-1161.

Johnson,N.P. and Mueller,J. (Perez-Trallero *et al.*, 2009). Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull. Hist Med. *76*, 105-115.

Kitikoon,P., Sreta,D., Na Ayudhya,S.N., Wongphatcharachai,M., Lapkuntod,J., Prakairungnamthip,D., Bunpapong,N., Suradhat,S., Thanawongnuwech,R., and Amonsin,A. (Sebastian *et al.*, 2009). Brief report: Molecular characterization of a novel reassorted pandemic H1N1 2009 in Thai pigs. Virus Genes *43*, 1-5.

Lakdawala,S.S., Lamirande,E.W., Suguitan,A.L., Jr., Wang,W., Santos,C.P., Vogel,L., Matsuoka,Y., Lindsley,W.G., Jin,H., and Subbarao,K. (Sebastian *et al.*, 2009). Eurasian-origin gene segments contribute to the transmissibility, aerosol release, and morphology of the 2009 pandemic H1N1 influenza virus. PLoS. Pathog. *7*, e1002443.

Lorusso,A., Vincent,A.L., Gramer,M.R., Lager,K.M., and Ciacci-Zanella,J.R. (Li *et al.*, 2009). Contemporary Epidemiology of North American Lineage Triple Reassortant Influenza A Viruses in Pigs. Curr. Top. Microbiol. Immunol.

Moreno,A., Di,T.L., Alborali,L., Vaccari,G., Barbieri,I., Falcone,E., Sozzi,E., Puzelli,S., Ferri,G., and Cordioli,P. (Khanna *et al.*, 2009). First Pandemic H1N1 Outbreak from a Pig Farm in Italy. Open. Virol. J. *4*, 52-56.

Moreno,A. *et al.* (Sebastian *et al.*, 2009). Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. Vet. Microbiol. *149*, 472-477.

Nelson,M.I., Vincent,A.L., Kitikoon,P., Holmes,E.C., and Gramer,M.R. (Li *et al.*, 2009). Evolution of novel reassortant A/H3N2 influenza viruses in North American swine and humans, 2009-2011. J. Virol. *86*, 8872-8878.

Neumann,G. and Kawaoka,Y. (Sebastian *et al.*, 2009). The first influenza pandemic of the new millennium. Influenza. Other Respi. Viruses. *5*, 157-166.

Pascua,P.N. *et al.* (Li *et al.*, 2009). Virulence and transmissibility of H1N2 influenza virus in ferrets imply the continuing threat of triple-reassortant swine viruses. Proc. Natl. Acad. Sci. U. S. A *109*, 15900-15905.

Pereda,A. *et al.* (Khanna *et al.*, 2009). Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg. Infect. Dis. *16*, 304-307.

Pereda,A. *et al.* (Sebastian *et al.*, 2009). Evidence of reassortment of pandemic H1N1 influenza virus in swine in Argentina: are we facing the expansion of potential epicenters of influenza emergence? Influenza. Other Respi. Viruses. *5*, 409-412.

Ronquist,F. and Huelsenbeck,J.P. (Burrel *et al.*, 2009). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. *19*, 1572-1574.

Schild,G.C., Oxford,J.S., Dowdle,W.R., Coleman,M., Pereira,M.S., and Chakraverty,P. (Vallings, 2010). Antigenic variation in current influenza A viruses: evidence for a high frequency of antigenic 'drift' for the Hong Kong virus. Bull. World Health Organ *51*, 1-11.

Song,M.S. *et al.* (Khanna *et al.*, 2009). Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea. J. Clin. Microbiol. *48*, 3204-3211.

Sreta,D. *et al.* (Khanna *et al.*, 2009). Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. Emerg. Infect. Dis. *16*, 1587-1590.

Starick,E. *et al.* (Sebastian *et al.*, 2009). Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. J. Gen. Virol. *92*, 1184-1188.

Stech,J., Stech,O., Herwig,A., Altmeppen,H., Hundt,J., Gohrbandt,S., Kreibich,A., Weber,S., Klenk,H.D., and Mettenleiter,T.C. (Khiabanian *et al.*, 2009). Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification. Nucleic Acids Res. *36*, e139.

Trebbien,R., Bragstad,K., Larsen,L.E., Nielsen,J., Botner,A., Heegaard,P.M., Fomsgaard,A., Viuff,B., and Hjulsager,C.K. (Zijtregtop *et al.*, 2009). Genetic and biological characterisation of an avian-like H1N2 swine influenza virus generated by reassortment of circulating avian-like H1N1 and H3N2 subtypes in Denmark. Virol. J. *10*, 290.

Webster,R.G., Bean,W.J., Gorman,O.T., Chambers,T.M., and Kawaoka,Y. (Steel *et al.*, 2010). Evolution and ecology of influenza A viruses. Microbiol. Rev. *56*, 152-179.

Welsh,M.D. *et al.* (Khanna *et al.*, 2009). Initial incursion of pandemic (H1N1) 2009 influenza A virus into European pigs. Vet. Rec. *166*, 642-645.

Yen,H.L. *et al.* (Sebastian *et al.*, 2009). Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proc. Natl. Acad. Sci. U. S. A *108*, 14264-14269.

Zell,R., Scholtissek,C., and Ludwig,S. (Li *et al.*, 2009). Genetics, Evolution, and the Zoonotic Capacity of European Swine Influenza Viruses. Curr. Top. Microbiol. Immunol.

Zhu H, Webby,R., Lam,T.Y., Smith,D.K., Peiris,J.M., and Guan,Y. (Li *et al.*, 2009). History of Swine Influenza Viruses in Asia. In: Swine Influenza, ed. J.Richt and R.Webby Springer, 57-68.

Zhu,H. *et al.* (Sebastian *et al.*, 2009). Novel reassortment of Eurasian avian-like and pandemic/2009 influenza viruses in swine: infectious potential for humans. J. Virol. *85*, 10432-10439.
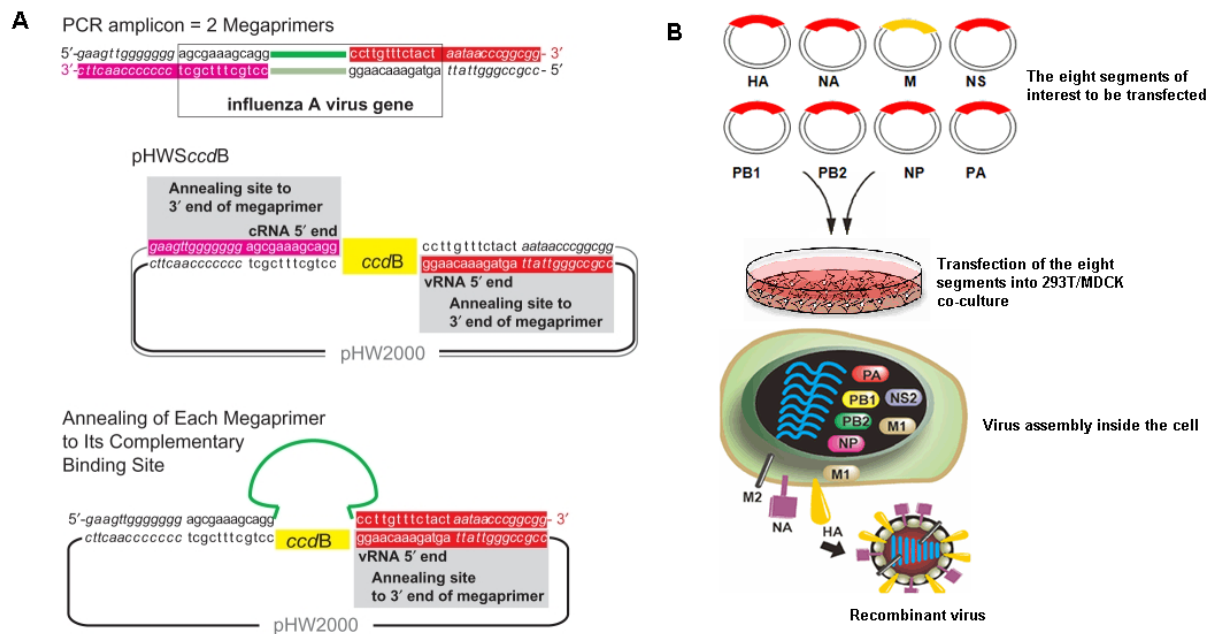
FIG 1 A schematic representation of the principles of reverse genetics. (A) Target primed amplification of the PCR amplicons and the pHWS*ccd*B plasmid where the strands of the PCR amplicon serve as megaprimers. Upon amplification the *ccd*B marker is replaced by the viral gene (Stech *et al.*, 2008). (B) The clones containing the eight different segments are transfected into a co-culture of 293T and MDCK cells and recombinant virus can accordingly be rescued. Modified from (http://www.aht.org.uk/cms-display/science_eqflu.html and http://www.nature.com/scitable/content/cells-growing-in-a-tissue-culture-14264811)
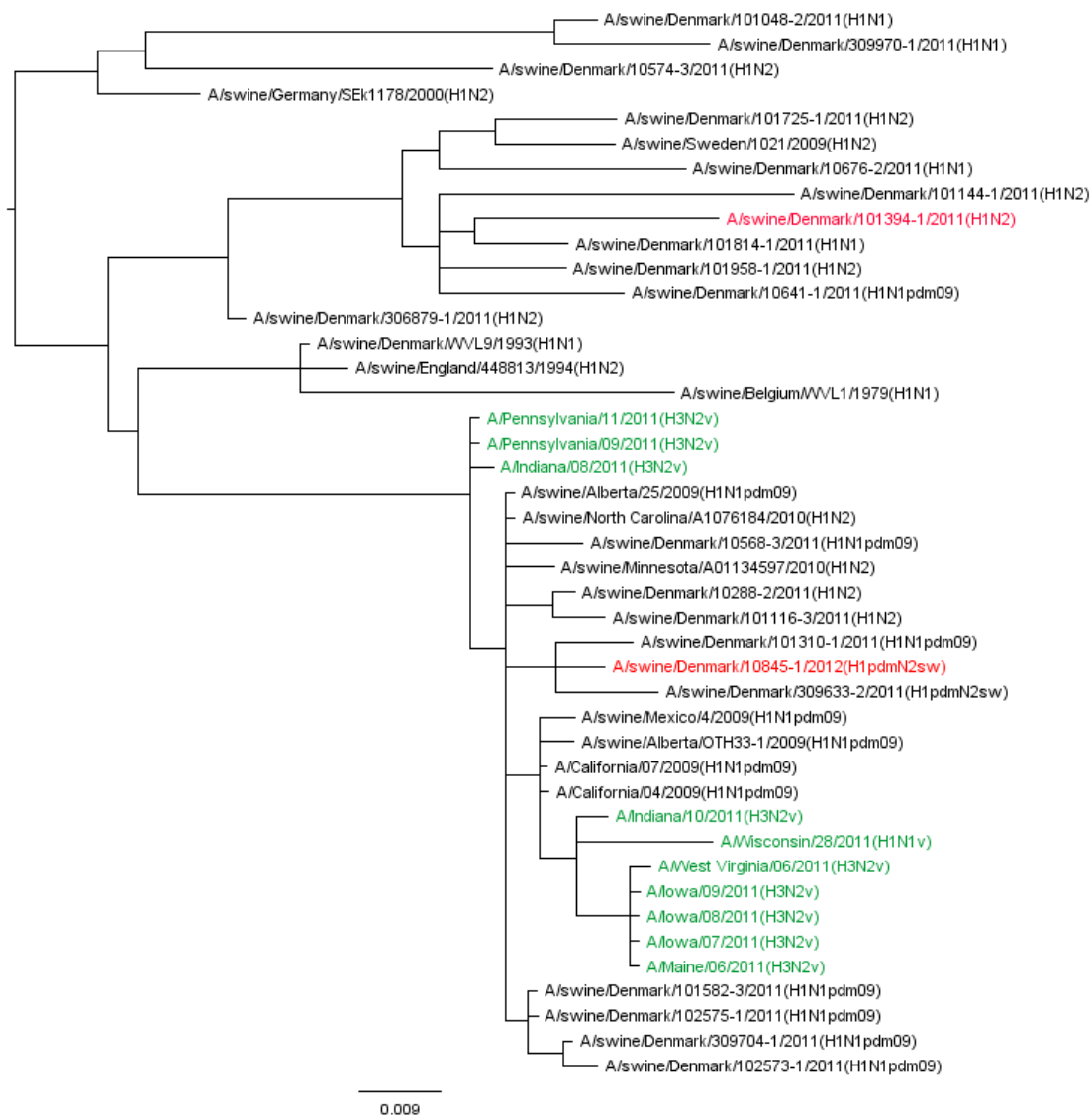
FIG 2 Phylogenetic tree of M sequences (position 29-920) was inferred using the Bayesian MCMC method integrated in Mr.Bayes 3.1 under the GTR+I+G model by running 2 million MCMCs with 25% burn-in and a sampling frequency of 2000. Scale bar indicates number of nucleotide substitutions per site. Tree was midpoint rooted for clarity. Variant influenza A viruses isolated from humans are colored green and the two swine influenza viruses used in this study are colored red.

Table 1 Primer sets used for the amplification of Danish swine influenza A virus genes

Gene	Forward primer	Reverse primer	RT tempe- rature/ °C	Annealing tempe- rature/ °C
PB2	**PHW-M-PB2-F2*:** *gaagttggggggg* agc**R**aaagcagg**TCAAATATATTC**	**PHW-PB2-2341r:** *ccgccgggttatt* agtagaaacaagg**TCGTTT**	55	52
PB1	**PHW-M-PB1-F2*:** *gaagttggggggg* agc**R**aaagcagg**CAAAC**	**PHW-PB1-2341r:** *ccgccgggttatt* agtagaaacaagg**CATTT**	52	52
PA	**PHW-M-PA-F2*:** *gaagttggggggg* agc**R**aaagcagg**TACTG**	**PHW-PA-2233r:** *ccgccgggttatt* agtagaaacaagg**TACTT**	52	52
HA	**PHW-HAf:** *gaagttggggggg* agcgaaagcagg**GG**	**PHW-M-HAr*:** *ccgccgggttatt* agtagaaacaagg**GTGTTTT**	55	55
NP	**PHW-M-NP-F2*:** *gaagttggggggg* agc**A**aaagcagg**GTAGATAATC**	**PHW-NPr:** *ccgccgggttatt* agtagaaacaagg**GTATTTTT**	58	54
NA	**PHW-N12458f:** *gaagttggggggg* agcgaaagcagg**AGT**	**PHW-M-N12458r*:** *ccgccgggttatt* agtagaaacaagg**AGTTTTTT**	54	58
M	**PHW-2-Mf*:** *gaagttggggggg* agcgaaagcagg**TAGATATTG**	**PHW-2-Mr*:** *ccgccgggttatt* agtagaaacaagg**TAGTTTTTTACTC**	50	56
NS	**PHW-M-H3NSF1*:** *gaagttggggggg* agc**A**aaagcagg**GTGACAAAGACA**	**PHW-H3NSR1*:** *ccgccgggttatt* agtagaaacaagg**GTGTTTTTTAT**	58	55

*Primers modified from (Stech *et al.*, 2008). The 5' ends of the primers are derived from the pHW2000 regions (italic), followed by influenza virus termini sequences (lower cases) and gene-specific nucleotides at the 3' end (uppercase bold). Nucleotides highlighted as uppercase bold in the influenza virus termini sequence, are nucleotides found to be prominent among Danish SIV sequences

Table 2 Amplified and/or verified plasmids for the establishment of a reverse genetics system

Segment	Strain	Amplified	Verified
PB1	A/swine/Denmark/101394-1/2011(H1N2)	x	-
PB2	A/swine/Denmark/101394-1/2011(H1N2)	x	x
PA	A/swine/Denmark/101394-1/2011(H1N2)	x	-
NP	A/swine/Denmark/101394-1/2011(H1N2)	x	x
HA	A/swine/Denmark/101394-1/2011(H1N2)	x	-
NA	A/swine/Denmark/101394-1/2011(H1N2)	x	x
M	A/swine/Denmark/101394-1/2011(H1N2)/	x	x
	A/swine/Denmark/10845-1/2012(H1pdmN2sw)	x	x
NS	A/swine/Denmark/101394-1/2011(H1N2)	x	x

DISCUSSION, CONCLUSIONS AND PERSPECTIVES

Pigs are one of the main hosts of IAV and have been considered mixing vessels of new reassortant viruses, since they can be infected with both human and avian IAVs (de Graaf and Fouchier, 2014;Scholtissek *et al.*, 1985). With the emergence of H1N1pdm09 as the first pandemic in this century containing both Eurasian avian-like and North American segments of swine-origin (Garten *et al.*, 2009;Smith *et al.*, 2009b), followed a renewed interest in the pigs as potential mixing vessels of new reassortant viruses with zoonotic potential and at the same time also highlighted the importance surveillance of IAV in swine. However, the impact of SIV infection on animal health is also an important aspect as well as the economic impact of SIV to the industry.

The main aim of this PhD study was to analyze the influenza ecology and evolution of the two most prevalent viruses having circulated among Danish pigs during the previous ten years and at the same time investigate the zoonotic potential of both enzootic viruses as well as newly emerged reassortants in the ferret model. Furthermore, in order to be able to investigate factors essential for replication as well as for determination of host range restrictions, a reverse genetics system was sought established for future detailed studies of these viruses.

Since 2011, a systematic passive surveillance of SIVs has been conducted in Denmark, which has given a more detailed insight in the different subtypes circulating in Danish pigs and has resulted in the detection of new reassortant SIVs. However, a thorough study of the evolution of Danish SIVs over a longer period of time has not previously been performed.

In my studies, Bayesian phylogeny was used to infer the genetic relationship between sequences of HA and NA sampled from a period of ten years. Traditionally ML analysis has been widely used for the estimation of genetic relationships among sequences, but in the recent years, the use of BI for these estimations have become more widely accepted (Holder and Lewis, 2003;Smith *et al.*, 2012). One of the advantages of using BI over ML, is that BI allows for the incorporation of prior information, which means that results obtained from a previous model can be used to inform the current model and hence improve the basis for the estimation, as well as the reliability of the outcome. In contrast to this, the more traditional ML method assumes that all priors are uniform and is thus a more rigid model

compared to BI (Holder and Lewis, 2003). The phylogenetic trees of the Danish H1 viruses inferred by this Bayesian approach showed no chronological drift as well as no specific clustering of the H1's in terms of their subtypes and the overall structure of the phylogenetic tree appeared to be random, suggesting that the drift of Danish H1 SIVs was not strongly driven by host immunity. This is in agreement with studies performed on H1 and H3 subtypes in humans (Bhatt *et al.*, 2011;Rambaut *et al.*, 2008;Wolf *et al.*, 2006). The immune pressure is believed to lead to the ladder-like phylogeny observed for H3N2, in which older strains are gradually replaced by newer ones (Smith *et al.*, 2004). In comparison, the human H1N1 does not show this ladder-like phylogeny, probably due to the weaker pressure exerted by the host immune system (Wolf *et al.*, 2006).

Further, as part of the evolution study, we estimated the tMRCA of the avian-like H1, but surprisingly our results did not correlate with previous estimates (Dunham *et al.*, 2009;Lycett *et al.*, 2012;Suzuki and Nei, 2002;Xu *et al.*, 2011), nor the time of the first detection of this virus in swine (Pensaert *et al.*, 1981). If our estimate is correct, the avian-like H1 must have circulated unnoticed for almost seventy years in either birds and/or pigs. Thus, one plausible hypothesis is that the virus emerged in birds around 1917 where it circulated and drifted for decades, before different viruses were introduced into the swine host which possibly could have been through several independent introductions. An alternate explanation is that the virus emerged in birds around 1917 and was shortly after introduced to swine, followed by decades of silent circulation, before the drifted viruses gained increased virulence and became swine pathogens. A combination of these two explanations is also possible. Indeed, it has previously been found that IAV subtypes could potentially have circulated undetected for a period of time before the initial detection, as seen with the wholly human H3N2 virus detected in swine in US (Nelson *et al.*, 2014), and the H1N1pdm09 (Smith *et al.*, 2009b). Until the pandemic in 2009, SIV surveillance was scarce or completely lacking and has previously also been based on serological evidence. Therefore an undetected circulation of the avian-like H1N1 in Danish and/or European pigs is not unlikely, especially if the virus was only circulating at low levels.

Considering the incongruence of the result obtained in this study and those obtained previously, several factors must be considered when estimating tMRCA, since this estimate depends on the method used, the choice of nucleotide substitution model, the amount and age of sequences used for the estimation, as well as the length of sequences (e.g. HA1 versus full length HA), just to mention some of the factors.

In my study a coalescent tree prior assuming a constant population size back through time was used. This prior was chosen since it has been found to be most suitable for trees describing the relationship among individuals in the same population or species (Kingman, 2000). The coalescent tree prior was also used in a recent study, but with the application of a different nucleotide substitution model (Lycett *et al.*, 2012) and their tMRCA also differed significantly from the one obtained in this study. Other studies (Dunham *et al.*, 2009;Xu *et al.*, 2011) have used the Bayesian skyline coalescent prior due to the ability of this model to describe the fluctuating population dynamics that are characteristic of IAV (Rambaut *et al.*, 2008) and also obtained tMRCAs differing significantly from the one in this study.

In conclusion, the means of estimating tMRCA for whole viruses or just selected genes can be undertaken by several different methods. Therefore, it is necessary to carefully select the models to be used based on the information available. A critical evaluation and perhaps reassessment of the obtained results by the use of different models is also extremely important and it will definitely be worthwhile to further analyze the obtained result from the Danish H1 SIV.

Since the 1980's, the pig production has increased dramatically which has led to more consistent infection chains and thereby also an increased spread of SIVs. With the introduction of the H1N1pdm09 to pigs, the number of new reassortants has increased (Breum *et al.*, 2013;Moreno *et al.*, 2011;Pascua *et al.*, 2013;Starick *et al.*, 2011;Starick *et al.*, 2012;Tremblay *et al.*, 2011). This increase could also be considered an artefact following enhanced surveillance as well as the development of more efficient tools for detection of IAVs in general, but it appears that especially segments from H1N1pdm09 is favored during reassortment (Stincarelli *et al.*, 2013). During the previous three years the Danish passive surveillance has detected several new reassortants and many of these are containing pandemic segments. Most new reassortants seem to be transient viruses, but few appear to have become established. My experiment in which the zoonotic potential of two reassortant and two enzootic SIVs were assessed showed interestingly that the H3N2 virus was efficiently transmitted between ferrets via aerosols. This finding further underlined the need to assess the zoonotic potential of both enzootic and reassortant viruses and in the particular case of the H3N2 virus, the need for a further characterization of this virus was also highlighted. No evolutionary studies regarding genetic and antigenic evolution of this particular virus in Denmark has been conducted and it would be interesting to analyze the evolutionary rate and the phylogeny of this virus as well to see if the phylogeny adapts the same ladder

like tree structure as the one observed for human H3N2 viruses. In terms of the ability of this virus to transmit via the airborne route, an antigenic characterization would be interesting in order to define the antigenic relatedness to currently circulating human H3N2 strains which could further be used to assess the zoonotic potential of this virus. A recent study showed that the difference between human H3N2 and North American swine H3N2 was profound enough that new introduction of human IAVs into pigs or vice versa would not be prevented by population immunity (Lewis *et al.*, 2014).

The risk of a zoonotic event caused by the swine H3N2 virus is most likely not impending. In the period from 2009 and until 2012, the swine H3N2 virus has not been detected in the Danish passive surveillance. In 2013 the swine H3N2 was detected in a single submission (unpublished data), but due to lack of genetic characterization of this subtype it is not certain if the virus is a new introduction or whether the swine H3N2 is circulating at very low levels in pigs. The low detection of the H3N2 in Danish pigs is not uncommon and several European countries report a low circulation of this subtype compared to the H1N1 and H1N2 subtypes (Harder *et al.*, 2013)(http://www.esnip3.eu/).

During my assessment of the zoonotic potential of the new reassortants, it was also observed that the efficiency of transmission among the ferrets seemed to correlate with the human-origin of the surface glycoproteins. Hence, the H3N2 virus having both surface glycoproteins of human-origin also was shown to be the most efficient virus for transmission between ferrets. It was also found that the H1pdmN2sw carrying a human-like HA was more efficiently transmitted compared to the H1avN2hu carrying the human-like NA. In light of a recent study, where the introduction of human IAVs were suggested to occur more frequently than previously assumed (Nelson *et al.*, 2014), these findings are highly important and vaccination of farmers is highly recommended for the limitation of this problem/interface. However, in pigs this problem would unfortunately not be limited significantly by vaccination, since vaccinated pigs can still be infected and shed virus (Romagosa *et al.*, 2011).

The human IAV vaccine strains are updated each year depending on the strains thought to be most prevalent in the forthcoming season. Since the introduction of the H1N1pdm09, this strain has largely (Neumann and Kawaoka, 2011)(if not completely), replaced the former seasonal H1N1 and has now also replaced the seasonal H1N1 in the current vaccines (http://www.cdc.gov/flu/about/season/vaccine-selection.htm). In comparison, SIV vaccines in Europe have only been updated once since the first vaccine (Gripovac VET) was licensed in the mid-1980s which is due to the requirement for very

laborious and stringent efficacy tests for the included strains that has to be performed before a new vaccine can be licensed (Van Reeth and Ma, 2012). The new Gripovac 3 vaccine now contains contemporary SIV strains with the H1N1 strain now with a H1 of avian origin, compared to the previous H1N1 that was a human strain and in addition to the H3N2 strain also contains a H1N2 strain. The inclusion of the H1N2 strain is of minor significance in a Danish perspective, since the H1 in this strain is of human origin and has not been detected in Denmark (Trebbien *et al.*, 2013). In contrast, and based on results from my evolution study, it is expected that the avian-like H1N1would provide enough cross-protection against the Danish avian-like H1N2 based on their high antigenic similarity. A problem that can be considered significant though, in light of the evolution of SIVs in recent years, is that it has not been considered necessary to include H1N1pdm09 in SIV vaccines (Van Reeth and Ma, 2012) and in Denmark, vaccination against this subtype is allowed only after applying for dispensation and only if the herd has already been tested positive for H1N1pdm09 (unpublished data).

Denmark is a country with a large export of live pigs to other countries, which includes the export of live pigs to Asia (Takemae *et al.*, 2011;Zhu H *et al.*, 2012) to be used for breeding. For an example, the extraordinary high population density in Hong Kong, combined with an expanding pig industry to which live pigs are imported from both US and Europe that also has live bird markets are all together creating the perfect opportunity for new reassortments to occur and thereby also the potential establishment of new pandemics. It is therefore highly important to continue and expand the surveillance of IAVs in Danish pigs in order to detect new reassortants as well as to monitor the evolution of enzootic viruses in regard to take appropriate precautions as well as developing new and more effective vaccines.

In conclusion, the results of these studies clearly showed that the overall ecology of IAVs is very complex and many aspects still remain to be elucidated. After the emergence of the H1N1pdm09, pigs have regained attention as possible sources of future pandemics and reverse zoonotic events have given rise to an increased number of reassortants and by that also an increased risk of new pandemics. Hence, gaining further insight into this important pathogen requires the consideration of several factors. It is therefore considered highly important that future studies of SIVs are focusing on the determinants of virulence and host range selection which can be analyzed using the reverse genetics system which was partly developed in this PhD project. The use of this system combined with the continued surveillance

strategy revealing new reassortants can then be used for assessment of zoonotic potential by analyzing growth kinetics in human cell lines as well as receptor binding preferences.

Elucidation of factors involved in host range selection and virulence may in the long run lead to the development of new and more efficient vaccines as well as the potential development of new antivirals – both factors that are beneficial not just to the general pig health but also seen from a human health aspect.

REFERENCES

WHO fact sheet, Influenza (seasonal). WHO . 2014. 21-7-2014.
   Ref Type: Electronic Citation
 (2012). Evaluation of rapid influenza diagnostic tests for influenza A (H3N2)v virus and updated case count--United States. MMWR Morb. Mortal. Wkly. Rep. *61*, 619-621.
 (2011). Swine-origin influenza A (H3N2) virus infection in two children--Indiana and Pennsylvania, July-August 2011. MMWR Morb. Mortal. Wkly. Rep. *60*, 1213-1215.
Abusugra,I.A., Linne,T., and Klingeborn,B. (1987). The provenance of the Swedish swine influenza H1N1 virus of 1983. Zentralbl. Veterinarmed. B *34*, 566-572.
Aebi,M., Bernasconi,R., Clerc,S., and Molinari,M. (2010). N-glycan structures: recognition and processing in the ER. Trends Biochem. Sci. *35*, 74-82.
Air,G.M. (2012). Influenza neuraminidase. Influenza. Other Respir. Viruses. *6*, 245-256.
Air,G.M. (1981). Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. Proc. Natl. Acad. Sci. U. S. A *78*, 7639-7643.
Alexander,D.J. (2000). A review of avian influenza in different bird species. Vet. Microbiol. *74*, 3-13.
Baigent,S.J. and McCauley,J.W. (2003). Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays *25*, 657-671.
Balint,A. *et al.* (2009). The first Swedish H1N2 swine influenza virus isolate represents an uncommon reassortant. Virol. J. *6*, 180.
Bantar,C. *et al.* (2009). Severe acute respiratory disease in the setting of an epidemic of swine-origin type A H1N1 influenza at a reference hospital in Entre Rios, Argentina. Clin. Infect. Dis. *49*, 1458-1460.
Barnard,D.L. (2009). Animal models for the study of influenza pathogenesis and therapy. Antiviral Res. *82*, A110-A122.
Bateman,A.C., Karamanska,R., Busch,M.G., Dell,A., Olsen,C.W., and Haslam,S.M. (2010). Glycan analysis and influenza A virus infection of primary swine respiratory epithelial cells: the importance of NeuAc{alpha}2-6 glycans. J. Biol. Chem. *285*, 34016-34026.
Baum,L.G. and Paulson,J.C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem. Suppl *40*, 35-38.
Belser,J.A., Katz,J.M., and Tumpey,T.M. (2011). The ferret as a model organism to study influenza A virus infection. Dis. Model. Mech. *4*, 575-579.
Bhatt,S., Holmes,E.C., and Pybus,O.G. (2011). The genomic rate of molecular adaptation of the human influenza A virus. Mol. Biol. Evol. *28*, 2443-2451.
Blake,T.A., Williams,T.L., Pirkle,J.L., and Barr,J.R. (2009). Targeted N-linked glycosylation analysis of H5N1 influenza hemagglutinin by selective sample preparation and liquid chromatography/tandem mass spectrometry. Anal. Chem. *81*, 3109-3118.
Blok,J. and Air,G.M. (1982). Variation in the membrane-insertion and "stalk" sequences in eight subtypes of influenza type A virus neuraminidase. Biochemistry *21*, 4001-4007.
Bochev,I. (2007). PORCINE RESPIRATORY DISEASE COMPLEX (PRDC):
   A REVIEW. I. ETIOLOGY, EPIDEMIOLOGY, CLINICAL
   FORMS AND PATHOANATOMICAL FEATURES. Bulg. J. Vet. Med *10*,
   131-146.
Bøtner,A. (1994). Svineinfluenza. Veterinær Information *6*, 24-27.

References

Bouvier,N.M. and Palese,P. (2008). The biology of influenza viruses. Vaccine *26 Suppl 4*, D49-D53.

Breum,S.O., Hjulsager,C.K., Trebbien,R., and Larsen,L.E. (2013). Influenza a virus with a human-like n2 gene is circulating in pigs. Genome Announc. *1*.

Brown,E.G. (2000a). Influenza virus genetics. Biomed. Pharmacother. *54*, 196-209.

Brown,I.H. (2000b). The epidemiology and evolution of influenza viruses in pigs. Vet. Microbiol. *74*, 29-46.

Brown,I.H., Done,S.H., Spencer,Y.I., Cooley,W.A., Harris,P.A., and Alexander,D.J. (1993). Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet. Rec. *132*, 598-602.

Brown,I.H., Harris,P.A., McCauley,J.W., and Alexander,D.J. (1998). Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J. Gen. Virol. *79 (Pt 12)*, 2947-2955.

Burrel,S., Roncin,L., Lafon,M.E., and Fleury,H. (2009). Oseltamivir susceptibility in south-western France during the 2007-8 and 2008-9 influenza epidemics and the ongoing influenza pandemic 2009. Euro. Surveill *14*.

Cai,Z., Zhang,T., and Wan,X.F. (2011). Concepts and applications for influenza antigenic cartography. Influenza. Other Respir. Viruses. *5 Suppl 1*, 204-207.

Campitelli,L., Donatelli,I., Foni,E., Castrucci,M.R., Fabiani,C., Kawaoka,Y., Krauss,S., and Webster,R.G. (1997). Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology *232*, 310-318.

Castro,J.M., del,P.M., and Simarro,I. (1988). Identification of H3N2 influenza virus isolated from pigs with respiratory problems in Spain. Vet. Rec. *122*, 418-419.

Castrucci,M.R., Donatelli,I., Sidoli,L., Barigazzi,G., Kawaoka,Y., and Webster,R.G. (1993). Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology *193*, 503-506.

Castrucci,M.R. and Kawaoka,Y. (1993). Biologic importance of neuraminidase stalk length in influenza A virus. J. Virol. *67*, 759-764.

Cate,T.R. (1987). Clinical manifestations and consequences of influenza. Am. J. Med. *82*, 15-19.

Caton,A.J., Brownlee,G.G., Yewdell,J.W., and Gerhard,W. (1982). The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell *31*, 417-427.

Chen,S.C. and Liao,C.M. (2010). Probabilistic indoor transmission modeling for influenza (sub)type viruses. J. Infect. *60*, 26-35.

Chou,H.H., Takematsu,H., Diaz,S., Iber,J., Nickerson,E., Wright,K.L., Muchmore,E.A., Nelson,D.L., Warren,S.T., and Varki,A. (1998). A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. Proc. Natl. Acad. Sci. U. S. A *95*, 11751-11756.

Colman,P.M., Varghese,J.N., and Laver,W.G. (1983). Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature *303*, 41-44.

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

Dawood,F.S., Jain,S., Finelli,L., Shaw,M.W., Lindstrom,S., Garten,R.J., Gubareva,L.V., Xu,X., Bridges,C.B., and Uyeki,T.M. (2009). Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. *360*, 2605-2615.

de Graaf,M. and Fouchier,R.A. (2014). Role of receptor binding specificity in influenza A virus transmission and pathogenesis. EMBO J. *33*, 823-841.

Deom,C.M. and Schulze,I.T. (1985). Oligosaccharide composition of an influenza virus hemagglutinin with host-determined binding properties. J. Biol. Chem. *260*, 14771-14774.

Duan,S., Boltz,D.A., Seiler,P., Li,J., Bragstad,K., Nielsen,L.P., Webby,R.J., Webster,R.G., and Govorkova,E.A. (2010). Oseltamivir-resistant pandemic H1N1/2009 influenza virus possesses lower transmissibility and fitness in ferrets. PLoS. Pathog. *6*, e1001022.

Dunham,E.J., Dugan,V.G., Kaser,E.K., Perkins,S.E., Brown,I.H., Holmes,E.C., and Taubenberger,J.K. (2009). Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses. J. Virol. *83*, 5485-5494.

Durrwald,R., Krumbholz,A., Baumgarte,S., Schlegel,M., Vahlenkamp,T.W., Selbitz,H.J., Wutzler,P., and Zell,R. (2010). Swine influenza A vaccines, pandemic (H1N1) 2009 virus, and cross-reactivity. Emerg. Infect. Dis. *16*, 1029-1030.

Felsenstein,J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. *17*, 368-376.

Fernandez-Sesma,A., Marukian,S., Ebersole,B.J., Kaminski,D., Park,M.S., Yuen,T., Sealfon,S.C., Garcia-Sastre,A., and Moran,T.M. (2006). Influenza virus evades innate and adaptive immunity via the NS1 protein. J. Virol. *80*, 6295-6304.

Fodor,E., Devenish,L., Engelhardt,O.G., Palese,P., Brownlee,G.G., and Garcia-Sastre,A. (1999). Rescue of influenza A virus from recombinant DNA. J. Virol. *73*, 9679-9682.

Fouchier,R.A., Munster,V., Wallensten,A., Bestebroer,T.M., Herfst,S., Smith,D., Rimmelzwaan,G.F., Olsen,B., and Osterhaus,A.D. (2005). Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. *79*, 2814-2822.

Franca,M., Stallknecht,D.E., and Howerth,E.W. (2013). Expression and distribution of sialic acid influenza virus receptors in wild birds. Avian Pathol. *42*, 60-71.

Fujiyoshi,Y., Kume,N.P., Sakata,K., and Sato,S.B. (1994). Fine structure of influenza A virus observed by electron cryo-microscopy. EMBO J. *13*, 318-326.

Gabriel,G., Dauber,B., Wolff,T., Planz,O., Klenk,H.D., and Stech,J. (2005). The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. U. S. A *102*, 18590-18595.

Gambaryan,A.S., Marinina,V.P., Tuzikov,A.B., Bovin,N.V., Rudneva,I.A., Sinitsyn,B.V., Shilov,A.A., and Matrosovich,M.N. (1998). Effects of host-dependent glycosylation of hemagglutinin on receptor-binding properties on H1N1 human influenza A virus grown in MDCK cells and in embryonated eggs. Virology *247*, 170-177.

Garten,R.J. *et al.* (2009). Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science *325*, 197-201.

Gaydos,J.C., Hodder,R.A., Top,F.H., Jr., Soden,V.J., Allen,R.G., Bartley,J.D., Zabkar,J.H., Nowosiwsky,T., and Russell,P.K. (1977). Swine influenza A at Fort Dix, New Jersey (January-February 1976). I. Case finding and clinical study of cases. J. Infect. Dis. *136 Suppl*, S356-S362.

Ge,H., Wang,Y.F., Xu,J., Gu,Q., Liu,H.B., Xiao,P.G., Zhou,J., Liu,Y., Yang,Z., and Su,H. (2010). Anti-influenza agents from Traditional Chinese Medicine. Nat. Prod. Rep. *27*, 1758-1780.

Gerhard,W., Yewdell,J., Frankel,M.E., and Webster,R. (1981). Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature *290*, 713-717.

Gibbs,J.S., Malide,D., Hornung,F., Bennink,J.R., and Yewdell,J.W. (2003). The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. J. Virol. *77*, 7214-7224.

References

Goodwin,R., Haque,S., Neto,F., and Myers,L.B. (2009). Initial psychological responses to Influenza A, H1N1 ("Swine flu"). BMC. Infect. Dis. *9*, 166.

Gourreau,J.M., Kaiser,C., Valette,M., Douglas,A.R., Labie,J., and Aymard,M. (1994). Isolation of two H1N2 influenza viruses from swine in France. Arch. Virol. *135*, 365-382.

Gramer,M.R., Lee,J.H., Choi,Y.K., Goyal,S.M., and Joo,H.S. (2007). Serologic and genetic characterization of North American H3N2 swine influenza A viruses. Can. J. Vet. Res. *71*, 201-206.

Gray,G.C., Bender,J.B., Bridges,C.B., Daly,R.F., Krueger,W.S., Male,M.J., Heil,G.L., Friary,J.A., Derby,R.B., and Cox,N.J. (2012). Influenza A(H1N1)pdm09 virus among healthy show pigs, United States. Emerg. Infect. Dis. *18*, 1519-1521.

Gregory,V., Lim,W., Cameron,K., Bennett,M., Marozin,S., Klimov,A., Hall,H., Cox,N., Hay,A., and Lin,Y.P. (2001). Infection of a child in Hong Kong by an influenza A H3N2 virus closely related to viruses circulating in European pigs. J. Gen. Virol. *82*, 1397-1406.

Ha,Y., Stevens,D.J., Skehel,J.J., and Wiley,D.C. (2001). X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc. Natl. Acad. Sci. U. S. A *98*, 11181-11186.

Hale,B.G., Randall,R.E., Ortin,J., and Jackson,D. (2008). The multifunctional NS1 protein of influenza A viruses. J. Gen. Virol. *89*, 2359-2376.

Harder,T.C., Grosse,B.E., Lange,E., Meiners,C., Dohring,S., Pesch,S., Noe,T., Grund,C., Beer,M., and Starick,E. (2013). Expanded cocirculation of stable subtypes, emerging lineages, and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany. J. Virol. *87*, 10460-10476.

Harris,A., Cardone,G., Winkler,D.C., Heymann,J.B., Brecher,M., White,J.M., and Steven,A.C. (2006). Influenza virus pleiomorphy characterized by cryoelectron tomography. Proc. Natl. Acad. Sci. U. S. A *103*, 19123-19127.

Harrison,S.C. (2008). Viral membrane fusion. Nat. Struct. Mol. Biol. *15*, 690-698.

Heinen,P.P., van Nieuwstadt,A.P., Pol,J.M., de Boer-Luijtze,E.A., van Oirschot,J.T., and Bianchi,A.T. (2000). Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection. Viral Immunol. *13*, 237-247.

Hoffmann,E., Neumann,G., Hobom,G., Webster,R.G., and Kawaoka,Y. (2000a). "Ambisense" approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. Virology *267*, 310-317.

Hoffmann,E., Neumann,G., Kawaoka,Y., Hobom,G., and Webster,R.G. (2000b). A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A *97*, 6108-6113.

Hoffmann,E. and Webster,R.G. (2000). Unidirectional RNA polymerase I-polymerase II transcription system for the generation of influenza A virus from eight plasmids. J. Gen. Virol. *81*, 2843-2847.

Holder,M. and Lewis,P.O. (2003). Phylogeny estimation: traditional and Bayesian approaches. Nat. Rev. Genet. *4*, 275-284.

Howden,K.J. *et al.* (2009). An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can. Vet. J. *50*, 1153-1161.

Hutchinson,E.C. and Fodor,E. (2013). Transport of the influenza virus genome from nucleus to nucleus. Viruses. *5*, 2424-2446.

Ito,T. *et al.* (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. *72*, 7367-7373.

Iwasaki,A. and Pillai,P.S. (2014). Innate immunity to influenza virus infection. Nat. Rev. Immunol. *14*, 315-328.

Jackson,D., Hossain,M.J., Hickman,D., Perez,D.R., and Lamb,R.A. (2008). A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc. Natl. Acad. Sci. U. S. A *105*, 4381-4386.

Jahangir,A., Ruenphet,S., Sultana,N., Shoham,D., and Takehara,K. (2012). Genetic Analysis of Avian Influenza Viruses: Cocirculation of Avian Influenza Viruses with Allele A and B Nonstructural Gene in Northern Pintail (Anas acuta) Ducks Wintering in Japan. Influenza. Res. Treat. *2012*, 847505.

Jiang,T. *et al.* (2010). Development of a real-time RT-PCR assay for a novel influenza A (H1N1) virus. J. Virol. Methods *163*, 470-473.

Johnsen,L.A. (1985). En kort litterarturoversigt samt sygdommens forløb og udbredelse i et praksisområde. Dan. Vet. Tidsskr. *68*, 193-201.

Johnson,N.P. and Mueller,J. (2002). Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull. Hist Med. *76*, 105-115.

Karasin,A.I., Carman,S., and Olsen,C.W. (2006). Identification of human H1N2 and human-swine reassortant H1N2 and H1N1 influenza A viruses among pigs in Ontario, Canada (2003 to 2005). J. Clin. Microbiol. *44*, 1123-1126.

Karasin,A.I., Landgraf,J., Swenson,S., Erickson,G., Goyal,S., Woodruff,M., Scherba,G., Anderson,G., and Olsen,C.W. (2002). Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. J. Clin. Microbiol. *40*, 1073-1079.

Karlsson Hedestam,G.B., Fouchier,R.A., Phogat,S., Burton,D.R., Sodroski,J., and Wyatt,R.T. (2008). The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nat. Rev. Microbiol. *6*, 143-155.

Khanna,M., Gupta,N., Gupta,A., and Vijayan,V.K. (2009). Influenza A (H1N1) 2009: a pandemic alarm. J. Biosci. *34*, 481-489.

Khiabanian,H., Trifonov,V., and Rabadan,R. (2009). Reassortment patterns in Swine influenza viruses. PLoS. One. *4*, e7366.

Kim,H.R., Lee,Y.J., Lee,K.K., Oem,J.K., Kim,S.H., Lee,M.H., Lee,O.S., and Park,C.K. (2010a). Genetic relatedness of H6 subtype avian influenza viruses isolated from wild birds and domestic ducks in Korea and their pathogenicity in animals. J. Gen. Virol. *91*, 208-219.

Kim,Y.C., Quan,F.S., Compans,R.W., Kang,S.M., and Prausnitz,M.R. (2010b). Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity. J. Control Release *142*, 187-195.

Kingman,J.F. (2000). Origins of the coalescent. 1974-1982. Genetics *156*, 1461-1463.

Kitikoon,P., Nilubol,D., Erickson,B.J., Janke,B.H., Hoover,T.C., Sornsen,S.A., and Thacker,E.L. (2006). The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet. Immunol. Immunopathol. *112*, 117-128.

Kitikoon,P., Vincent,A.L., Janke,B.H., Erickson,B., Strait,E.L., Yu,S., Gramer,M.R., and Thacker,E.L. (2009). Swine influenza matrix 2 (M2) protein contributes to protection against infection with different H1 swine influenza virus (SIV) isolates. Vaccine *28*, 523-531.

Koel,B.F. *et al.* (2013). Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science *342*, 976-979.

Koen (1919). A practical method for field diagnosis of swine diseases. Am. J. Vet. Med. *14*, 468-470.

References

Kornfeld,R. and Kornfeld,S. (1985). Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. *54*, 631-664.

Krueger,W.S. and Gray,G.C. (2012). Swine Influenza Virus Infections in Man. Curr. Top. Microbiol. Immunol.

Kuiken,T., Holmes,E.C., McCauley,J., Rimmelzwaan,G.F., Williams,C.S., and Grenfell,B.T. (2006). Host species barriers to influenza virus infections. Science *312*, 394-397.

Kuntz-Simon,G. and Madec,F. (2009). Genetic and antigenic evolution of swine influenza viruses in Europe and evaluation of their zoonotic potential. Zoonoses. Public Health *56*, 310-325.

Kyriakis,C.S., Gramer,M.R., Barbe,F., Van,D.J., and Van,R.K. (2010). Efficacy of commercial swine influenza vaccines against challenge with a recent European H1N1 field isolate. Vet. Microbiol. *144*, 67-74.

Kyriakis,C.S., Rose,N., Foni,E., Maldonado,J., Loeffen,W.L., Madec,F., Simon,G., and Van,R.K. (2013). Influenza A virus infection dynamics in swine farms in Belgium, France, Italy and Spain, 2006-2008. Vet. Microbiol. *162*, 543-550.

Laver,W.G. (1971). Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. Virology *45*, 275-288.

Lewis,N.S., Anderson,T.K., Kitikoon,P., Skepner,E., Burke,D.F., and Vincent,A.L. (2014). Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J. Virol. *88*, 4752-4763.

Li,I.W. *et al.* (2009). Differential susceptibility of different cell lines to swine-origin influenza A H1N1, seasonal human influenza A H1N1, and avian influenza A H5N1 viruses. J. Clin. Virol. *46*, 325-330.

Li,Q., Sun,X., Li,Z., Liu,Y., Vavricka,C.J., Qi,J., and Gao,G.F. (2012). Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. Proc. Natl. Acad. Sci. U. S. A *109*, 18897-18902.

Loeffen,W.L., Kamp,E.M., Stockhofe-Zurwieden,N., van Nieuwstadt,A.P., Bongers,J.H., Hunneman,W.A., Elbers,A.R., Baars,J., Nell,T., and van Zijderveld,F.G. (1999). Survey of infectious agents involved in acute respiratory disease in finishing pigs. Vet. Rec. *145*, 123-129.

Lopez,C.H., Roca,R.F., and Daunis,J.V. (2009). [Pneumonia and the acute respiratory distress syndrome due to influenza A (H1N1) virus]. Med. Intensiva. *33*, 455-458.

Lorusso,A., Vincent,A.L., Gramer,M.R., Lager,K.M., and Ciacci-Zanella,J.R. (2012). Contemporary Epidemiology of North American Lineage Triple Reassortant Influenza A Viruses in Pigs. Curr. Top. Microbiol. Immunol.

Lycett,S.J., Baillie,G., Coulter,E., Bhatt,S., Kellam,P., McCauley,J.W., Wood,J.L., Brown,I.H., Pybus,O.G., and Leigh Brown,A.J. (2012). Estimating reassortment rates in co-circulating Eurasian swine influenza viruses. J. Gen. Virol. *93*, 2326-2336.

Ma,W., Lager,K.M., Vincent,A.L., Janke,B.H., Gramer,M.R., and Richt,J.A. (2009). The role of swine in the generation of novel influenza viruses. Zoonoses. Public Health *56*, 326-337.

Maher,J.A. and DeStefano,J. (2004). The ferret: an animal model to study influenza virus. Lab Anim (NY) *33*, 50-53.

Malby,R.L., Tulip,W.R., Harley,V.R., Kimm-Breschkin,J.L., Laver,W.G., Webster,R.G., and Colman,P.M. (1994). The structure of a complex between the NC10 antibody and influenza virus neuraminidase and comparison with the overlapping binding site of the NC41 antibody. Structure. *2*, 733-746.

Marozin,S., Gregory,V., Cameron,K., Bennett,M., Valette,M., Aymard,M., Foni,E., Barigazzi,G., Lin,Y., and Hay,A. (2002). Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J. Gen. Virol. *83*, 735-745.

Matrosovich,M., Tuzikov,A., Bovin,N., Gambaryan,A., Klimov,A., Castrucci,M.R., Donatelli,I., and Kawaoka,Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. *74*, 8502-8512.

Matsuoka,Y., Swayne,D.E., Thomas,C., Rameix-Welti,M.A., Naffakh,N., Warnes,C., Altholtz,M., Donis,R., and Subbarao,K. (2009). Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. J. Virol. *83*, 4704-4708.

McAuley,J.L., Hornung,F., Boyd,K.L., Smith,A.M., McKeon,R., Bennink,J., Yewdell,J.W., and McCullers,J.A. (2007). Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host. Microbe *2*, 240-249.

McAuley,J.L., Zhang,K., and McCullers,J.A. (2010). The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. J. Virol. *84*, 558-564.

Metreveli,G. *et al.* (2011). Comparison of two H1N2 swine influenza A viruses from disease outbreaks in pigs in Sweden during 2009 and 2010. Virus Genes *42*, 236-244.

Min,J.Y. and Krug,R.M. (2006). The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. Proc. Natl. Acad. Sci. U. S. A *103*, 7100-7105.

Miwa,Y., Piao,F.Z., Goto,H., and Noro,S. (1987). Isolation of human (H3N2) influenza virus and prevalence of the virus-antibody in swine. Nihon Juigaku. Zasshi *49*, 1168-1170.

Moreno,A., Di,T.L., Alborali,L., Vaccari,G., Barbieri,I., Falcone,E., Sozzi,E., Puzelli,S., Ferri,G., and Cordioli,P. (2010). First Pandemic H1N1 Outbreak from a Pig Farm in Italy. Open. Virol. J. *4*, 52-56.

Moreno,A. *et al.* (2011). Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. Vet. Microbiol. *149*, 472-477.

Nakajima,S., Cox,N.J., and Kendal,A.P. (1981). Antigenic and genomic analyses of influenza A(H1N1) viruses from different regions of the world, February 1978 to March 1980. Infect. Immun. *32*, 287-294.

Nardelli,L., Pascucci,S., Gualandi,G.L., and Loda,P. (1978). Outbreaks of classical swine influenza in Italy in 1976. Zentralbl. Veterinarmed. B *25*, 853-857.

Nayak,D.P., Balogun,R.A., Yamada,H., Zhou,Z.H., and Barman,S. (2009). Influenza virus morphogenesis and budding. Virus Res. *143*, 147-161.

Nelli,R.K., Kuchipudi,S.V., White,G.A., Perez,B.B., Dunham,S.P., and Chang,K.C. (2010). Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC. Vet. Res. *6*, 4.

Nelson,M.I., Vincent,A.L., Kitikoon,P., Holmes,E.C., and Gramer,M.R. (2012). Evolution of novel reassortant A/H3N2 influenza viruses in North American swine and humans, 2009-2011. J. Virol. *86*, 8872-8878.

Nelson,M.I., Wentworth,D.E., Culhane,M.R., Vincent,A.L., Viboud,C., LaPointe,M.P., Lin,X., Holmes,E.C., and Detmer,S.E. (2014). Introductions and evolution of human-origin seasonal influenza A viruses in multinational swine populations. J. Virol.

Neumann,G. and Kawaoka,Y. (2011). The first influenza pandemic of the new millennium. Influenza. Other Respi. Viruses. *5*, 157-166.

References

Neumann,G. *et al.* (1999). Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A *96*, 9345-9350.

Nicholls,J.M. (2013). The battle between influenza and the innate immune response in the human respiratory tract. Infect. Chemother. *45*, 11-21.

Nicholls,J.M., Bourne,A.J., Chen,H., Guan,Y., and Peiris,J.S. (2007). Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respir. Res. *8*, 73.

Nicholls,J.M., Chan,R.W., Russell,R.J., Air,G.M., and Peiris,J.S. (2008). Evolving complexities of influenza virus and its receptors. Trends Microbiol. *16*, 149-157.

Nicoll,A., Ciancio,B.C., Lopez,C., V, Molbak,K., Pebody,R., Pedzinski,B., Penttinen,P., van der,S.M., Snacken,R., and Van,K. (2012). Influenza-related deaths--available methods for estimating numbers and detecting patterns for seasonal and pandemic influenza in Europe. Euro. Surveill *17*.

Nobusawa,E., Aoyama,T., Kato,H., Suzuki,Y., Tateno,Y., and Nakajima,K. (1991). Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology *182*, 475-485.

Nobusawa,E. and Sato,K. (2006). Comparison of the mutation rates of human influenza A and B viruses. J. Virol. *80*, 3675-3678.

Obenauer,J.C. *et al.* (2006). Large-scale sequence analysis of avian influenza isolates. Science *311*, 1576-1580.

Ohuchi,M., Ohuchi,R., Feldmann,A., and Klenk,H.D. (1997). Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. J. Virol. *71*, 8377-8384.

Ottis,K., Sidoli,L., Bachmann,P.A., Webster,R.G., and Kaplan,M.M. (1982). Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. Arch. Virol. *73*, 103-108.

Palese,P. and Compans,R.W. (1976). Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. J. Gen. Virol. *33*, 159-163.

Pascua,P.N., Lim,G.J., Kwon,H.I., Park,S.J., Kim,E.H., Song,M.S., Kim,C.J., and Choi,Y.K. (2013). Emergence of H3N2pM-like and novel reassortant H3N1 swine viruses possessing segments derived from the A (H1N1)pdm09 influenza virus, Korea. Influenza. Other Respir. Viruses. *7*, 1283-1291.

Pascua,P.N. *et al.* (2012). Virulence and transmissibility of H1N2 influenza virus in ferrets imply the continuing threat of triple-reassortant swine viruses. Proc. Natl. Acad. Sci. U. S. A *109*, 15900-15905.

Pearce,M.B. *et al.* (2012). Pathogenesis and transmission of swine origin A(H3N2)v influenza viruses in ferrets. Proc. Natl. Acad. Sci. U. S. A *109*, 3944-3949.

Peiris,J.S., Guan,Y., Markwell,D., Ghose,P., Webster,R.G., and Shortridge,K.F. (2001). Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J. Virol. *75*, 9679-9686.

Pensaert,M., Ottis,K., Vandeputte,J., Kaplan,M.M., and Bachmann,P.A. (1981). Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man. Bull. World Health Organ *59*, 75-78.

Perez,D.R. and Donis,R.O. (1998). The matrix 1 protein of influenza A virus inhibits the transcriptase activity of a model influenza reporter genome in vivo. Virology *249*, 52-61.

Perez,L.J., Perera,C.L., Vega,A., Frias,M.T., Rouseaux,D., Ganges,L., Nunez,J.I., and az de,A.H. (2013). Isolation and complete genomic characterization of pandemic H1N1/2009 influenza viruses from Cuban swine herds. Res. Vet. Sci.

Perez-Trallero,E., Pineiro,L., Vicente,D., Montes,M., and Cilla,G. (2009). Residual immunity in older people against the influenza A(H1N1)--recent experience in northern Spain. Euro. Surveill *14*.

Pleschka,S. (2012). Overview of Influenza Viruses. In: Swine Influenza, ed. J.Richt and R.Webby Springer, 1-20.

Portela,A. and Digard,P. (2002). The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J. Gen. Virol. *83*, 723-734.

Potter,C.W. (2001). A history of influenza. J. Appl. Microbiol. *91*, 572-579.

Rahier,J.F., Yazdanpanah,Y., Viget,N., Travis,S., and Colombel,J.F. (2010). Review article: influenza A (H1N1) virus in patients with inflammatory bowel disease. Aliment. Pharmacol. Ther. *31*, 5-10.

Rambaut,A., Pybus,O.G., Nelson,M.I., Viboud,C., Taubenberger,J.K., and Holmes,E.C. (2008). The genomic and epidemiological dynamics of human influenza A virus. Nature *453*, 615-619.

Rannala,B. and Yang,Z. (1996). Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol. *43*, 304-311.

Reid,A.H., Fanning,T.G., Hultin,J.V., and Taubenberger,J.K. (1999). Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc. Natl. Acad. Sci. U. S. A *96*, 1651-1656.

Reuman,P.D., Keely,S., and Schiff,G.M. (1989). Assessment of signs of influenza illness in the ferret model. J. Virol. Methods *24*, 27-34.

Roberts,P.C., Garten,W., and Klenk,H.D. (1993). Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J. Virol. *67*, 3048-3060.

Rogers,G.N. and Paulson,J.C. (1983). Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology *127*, 361-373.

Romagosa,A., Allerson,M., Gramer,M., Joo,H.S., Deen,J., Detmer,S., and Torremorell,M. (2011). Vaccination of influenza a virus decreases transmission rates in pigs. Vet. Res. *42*, 120.

Russell,R.J., Haire,L.F., Stevens,D.J., Collins,P.J., Lin,Y.P., Blackburn,G.M., Hay,A.J., Gamblin,S.J., and Skehel,J.J. (2006). The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature *443*, 45-49.

Saitou,N. and Nei,M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406-425.

Scalera,N.M. and Mossad,S.B. (2009). The first pandemic of the 21st century: a review of the 2009 pandemic variant influenza A (H1N1) virus. Postgrad. Med. *121*, 43-47.

Schnitzler,S.U. and Schnitzler,P. (2009). An update on swine-origin influenza virus A/H1N1: a review. Virus Genes *39*, 279-292.

Scholtissek,C., Burger,H., Bachmann,P.A., and Hannoun,C. (1983). Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. Virology *129*, 521-523.

Scholtissek,C., Burger,H., Kistner,O., and Shortridge,K.F. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology *147*, 287-294.

Schultz,U., Fitch,W.M., Ludwig,S., Mandler,J., and Scholtissek,C. (1991). Evolution of pig influenza viruses. Virology *183*, 61-73.

References

Schwahn,A.B., Wong,J.W., and Downard,K.M. (2009). Signature peptides of influenza nucleoprotein for the typing and subtyping of the virus by high resolution mass spectrometry. Analyst *134*, 2253-2261.

Sebastian,M.R., Lodha,R., and Kabra,S.K. (2009). Swine origin influenza (swine flu). Indian J. Pediatr. *76*, 833-841.

Shapiro,G.I., Gurney,T., Jr., and Krug,R.M. (1987). Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J. Virol. *61*, 764-773.

Shapiro,G.I. and Krug,R.M. (1988). Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J. Virol. *62*, 2285-2290.

Shin,W.J. and Seong,B.L. (2013). Recent advances in pharmacophore modeling and its application to anti-influenza drug discovery. Expert. Opin. Drug Discov.

Shinya,K., Ebina,M., Yamada,S., Ono,M., Kasai,N., and Kawaoka,Y. (2006). Avian flu: influenza virus receptors in the human airway. Nature *440*, 435-436.

Shinya,K., Hamm,S., Hatta,M., Ito,H., Ito,T., and Kawaoka,Y. (2004). PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology *320*, 258-266.

Shope,R.E. (1931a). SWINE INFLUENZA : III. FILTRATION EXPERIMENTS AND ETIOLOGY. J. Exp. Med. *54*, 373-385.

Shope,R.E. (1931b). SWINE INFLUENZA : I. EXPERIMENTAL TRANSMISSION AND PATHOLOGY. J. Exp. Med. *54*, 349-359.

Shope,R.E. (1934). THE INFECTION OF FERRETS WITH SWINE INFLUENZA VIRUS. J. Exp. Med. *60*, 49-61.

Shortridge,K.F. and Webster,R.G. (1979). Geographical distribution of swine (Hsw1N1) and Hong Kong (H3N2) influenza virus variants in pigs in Southeast Asia. Intervirology *11*, 9-15.

Silverman,R.H. (2007). Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. J. Virol. *81*, 12720-12729.

Skehel,J.J. and Wiley,D.C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. *69*, 531-569.

Skowronski,D.M., Moser,F.S., Janjua,N.Z., Davoudi,B., English,K.M., Purych,D., Petric,M., and Pourbohloul,B. (2013). H3N2v and other influenza epidemic risk based on age-specific estimates of sero-protection and contact network interactions. PLoS. One. *8*, e54015.

Smith,D.J., Lapedes,A.S., de Jong,J.C., Bestebroer,T.M., Rimmelzwaan,G.F., Osterhaus,A.D., and Fouchier,R.A. (2004). Mapping the antigenic and genetic evolution of influenza virus. Science *305*, 371-376.

Smith,G.J., Bahl,J., and Vijaykrishna,D. (2012). Genetic analysis. Methods Mol. Biol. *865*, 207-227.

Smith,G.J., Bahl,J., Vijaykrishna,D., Zhang,J., Poon,L.L., Chen,H., Webster,R.G., Peiris,J.S., and Guan,Y. (2009a). Dating the emergence of pandemic influenza viruses. Proc. Natl. Acad. Sci. U. S. A *106*, 11709-11712.

Smith,G.J. *et al.* (2009b). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature *459*, 1122-1125.

Smith,W., Andrewes,C.H., and Laidlaw,P.P. (1933). A VIRUS OBTAINED FROM INFLUENZA PATIENTS. Lancet *222*, 66-68.

Sorensen,K.J., Have,P.D., Ronsholt,L., Hage,J.M., Westergaard, and Pedersen,K.B. (1981). Influenza hos svin i Danmark. Dan. Vet. Tidsskr. *64*, 826-829.

Sriwilaijaroen,N., Kondo,S., Yagi,H., Takemae,N., Saito,T., Hiramatsu,H., Kato,K., and Suzuki,Y. (2011). N-glycans from porcine trachea and lung: predominant NeuAcalpha2-6Gal could be a selective pressure for influenza variants in favor of human-type receptor. PLoS. One. *6*, e16302.

Sriwilaijaroen,N. and Suzuki,Y. (2012). Molecular basis of the structure and function of H1 hemagglutinin of influenza virus. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. *88*, 226-249.

Starick,E. *et al.* (2011). Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. J. Gen. Virol. *92*, 1184-1188.

Starick,E., Lange,E., Grund,C., Grosse,B.E., Dohring,S., Maas,A., Noe,T., Beer,M., and Harder,T.C. (2012). Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. J. Gen. Virol. *93*, 1658-1663.

Steain,M.C., Dwyer,D.E., Hurt,A.C., Kol,C., Saksena,N.K., Cunningham,A.L., and Wang,B. (2009). Detection of influenza A H1N1 and H3N2 mutations conferring resistance to oseltamivir using rolling circle amplification. Antiviral Res. *84*, 242-248.

Stech,J., Stech,O., Herwig,A., Altmeppen,H., Hundt,J., Gohrbandt,S., Kreibich,A., Weber,S., Klenk,H.D., and Mettenleiter,T.C. (2008). Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification. Nucleic Acids Res. *36*, e139.

Stech,J., Xiong,X., Scholtissek,C., and Webster,R.G. (1999). Independence of evolutionary and mutational rates after transmission of avian influenza viruses to swine. J. Virol. *73*, 1878-1884.

Steel,J., Staeheli,P., Mubareka,S., Garcia-Sastre,A., Palese,P., and Lowen,A.C. (2010). Transmission of pandemic H1N1 influenza virus and impact of prior exposure to seasonal strains or interferon treatment. J. Virol. *84*, 21-26.

Stevens,J., Corper,A.L., Basler,C.F., Taubenberger,J.K., Palese,P., and Wilson,I.A. (2004). Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science *303*, 1866-1870.

Stincarelli,M., Arvia,R., De Marco,M.A., Clausi,V., Corcioli,F., Cotti,C., Delogu,M., Donatelli,I., Azzi,A., and Giannecchini,S. (2013). Reassortment ability of the 2009 pandemic H1N1 influenza virus with circulating human and avian influenza viruses: public health risk implications. Virus Res. *175*, 151-154.

Sun,S., Wang,Q., Zhao,F., Chen,W., and Li,Z. (2011). Glycosylation site alteration in the evolution of influenza A (H1N1) viruses. PLoS. One. *6*, e22844.

Suzuki,Y., Ito,T., Suzuki,T., Holland,R.E., Jr., Chambers,T.M., Kiso,M., Ishida,H., and Kawaoka,Y. (2000). Sialic acid species as a determinant of the host range of influenza A viruses. J. Virol. *74*, 11825-11831.

Suzuki,Y., Nagao,Y., Kato,H., Matsumoto,M., Nerome,K., Nakajima,K., and Nobusawa,E. (1986). Human influenza A virus hemagglutinin distinguishes sialyloligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. Specificity for oligosaccharides and sialic acids and the sequence to which sialic acid is attached. J. Biol. Chem. *261*, 17057-17061.

Suzuki,Y. and Nei,M. (2002). Origin and evolution of influenza virus hemagglutinin genes. Mol. Biol. Evol. *19*, 501-509.

Takemae,N., Parchariyanon,S., Ruttanapumma,R., Hiromoto,Y., Hayashi,T., Uchida,Y., and Saito,T. (2011). Swine influenza virus infection in different age groups of pigs in farrow-to-finish farms in Thailand. Virol. J. *8*, 537.

References

Tashiro,M., Kimm-Breschkin,J.L., Saito,T., Klimov,A., Macken,C., Zambon,M., and Hayden,F.G. (2009). Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996-2007. Antivir. Ther. *14*, 751-761.

Taubenberger,J.K. and Kash,J.C. (2010). Influenza virus evolution, host adaptation, and pandemic formation. Cell Host. Microbe *7*, 440-451.

Taubenberger,J.K. and Morens,D.M. (2006). 1918 Influenza: the mother of all pandemics. Emerg. Infect. Dis. *12*, 15-22.

Taubenberger,J.K. and Morens,D.M. (2008). The pathology of influenza virus infections. Annu. Rev. Pathol. *3*, 499-522.

Teijaro,J.R., Walsh,K.B., Long,J.P., Tordoff,K.P., Stark,G.V., Eisfeld,A.J., Kawaoka,Y., Rosen,H., and Oldstone,M.B. (2014). Protection of ferrets from pulmonary injury due to H1N1 2009 influenza virus infection: immunopathology tractable by sphingosine-1-phosphate 1 receptor agonist therapy. Virology *452-453*, 152-157.

Tharakaraman,K., Raman,R., Stebbins,N.W., Viswanathan,K., Sasisekharan,V., and Sasisekharan,R. (2013). Antigenically intact hemagglutinin in circulating avian and swine influenza viruses and potential for H3N2 pandemic. Sci. Rep. *3*, 1822.

Tong,S. *et al.* (2012). A distinct lineage of influenza A virus from bats. Proc. Natl. Acad. Sci. U. S. A *109*, 4269-4274.

Tong,S. *et al.* (2013). New world bats harbor diverse influenza A viruses. PLoS. Pathog. *9*, e1003657.

Trebbien,R., Bragstad,K., Larsen,L.E., Nielsen,J., Botner,A., Heegaard,P.M., Fomsgaard,A., Viuff,B., and Hjulsager,C.K. (2013). Genetic and biological characterisation of an avian-like H1N2 swine influenza virus generated by reassortment of circulating avian-like H1N1 and H3N2 subtypes in Denmark. Virol. J. *10*, 290.

Trebbien,R., Larsen,L.E., and Viuff,B.M. (2011). Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol. J. *8*, 434.

Tremblay,D., Allard,V., Doyon,J.F., Bellehumeur,C., Spearman,J.G., Harel,J., and Gagnon,C.A. (2011). Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine. J. Clin. Microbiol. *49*, 4386-4390.

Tulip,W.R., Varghese,J.N., Laver,W.G., Webster,R.G., and Colman,P.M. (1992). Refined crystal structure of the influenza virus N9 neuraminidase-NC41 Fab complex. J. Mol. Biol. *227*, 122-148.

Tumova,B., Veznikova,D., Mensik,J., and Stumpa,A. (1980). Surveillance of influenza in pig herds in Czechoslovakia in 1974--1979. 1. Introduction of influenza epidemic A (H3N2) viruses into pig herds. Zentralbl. Veterinarmed. B *27*, 517-523.

Vallings,R. (2010). A case of chronic fatigue syndrome triggered by influenza H1N1 (swine influenza). J. Clin. Pathol. *63*, 184-185.

van de Sandt,C.E., Kreijtz,J.H., and Rimmelzwaan,G.F. (2012). Evasion of influenza A viruses from innate and adaptive immune responses. Viruses. *4*, 1438-1476.

Van Reeth,K., Labarque,G., and Pensaert,M. (2006). Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. Viral Immunol. *19*, 373-382.

Van Reeth,K. and Ma,W. (2012). Swine Influenza Virus Vaccines: To Change or Not to Change-That's the Question. Curr. Top. Microbiol. Immunol.

van Riel,D., Munster,V.J., de,W.E., Rimmelzwaan,G.F., Fouchier,R.A., Osterhaus,A.D., and Kuiken,T. (2007). Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am. J. Pathol. *171*, 1215-1223.

Varghese,J.N., Laver,W.G., and Colman,P.M. (1983). Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. Nature *303*, 35-40.

Varki,N.M. and Varki,A. (2007). Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest *87*, 851-857.

Venkatramani,L., Bochkareva,E., Lee,J.T., Gulati,U., Graeme,L.W., Bochkarev,A., and Air,G.M. (2006). An epidemiologically significant epitope of a 1998 human influenza virus neuraminidase forms a highly hydrated interface in the NA-antibody complex. J. Mol. Biol. *356*, 651-663.

Vigerust,D.J. and Shepherd,V.L. (2007). Virus glycosylation: role in virulence and immune interactions. Trends Microbiol. *15*, 211-218.

Vijaykrishna,D. *et al.* (2011). Long-term evolution and transmission dynamics of swine influenza A virus. Nature *473*, 519-522.

Vincent,A.L., Ma,W., Lager,K.M., Gramer,M.R., Richt,J.A., and Janke,B.H. (2009). Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. Virus Genes *39*, 176-185.

Vincent,A.L., Ma,W., Lager,K.M., Janke,B.H., and Richt,J.A. (2008). Swine influenza viruses a North American perspective. Adv. Virus Res. *72*, 127-154.

Wang,C.C. *et al.* (2009). Glycans on influenza hemagglutinin affect receptor binding and immune response. Proc. Natl. Acad. Sci. U. S. A *106*, 18137-18142.

Wang,X., Li,M., Zheng,H., Muster,T., Palese,P., Beg,A.A., and Garcia-Sastre,A. (2000). Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J. Virol. *74*, 11566-11573.

Webby,R.J., Rossow,K., Erickson,G., Sims,Y., and Webster,R. (2004). Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res. *103*, 67-73.

Webby,R.J., Swenson,S.L., Krauss,S.L., Gerrish,P.J., Goyal,S.M., and Webster,R.G. (2000). Evolution of swine H3N2 influenza viruses in the United States. J. Virol. *74*, 8243-8251.

Webster,R.G., Bean,W.J., Gorman,O.T., Chambers,T.M., and Kawaoka,Y. (1992). Evolution and ecology of influenza A viruses. Microbiol. Rev. *56*, 152-179.

Webster,R.G. and Govorkova,E.A. (2014). Continuing challenges in influenza. Ann. N. Y. Acad. Sci.

Webster,R.G. and Laver,W.G. (1980). Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology *104*, 139-148.

Wiley,D.C. and Skehel,J.J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. *56*, 365-394.

Wiley,D.C., Wilson,I.A., and Skehel,J.J. (1981). Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature *289*, 373-378.

Wolf,Y.I., Viboud,C., Holmes,E.C., Koonin,E.V., and Lipman,D.J. (2006). Long intervals of stasis punctuated by bursts of positive selection in the seasonal evolution of influenza A virus. Biol. Direct. *1*, 34.

Wu,Y., Wu,Y., Tefsen,B., Shi,Y., and Gao,G.F. (2014). Bat-derived influenza-like viruses H17N10 and H18N11. Trends Microbiol. *22*, 183-191.

Wu,Z.L., Ethen,C., Hickey,G.E., and Jiang,W. (2009). Active 1918 pandemic flu viral neuraminidase has distinct N-glycan profile and is resistant to trypsin digestion. Biochem. Biophys. Res. Commun. *379*, 749-753.

References

Xu,J., Christman,M.C., Donis,R.O., and Lu,G. (2011). Evolutionary dynamics of influenza A nucleoprotein (NP) lineages revealed by large-scale sequence analyses. Infect. Genet. Evol. *11*, 2125-2132.

Xu,Q., Wang,W., Cheng,X., Zengel,J., and Jin,H. (2010). Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. J. Virol. *84*, 4936-4945.

Yang,Z. and Nielsen,R. (2000). Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. Mol. Biol. Evol. *17*, 32-43.

Yen,H.L. *et al.* (2011). Transmissibility of pandemic H1N1 and genetically related swine influenza viruses in ferrets. Influenza. Other Respi. Viruses. *5 Suppl 1*, 85-87.

York,A. and Fodor,E. (2013). Biogenesis, assembly, and export of viral messenger ribonucleoproteins in the influenza A virus infected cell. RNA. Biol. *10*, 1274-1282.

Yus,E., Sanjuan,M.L., Garcia,F., Castro,J.M., and Simarro,I. (1992). Influenza A viruses: epidemiologic study in fatteners in Spain (1987-89). Zentralbl. Veterinarmed. B *39*, 113-118.

Zamarin,D., Garcia-Sastre,A., Xiao,X., Wang,R., and Palese,P. (2005). Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS. Pathog. *1*, e4.

Zell,R., Scholtissek,C., and Ludwig,S. (2012). Genetics, Evolution, and the Zoonotic Capacity of European Swine Influenza Viruses. Curr. Top. Microbiol. Immunol.

Zhang,X.M., Herbst,W., Lange-Herbst,H., and Schliesser,T. (1989). Seroprevalence of porcine and human influenza A virus antibodies in pigs between 1986 and 1988 in Hassia. Zentralbl. Veterinarmed. B *36*, 765-770.

Zhou,N.N., Senne,D.A., Landgraf,J.S., Swenson,S.L., Erickson,G., Rossow,K., Liu,L., Yoon,K., Krauss,S., and Webster,R.G. (1999). Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J. Virol. *73*, 8851-8856.

Zhu H, Webby,R., Lam,T.Y., Smith,D.K., Peiris,J.M., and Guan,Y. (2012). History of Swine Influenza Viruses in Asia. In: Swine Influenza, ed. J.Richt and R.Webby Springer, 57-68.

Zijtregtop,E.A., Wilschut,J., Koelma,N., Van Delden,J.J., Stolk,R.P., Van,S.J., Broer,J., Wolters,B., Postma,M.J., and Hak,E. (2009). Which factors are important in adults' uptake of a (pre)pandemic influenza vaccine? Vaccine *28*, 207-227.