Swine Influenza A virus subtype H1N2 in Sweden

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Cover: The influenza A virus genome is segmented, and when two or more different virus subtypes infect a single cell new strains can unexpectedly be produced by reassortment. This is associated with the emergence of pandemic influenza viruses. This process is able to introduce new proteins, which can drastically change the biology of the virion. Pigs make particularly good cauldrons for this type of genetic mixing. In this diagram, you can see how the purple and green types give rise to a mixed new blue type.

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Abstract

The influenza A virus subtypes H1N1, H1N2 and H3N2 are prevalent in pig populations worldwide. All scientific data point towards swine as the key host species when new human influenza pandemics arise. All previous pandemics have been suggested to evolve in pigs from viral genes of avian, human and porcine origin. Therefore, it is of major importance to monitor the evolution of swine influenza viruses in pigs, and in particular monitor hallmarks of species adaptation to humans.

The scope of this project was to increase the understanding of the genetics of swine influenza virus (SIV), with special emphasis on its zoonotic potential, and to investigate the importance of different viral gene markers for species specificity and adaptation.

Since clinical manifestation of swine influenza is rare in Sweden, and SIV strains are of particular concern due to the novel human H1N1 epidemic, viruses were isolated in primary swine kidney or Madin Darby Canine Kidney (MDCK) cells, based on standard protocols and the isolates were subjected to full genome sequencing and comparative sequence analysis of the viral genomes.

The results describe the analysis of the whole genome sequences from two swine influenza viruses isolated from Sweden in 2009 and 2010. Moreover, this study demonstrates, for the first time, natural reassortment in H1N2 viruses in the pig populations of Sweden. Biological characterization of the two viruses revealed a weaker growing potential, compared to the Swedish 2002 H1N1 isolate. Sequence comparison revealed significant differences between the two consecutive H1N2 isolates. The most remarkable of these was a truncated coding region for PB1-F2 in the earlier isolates and a full length coding region in the more recent isolates.

In order to determine the effect of these viruses on the swine industry and on influenza ecology, further surveillance investigations and detailed analyses are needed.

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Dedication

To my family

უძღვნი ჩემს ოჯახს და ჩემს მეგობრებს რომლებიც ამ ხნის განმავლობაში იყვნენ "მიტოვებულნი" ჩემს მიერ და განიცდიდნენ ჩემს მიერ მოკლებულ სითბოსა და სიყვარულს. მათ ვინც ამ ხნის მანძილზე იდგა ჩემი ოჯახის გვერდით და ეხმარებოდა.
დიდი სიყვარულითა და პატივისცემით გიორგი!

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I   Bálint A, **Metreveli G,** Widén F, Zohari S, Berg M, Isaksson M, Renström LH, Wallgren P, Belák S, Segall T, Kiss I. (2009) The first Swedish H1N2 swine influenza virus isolate represents an uncommon reassortant. Virology Journal 6:180

II   **Metreveli G,** Emmoth E, Zohari S, Bálint Á, Widén F, Muradrasoli S, Wallgren P, Belák S, LeBlanc N, Berg M, Kiss I. (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.

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Abbreviations

AA	Amino acid
AI	Avian influenza
CPE	Cytopathic effects
cRNA	Complementary RNA
FPK	Primary foetal porcine kidney
H1N1pdm	The 2009 H1N1 pandemic
HA	Haemagglutinin
IV	Influenza virus
M1	Matrix protein 1
M2	Transmembrane protein
mRNA	Messenger RNA
NA	Neuraminidase
NEP/ NS2	Nuclear export protein/ Non-structural protein 2
NP	Nucleoprotein
NTR	Non-translated region
PA	Acidic polymerase protein

PB1	Basic polymerase protein 1
PB2	Basic polymerase protein 2
RG	Reverse genetics
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Sialic acid
SI	Swine influenza
SIV	Swine influenza virus
TCID 50	50% Tissue culture infectious dose
vRNA	Viral RNA
vRNPs	Viral ribonucleoproteins

1 Introduction

1.1 History and epidemiological evolution of European swine influenza viruses

Influenza is a highly contagious acute respiratory illness that appears to have caused serious disease in humans since ancient times. It is a viral illness of global importance, caused by the influenza virus. Influenza viruses were probably responsible for the disease described by Hippocrates in 412 BC (Hoehling, 1961), and are still causing significant mortality and morbidity throughout the world. Influenza viruses can infect many animal species and some of these viruses may give rise to pandemic strains in humans. One recent example is the case of the 2009 H1N1 pandemic (H1N1pdm). Most threatening is the possibility of another pandemic appearing, similar to the Spanish influenza of 1918, which cost more than 40 million lives (Palese, 2004; Johnson & Mueller, 2002; Hoehling, 1961).

Influenza viruses have been isolated from avian species and mammals, including humans and pigs. Swine influenza viruses (SIV) cause respiratory disease in pigs and can have a significant economic impact in an affected herd. Swine influenza (SI) was first clinically recognized at the time of the Spanish influenza pandemic of 1918 but the virus was not isolated and identified by Shope until 1930. It was shown to be an influenza A virus of the H1N1 antigenic subtype called classical H1N1 (Shope, 1931). It was probably exactly the same virus affecting both humans and pigs. Nowadays three main subtypes of SIV are prevalent in pig populations worldwide, H1N1, H3N2 and H1N2 (Karasin *et al.*, 2002; Gourreau *et al.*, 1994), responsible for a highly contagious respiratory disease in pigs (Brown, 2000). The origin and nature of SIVs differ on different continents (Olsen *et al.*, 2005). These differences have

implication for the diagnosis and control of SIV, e.g. the strains used as antigens in vaccines and diagnostic test are different in Europe and in the United States. Also, the influenza virus can be one of many components in association with the porcine respiratory disease complex, of multifactorial aetiology (Hansen *et al.*, 2010). In the epidemic form of the disease SIV moves swiftly in all parts of the swine unit as it is readily transmitted by contact with respiratory secretions as well as by aerosol. Infection causes coughing, sneezing, nasal discharge, fever, lethargy, breathing difficulty and depressed appetite, and can lead to morbidity rates of up to 100% (Olsen *et al.*, 2006). Mortality is on the other hand low and recovery is unusually rapid, occurring within 7–10 days.

1.1.1 Classical H1N1

In Europe, SIVs were first isolated in Great Britain between 1938 and 1940 (Blakemore & Gledhill, 1941; Lamont, 1938). Nucleotide sequence analysis has shown that all eight genome segments of these strains were more closely related to the early human H1N1 strains A/Wisconsin/33 and A/Puerto Rico/8/34 than to the classical swine H1N1 virus (A/Sw/Iowa/1930), which was isolated in pigs in the US by Shope (Neumeier *et al.*, 1994). In 1950, the classical swine H1N1 virus was isolated in Czechoslovakia but after that no SIV detection was reported in Europe for more than two decades (Dunham *et al.*, 2009; Kuntz-Simon & Madec, 2009). Classical swine H1N1 viruses circulated in North America but were absent in Europe until 1976, when they were isolated from pigs imported to Italy from the United States (Nardelli *et al.*, 1978). After that, H1N1 spread and became established in other European countries, e.g. in the Netherlands (Masurel *et al.*, 1983), Denmark (Johnsen, 1985; Sorensen *et al.*, 1981), and southern Sweden in 1983 (Abusugra *et al.*, 1987) and in the United Kingdom (Roberts *et al.*, 1987). In North America and Asia the classical swine H1N1 is still the most common isolate but in Europe the H1N1 in pigs is now predominantly of avian origin (Guan *et al.*, 1996; Hinshaw *et al.*, 1978).

1.1.2 "Avian-like" H1N1'

The predominant H1N1 SIVs in Europe are of wholly avian genome origin, and were introduced from wild ducks to the pig population in 1979 (Campitelli *et al.*, 1997; Pensaert, 1981). They are called "avian-like" swine H1N1 viruses and are antigenically distinguishable from classical swine H1N1 influenza viruses. These "avian-like" viruses have a selective advantage over classical swine H1N1 viruses that are antigenically related, as in Europe they have replaced the classical SI virus (Brown, 2000; Campitelli *et al.*, 1997). After two

years of circulation of the "avian-like" viruses in the pig population in Great Britain, the classical H1N1 virus disappeared as a clinical entity.

1.1.3 Human-like H3N2

In the early 1970s, H3N2 viruses resembling the H3N2 strain responsible for the human 1968 Hong Kong influenza pandemic were introduced into European pigs (Harkness *et al.*, 1972). In 1984 a reassortment event occurred, resulting in a new strain containing human-like H3N2 "Hong Kong influenza virus" surface genes (HA, NA) and the internal genes of "avian-like" H1N1 (Campitelli *et al.*, 1997), and this replaced the original human-like swine H3N2 "Hong Kong influenza 1968" strain in circulation among pig populations in European countries in the 1990s (Kuntz-Simon & Madec, 2009). The occurrence and prevalence of SIVs vary among the different regions in Europe, but the avian origin H1N1 appears to be the most predominant subtype, followed by reassortant H3N2 and to a lower extent the H1N2 subtypes, which comprise viruses of diverse genetic constellations.

1.1.4 H1N2

As a result of further reassortment events, the first European SI H1N2 subtype was reported in France in 1987 (Gourreau *et al.*, 1994). This virus was a reassortant between the avian-like swine H1N1 virus and the European reassortant human-like swine H3N2. This virus has the haemagglutinin (HA) gene of avian and the neuraminidase (NA) gene of human origin. The first European SI H1N2 subtype did not become widespread in the European pig populations. However, another distinct lineage of H1N2 virus did become widespread in European pig populations, by a reassortant variant of "human-like" H1N2 virus identified in the UK in 1994. This virus spread to Belgium and other European countries during the next few years and became endemic (Marozin *et al.*, 2002; Van Reeth *et al.*, 2000; Brown *et al.*, 1995). This reassortant was the result of a reassortment between a human H1N1 virus from the 1980s (HA protein), the "human-like" swine H3N2 virus (NA protein) and the "avian-like swine" H1N1 SIV, from which it "inherited" the internal protein genes (Brown *et al.*, 1998). These viruses are genetically and antigenically different from viruses of the same subtype that had been present in pigs in France in 1987. Since 1998 reassortant H1N2 SIVs have been isolated in Italy and France (Kuntz-Simon & Madec, 2009; Marozin *et al.*, 2002). They are similar to the prototype H1N2 strains; the difference is that instead of a human HA, they have the "avian-like" HA from H1N1 SIVs.
In Denmark an H1N2 influenza subtype was discovered for the first time in 2003. All H1N2 viruses found in Denmark are reassortants of "avian-like"

H1N1 and "human-like" H3N2 SIVs (Hjulsager *et al.*, 2006). In 2009 and 2010 there were the first reported isolations and demonstrations of natural reassortants of H1N2 viruses in pigs in Sweden (Balint *et al.*, 2009). The characterized Swedish isolates possessed avian-like SIV H1N1 HA and European H3N2 SIV-like NA (Metreveli *et al.*, 2011).

1.1.5 Epidemiology

Continuous circulation of influenza A viruses in pigs can result in the production of new reassortant viruses. These reassortants cannot always become established in an immunologically naïve pig population. In Europe, other subtypes have sporadically been isolated and then disappeared from pigs. For example, subtype H1N7 (human and equine) in 1992 from England (Brown *et al.*, 1997) and H3N1 reported from Italy (Moreno *et al.*, 2009) have been isolated but have not been established in the pig population. These novel subtypes have not been widespread either in Great Britain or in the Italian pig population. This could be explained by the budding interplay activities of H3 and N1 and H1 and N7 proteins are not being as well functionally balanced as are the H1 and N2 proteins. In North America and Asia, many other subtypes have been described in pigs (H4N6, H3N3, H9N2, H5N1, H2N3), but no evidence has been found of such viruses circulating in European pigs. It is widely accepted that wild aquatic birds are reservoirs of all influenza A viruses for other species (Fouchier *et al.*, 2005). Due to their susceptibility to both human and avian influenza A viruses (Ito *et al.*, 1998; Webster *et al.*, 1992), swine have been postulated to play an important role in interspecies transmission, by acting as a mixing vessel for reassortment between viruses specific to different host species (Scholtissek, 1994), which is an important mechanism in the evolution of human pandemics (Webster *et al.*, 1992). This has been verified in Europe, in Italian pigs, when (Castrucci *et al.*, 1993) detected reassortment of human and avian viruses, and it is best exemplified by the appearance of the 2009 H1N1pdm influenza virus (Fig 1). Pigs were found to be susceptible to the 2009 H1N1pdm, which was transmitted to pigs in several countries.

The classical swine influenza virus lineage became established in domestic pigs in 1918-1920, similar to the 1918 pandemic H1N1virus. In 1979 "avian-like" H1N1 virus appeared in European pigs and was circulating with the classical swine H1N1 viruses. In 1998, a North American triple H3N2 reassortant virus was reported in the United States, derived from three different origins: an avian virus that circulated in wild birds, classical swine H1N1 viruses, and a human H3N2 influenza virus. This triple reassortant swine origin influenza virus became predominant in the North American pig population.

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These are the viruses that provided genomic segments for the 2009 H1N1pdm virus. This 2009 H1N1pdm virus has PB2 and PA segments from North American avian viruses: the PB1 segment is from the human H3N2 viruses, (from the 1968 pandemic, whose PB1 segment is of avian origin), the HA (of the H1 subtype), NP and NS segments are from classical swine H1N1 viruses (also avian origin), and the NA (of the N1 subtype) and M segments are from Eurasian 1979 "avian-like" swine viruses.
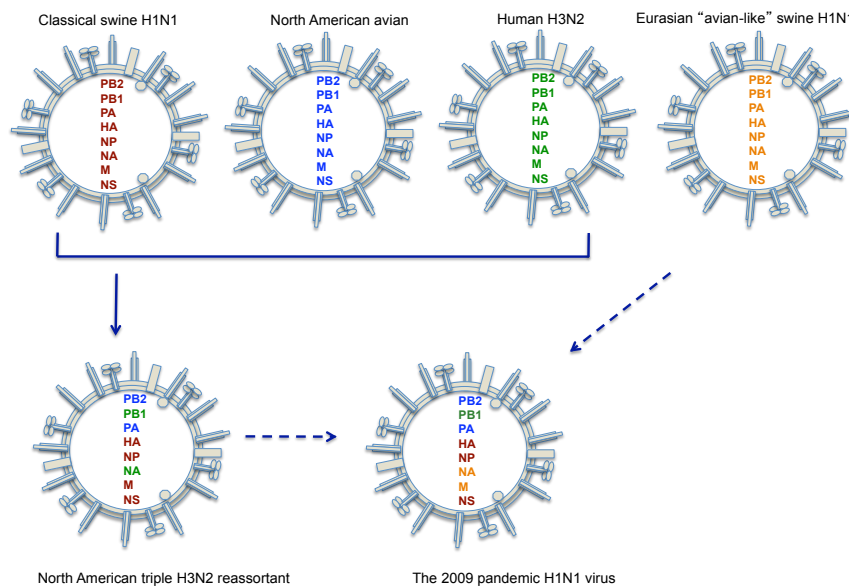
Figure 1. Emergence of the 2009 pandemic H1N1 virus. This 2009 H1N1pdm virus has PB2 and PA segments from North American avian viruses, the PB1 segment is from the human H3N2 viruses, (from the 1968 pandemic whose PB1 segment is of avian origin), the HA (of the H1 subtype), NP and NS are segments are from classical swine H1N1 viruses (also avian origin), and the NA (of the N1 subtype) and M segments are from Eurasian 1979 "avian-like" swine viruses.

The circulation of SIVs in Asia is more complex than it is elsewhere. Some SIVs of the North American and European lineages have been found and detected in Asia; this could be the reason of importation of live pigs from these regions but there are several lineages that are found only in Asia. It is not so surprising, therefore, that the circulation of entirely avian influenza (AI) viruses in pigs in nature is a relatively rare event. Still, there is circumstantial evidence that the genes of avian viruses may persist after reassortment with one or more influenza viruses endemic in pigs. As an example, H3N2 and H1N2 influenza viruses carrying mixtures of avian, swine and human influenza

virus genes have become enzootic in Europe. This probably means that genetic reassortments or mutations are needed for successful transmission of AI viruses between pigs. Therefore it is of major importance to monitor the evolution of SI in pigs, and in particular monitor the hallmarks of species adaptation to humans.

1.2 Influenza virus biology, genome structure and organization

1.2.1 Classification

Influenza viruses A, B and C together with Thogotovirus, Isavirus and newly discovered Quaranfilvirus are genera in the family of Orthomyxoviridae (Perez *et al.*, 2011). The name of the family is derived from Greek – *orthos*, which means correct or right and *myxa*, which means mucus. The influenza A, B and C viruses have antigenic differences in their nucleoproteins (NP) and matrix proteins (M) (Lamb & Krug, 2001). Influenza type B and C occur in humans, although not exclusively; influenza C virus has been isolated from swine (Yuanji & Desselberger, 1984) and influenza B virus from seals (Osterhaus *et al.*, 2000). Influenza A viruses have been isolated from a large variety of animals, including humans, pigs, mink, horses, dogs, sea mammals and birds (Webster *et al.*, 1992; Alexander, 1982) and can cause serious disease with pandemic characteristics.

Influenza A viruses are further divided into subtypes based on the antigenic properties of the external glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Sixteen antigenically different HAs (H1–H16) and nine different NAs (N1–N9) have been identified and their combination designates the subtype of the virus (Fouchier *et al.*, 2005; Alexander, 2000). Recently a new subtype H17 was discovered in Central American bats (Gewin, 2012). It is widely accepted that wild aquatic birds like ducks, gulls, and seabirds, are the most important reservoir of these viruses, because influenza viruses of all possible subtypes have been identified in this population. It is thought that they are the source of viruses that infect other avian and mammalian species. Influenza virus strains are given names according to their type (A, B or C), host (from where the virus was isolated), geographic location, number of the isolate, the year of isolation and HA and NA subtypes, for example: the first isolate of an H1N2 subtype virus isolated from pig in Sweden in 2009 is named: A/swine/Sweden/1021/2009(H1N2).

1.2.2 Virion structure

Influenza A virions are enveloped and pleomorphic. Their shape varies from small and spherical (cell culture isolates) with a diameter of about 100 nm, to

long and filamentous of more than 300 nm, which was observed especially in fresh clinical isolates (Chu *et al.*, 1949) and in preparations of viruses with specific M1 or M2 proteins (Elleman & Barclay, 2004; Bourmakina & Garcia-Sastre, 2003). The influenza A virus genome consists of eight single stranded, negative-sense RNAs that encode for 10–11 proteins, and eight are packaged into the infectious, enveloped virion (Palese & Shaw, 2007). The influenza A virus particle has a lipid membrane, derived from the host cell during the viral budding process (Cheung & Poon, 2007; Lamb & Krug, 2001; Webster *et al.*, 1992), and this where three viral proteins HA, NA and transmembrane protein M2 (Lamb & Krug, 2001) are inserted. The HA and NA are spike glycoproteins that are rod-shape and mushroom-shaped, respectively (Fig. 2).
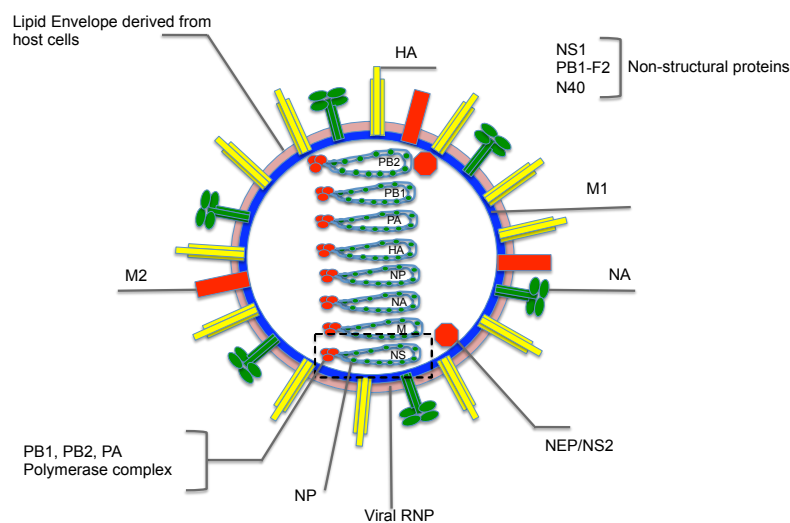
Figure 2. Schematic graph of influenza A viruses. The genome consists of eight single-stranded RNAs that interact with nucleoprotein (NP) and components of the polymerase complex (PB2, PB1, PA). See text for details.

The HA protein exists as a homotrimer, and it plays important roles in the influenza virus life cycle by mediating through receptor binding and membrane fusion. The cleavability of the HA protein by the host cell proteases determine the pathogenicity of influenza viruses. The NA protein is a homotetramer, and it plays a role in the release of virions by destroying receptors of the host and viral membranes. This is necessary for progeny virions to be released from the cell surface. The transmembrane protein M2 functions as an ion channel

(Holsinger *et al.*, 1994; Wang *et al.*, 1994), which has a role in virus entry, assembly and budding. Under the viral lipid envelope there is an M1 protein layer (Ruigrok *et al.*, 1989). The ribonucleoprotein (RNP) complex is made up of the viral RNA segments, three polymerase proteins (basic polymerase protein 2 (PB2), basic polymerase protein 1 (PB1) and acidic polymerase protein (PA) and the nucleoprotein (NP) (Shaw & Palese, 2008). The nuclear export protein (NEP; also called non-structural protein 2, NS2) is also present inside the viral particle (Palese & Shaw, 2006) and it functions as a nuclear export protein for vRNA in infected cells (O'Neill *et al.*, 1998). Inside of the influenza A virion, all eight genomic RNAs (vRNA) are individually bound to the nucleoprotein (NP) and to the influenza virus RNA polymerase to form RNP complexes (Lamb & Choppin, 1983). Influenza A viruses increase the coding capacity of their genomes by splicing and using alternative open reading frames. Some viral isolates can express the PB1-F2 and PB1 N40 proteins encoded within the PB1 gene (Wise *et al.*, 2009; Chen *et al.*, 2001). The M and NS gene mRNAs are spliced, and thus can encode the M2 and the NS2 proteins, respectively (Lamb & Choppin, 1981; Lamb & Choppin, 1979). The PB1-F2 and NS1 proteins are the only non-structural proteins. Each viral gene has non-translated (NTR) regions at both the 5' and 3' ends that contain replication, transcription, and packaging signals. The 13 and 12 nucleotides (nt) at the 5'and 3' ends, respectively, are conserved among all segments of influenza A viruses (Palese & Shaw, 2006). The sizes of the viral RNA segments and the proteins encoded are shown in Table 1.

Table 1. *Genome segments of influenza A virus with encoded proteins, number of nucleotides and amino acids and function of virus protein.*

Genome segments	Virus encoded	Nucleotide	Amino acid	Functions
1	PB2	2341	759	Polymerase
2	PB1, PB1-F2/NP40	2341	757	Transcriptase, cell apoptosis
3	PA	2233	716	RNP release, ssRNA synthesis
4	HA	1778	566	Receptorbinding
5	NP	1565	498	Endocapsidate RNA (RNP)
6	NA	1423	454	Enzyme cleave sialic acid receptor
7	M	1027	252, 97	Capsid formation, ion channel
8	NS, NEP	890	230, 121	mRNA splicing and transport control, RNP nuclear transport

1.3 Replication of influenza A viruses

Replication of influenza A viruses can be divided into the following fundamental steps (Fig. 3).

1.3.1 Attachment and entry

To initiate infection and replication, the HA protein on the virus surface attaches to the host cell receptors, containing terminal α-2,6 linked or α-2,3 linked sialic acid (α-2,6-SA or α-2,3-SA). Avian and equine influenza viruses recognize mainly α-2,3-SA receptors (Gambotto *et al.*, 2008; Horimoto & Kawaoka, 2005; Connor *et al.*, 1994). Humans have both type of receptors but human viruses can mainly recognize α-2,6-SA receptors (Horimoto & Kawaoka, 2006). Pigs, on their tracheal epithelial cells, have both (α-2, 6-SA or α-2, 3-SA) types of receptors, which may explain why they are susceptible to viruses from human and avian origin (Webster *et al.*, 1992).
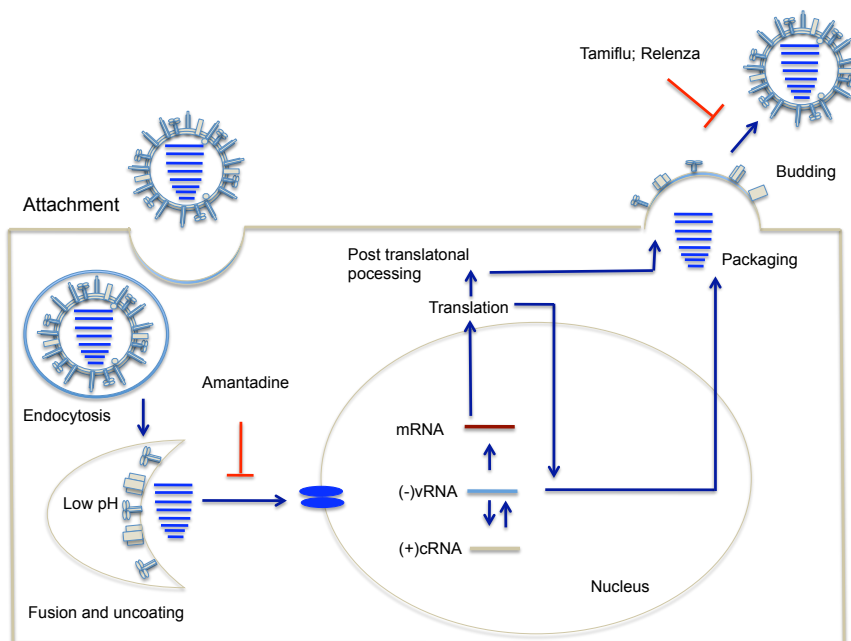
Figure 3. To initiate infection and replication, the HA protein on the virus surface attaches to the host cell receptors. After cell attachment, the virus enters via receptor-mediated endocytosis, the viral RNPs (vRNP) are released into the cytoplasm and transported to the nucleus where viral RNA (vRNA) synthesis takes place. vRNAs are used as templates by the viral RNA polymerase, and synthesize two kinds of positive-strand RNAs. Complementary RNAs (cRNAs) are full-length copies of the vRNAs, and viral mRNAs are capped and polyadenylated and are exported to the cytoplasm for translation. Once they are translated, into some of the viral proteins are

imported back to the nucleus to facilitate replication and transcription. After that newly synthesized RNPs are exported to the cytoplasm for packaging with the help of M1 and NEP. Viral HA, NA and M2 are matured in the Golgi apparatus (Golgi) and collecting at the plasma membrane where, with help of M1, the production of viral particles begins. As a consequence, budding starts and the progeny virus is released from the cell by the activity of NA that is destroys receptors of the host and viral membranes. This is necessary for progeny virions to be released from the cell surface.

1.3.2 Fusion and uncoating

After cell attachment, the virus enters by the receptor-mediated endocytosis of the virus particle via clathrin-coated pits (this process is dependent on a cellular GTPase) (Cross *et al.*, 2001). The endocytosis formed vesicles with the viral particles fuse to the endosomes in the cytoplasm (Skehel & Wiley, 2000). Influenza viruses need low pH to fuse with endosomal membranes. After binding to the host cell surface and undergoing endocytosis, the low pH of the endosome activates fusion of the viral membrane with that of the endosome. The low pH (5.0) in the endosomes results in conformational changes of the HA molecules, and the HA monomers are cleaved by trypsin-like enzymes into HA1 (globular domain) and HA2 (transmembrane domain) but are still attached by disulfide bonds (Skehel *et al.*, 1982). The concerted structural change of the HA molecules opens up a pore, which releases the viral RNPs into the cytoplasm of the cell. Successful uncoating is dependent on the presence of the M2 protein as well, which has ion channel activity (Pinto *et al.*, 1992). It has been shown that amantadine and rimantadine can block both the M2 protein ion channel activity (Fig. 3) and uncoating and thus act as anti-influenza drugs (Chizhmakov *et al.*, 1996; Wang *et al.*, 1993; Sugrue & Hay, 1991). The HA-mediated fusion of the viral membrane with the endosomal membrane and the M2-mediated release of the RNP result in the appearance of free RNP complexes in the cytoplasm. This completes the uncoating process (Martin & Helenius, 1991).

1.3.3 Transcription and replication

After the uncoating, the viral ribonucleoproteins (vRNPs) are transported into the nucleus (Helenius, 1992), where viral RNA synthesis takes place. Viral RNAs (vRNAs) are used as templates by the viral RNA polymerase, and produce two kinds of positive-strand RNAs (Krug *et al.*, 1989). Complementary RNAs (cRNAs) are full-length copies of the vRNAs, and mRNAs are capped and polyadenylated (Shih & Krug, 1996; Plotch *et al.*, 1981). The addition of the poly (A) tail occurs at a stretch of uridine residues close to the 5' end of the vRNAs. The mRNAs located at the 5' end of the

vRNAs do not have genetic information. Amplification of the vRNA is done via copying of the full-length cRNAs into new full-length vRNA molecules.

1.3.4 Assembly and release

The mRNA travels from the nucleus into the cytoplasm and it's translated into proteins. The translated viral proteins that are needed for replication and transcription are imported back into the nucleus again. After that, newly synthesized RNPs are exported to the cytoplasm for packaging, with help of the M1 and NEP. Viral HA, NA and M2 are matured in the Golgi apparatus (Golgi) and collect at the plasma membrane, where with help of M1, the production of viral particles begins. As a consequence, budding starts and the progeny virus is released from the cell by the activity of NA that is destroys receptors of the host and viral membranes. This is necessary for progeny virions to be released from the cell surface. The NA is also the main target of the antiviral drugs zanamivir and oseltamivir. These inhibitors interfere with the activity of the NA protein (Fig. 3) that is crucial for efficient release from the cell surface and subsequent spread to other cells.

1.4 Antigenic variation of influenza A viruses

1.4.1 Drift

Using two different mechanisms influenza A viruses continuously change their genetic form/antigenicity. The viral RNA polymerase does not have proof reading activity when synthesizing the genome. This results in point mutations, called genetic drift (Both *et al.*, 1983), the rate of mutations being about one nucleotide change for one copied genome (Drake, 1993). The highest evolutionary rates are observed in the HA and NA proteins. The antigenicity that the population develops drives the selection of the generated mutants and this is called antigenic drift. Antigenic drift is associated with seasonal epidemics, where new strains have amino acid changes in the HA and NA genes. These changes can overcome the existing immunity in humans, leading to seasonal influenza epidemics.

1.4.2 Shift

The influenza A virus genome is segmented, and when two or more different virus subtypes infect a single cell new strains can unexpectedly be produced by reassortment leading to antigenic shift. This is associated with the emergence of pandemic influenza viruses. This process is able to introduce new proteins, which can drastically change the biology of the virion. For example: it has played a huge role in the 1957 and 1968 pandemics, appearing with new PB1,

HA and/or NA proteins to which the human population was not immune (Scholtissek *et al.*, 1978). The best example of this is the 2009 H1N1pdm (Fig.1).

1.5   Reverse Genetics

Because influenza viruses are negative strand RNA viruses, introduction of genomic RNAs into cells does not result in the formation of an infectious virus (as is the case for positive strand RNA viruses). Experiments that have lead to the genetic engineering of influenza viruses have involved the reconstitution of functional RNP complexes in vitro (Parvin *et al.*, 1989; Honda *et al.*, 1987) and transfection of functional RNPs into cells. Reverse genetics (RG) for negative-strand RNA viruses, developed for influenza viruses (Enami *et al.*, 1990; Luytjes *et al.*, 1989), has dramatically changed our understanding of the replication cycles of these viruses. This methodology has allowed genetic manipulation of viral genomes in order to generate new viruses and elucidate their biological properties (Fodor *et al.*, 1999). In many cases, the definitive role of a gene/domain (or even a single amino acid) can only be explored by introducing appropriate mutations into the genome of the virus and then analyzing the phenotype of the rescued virus. These techniques are currently being used in several research areas including: i) characterization of virus-encoded virulence factors, ii) identification of virus-encoded antagonists of the interferon system, iii) virus replication and gene expression, iv) immune regulation of influenza replication, and v) vaccine development. For example, the live attenuated pandemic H1N1 2009 vaccine was made from a plasmid-generated strain, into which HA gene mutations were introduced to give high yields without changing the antigenicity of the strain (Chen *et al.*, 2010) .

2   Aims of the study

- The purpose of this study was to study an uncommon SIV H1N2 subtype, isolated from a pig in a multisite herd, which was detected for the first time in Sweden during the winter of 2008-2009 (Paper I)

- In paper II, two consecutive SIV H1N2 isolates were compared, which adds further information about influenza epidemiology, and supports the necessity of surveillance for influenza viruses in pigs

3 Materials and Methods

3.1 Clinical samples

Lung or nasal swab samples from infected pigs were received by the National Veterinary Institute (SVA) for diagnostic investigation (Paper I & II). The sow pool was centrally located in Sweden at Moholm, 400 km from the southern coastline and 200 km from the western coastline, and the herd was located in Staffanstorp in southern Sweden.

3.2 Virus isolation

The virus was isolated from the clinical material by infecting primary swine kidney cells or Madin Darby Canine kidney (MDCK) cells, following standard cell culture procedures (Papers I, II). Viral growth was determined by the observation of cytopathic effects (cpe), after 2– 4 days of incubation. The virus was aliquoted and stored at -70 ºC before use.

3.3 RNA isolation and genomic sequence analysis

Genetic analyses were conducted on the clinical material and on the MDCK cell isolates. Total RNA was prepared from virus-infected MDCK cells by the Qiagen RNeasy Mini kit, according to the manufacturer's instructions (Qiagen, Hilden Germany) and amplified by one-step RT-PCR (QIAGEN One Step RT-PCR Kit) using primers described previously (Kiss *et al.*, 2008). Amplified products were purified using the Purification kit from Promega and sequenced using the fluorescent dye terminator method with an ABI PRISM Big DyeTM Terminator Cycle Sequencing v3.1 Ready Reaction kit (Perkin Elmer, Waltham, MA, USA) on an ABI PRISM 310 genetic analyzer according to the manufacturer's recommendations (Applied Biosystems). Both strands of the

amplicons were sequenced with the same forward and reverse primers as used for the amplification. Multiple alignments of the DNA sequences of each gene were performed using CLC, Main Workbench 5.0.2 (CLC bio A/S, Aarhus, Denmark). The data were transformed to MEGA format, and distance-based phylogenetic trees were generated using the MEGA vs 4.0 software (Tamura *et al.*, 2007) with 1,000 bootstrap replicates. The phylogenetic trees were constructed with the Neighbour-joining method using the Kimura-two-parameter model. The results were verified by using the character based method maximum parsimony, which showed similar topologies.

3.4   Virus titration

Viruses were analyzed by end-point titration through cpe using 96-well plates containing MDCK cells, in tenfold dilutions assaying eight replicates of 50μl per dilution, essentially according to (Stallknecht *et al.*, 1990). The virus titres after 6–8 days were calculated according to (Kärber, 1931) and expressed as log10 tissue culture infectious dose $(TCID)_{50}$ ml^{-1}. Haemagglutination (HA) titrations were performed using 0.5% chicken erythrocytes according to (Abusugra *et al.*, 1987) (Paper II).

3.5   Biological titration assay and virus growth curves

The plates were incubated up to 48 h, with microscopic evaluation of onset of cpe every 12 h. For the growth curves, primary foetal porcine kidney (FPK) cells were grown to about 80% confluency in Leighton tubes, using EMEM supplemented with non-essential amino acids (NEA) and containing 10% FBS. The tubes were inoculated with 50 μl of the three viruses, calculated to contain 10^4 $TCID_{50}$. Two tubes were frozen at -70 °C at regular intervals up to 216 h, with start at 0 h and subsequently from 48 h where onset of cpe could be seen (Paper II).

3.6   Neuraminidase activity and inhibition assay

The NA enzyme activity and drug inhibition assays were based on (Potier *et al.*, 1979), with methylumbelliferone nacetylneuraminic acid (MUNANA) as the substrate. The avian influenza strains, which have been shown to be oseltamivir resistant and oseltamivir susceptible, were used as positive controls. The NA activity was plotted using GraphPad Prism Version 5 software. The oseltamivir resistant and oseltamivir susceptible H1N1 strains were kindly provided by Prof. Björn Olsen at Uppsala University, Department of Medical Sceinces, Section of infectious Diseases (PaperII).

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4 Results and discussion

4.1 Papers I & II

In this thesis the analysis of the whole genome sequences from two SIV isolates from Sweden in 2009 and 2010 were done (Metreveli *et al.*, 2011). The sequence comparison revealed significant differences between the two consecutive H1N2 isolates. The most remarkable of these was a truncated coding region for PB1-F2 in the earlier isolates and a full-length coding region in the more recent isolates. The majority of avian influenza viruses and many SIVs harbour a 90 amino acid (aa) (full length) protein, while human viruses collected since 1950 have a 57 aa truncated PB1-F2 protein. The minority of the European SIVs have stop codons after 11, 25 and 43 aa (Zell *et al.*, 2007). The 2009 Swedish H1N2 virus coded for a 57 aa truncated version of this protein, while the 2010 isolate coded for the 90 aa full length protein.

Another protein that has implications in the host range of influenza viruses, due to its role in host immune evasion, is the non-structural protein (NS1). The NS1 sequence of sw- H1N2-2009 and 2010 has an unusual PDZ binding domain (RPKV) at the C-terminal of the protein. It has recently been shown that the C-terminal four residues of the NS1 protein is a PDZ ligand domain of the X-S/T X-V type and may represent a virulence determinant (Ghedin *et al.*, 2005). This particular PDZ domain has been reported in only three cases, all in human isolates, but never in swine isolates. To our knowledge, this is the first reported isolation of this unusual PDZ binding domain in a pig population. More studies are needed to determine the exact function of the PDZ motif, and may help to determine and understand the function of the PDZ binding domain. One recent study (Moreno *et al.*, 2011) has shown that novel H1N2 swine influenza reassortants have derived from the 2009 H1N1pdm, with 7 genes well adapted to humans and the NA gene closely related to the Swedish 2009-2010 H1N2 SIVs (Moreno *et al.*, 2012). This new Italian H1N2 reassortant

virus confirms the function of swine as mixing vessels for animal and human influenza, resulting in reassortment and host adaptation. This shows that the introduction of the 2009 H1N1pdm in the swine population has given opportunities for reassortment facilitating the alteration of transmissibility and increased virulence in the 2009 H1N1pdm virus. Genetic reassortment involving a virus having genes specific for replication in a new host, e.g. humans, can result in interspecies transmission. If the 2009 H1N1pdm is established in the swine population, reassortment could occur between influenza viruses both of mammal and avian origin, resulting in new variants with key molecular determinants of pathogenicity that are concern for public health (Neumann *et al.*, 2009). All of these facts could have a great impact in southern Sweden where most of swine and poultry are raised and where we have diagnosed for the first time these uncommon avian-like reassortant H1N2 SIV variants. Clearly, it is very important to analyze the whole genome of an IV isolate and to identify amino acid changes, and possibly their effect on the virus's life cycle. It has been recognized that it is vital for a "successful" reassortant to obtain functionally compatible gene segments. For example, the interaction of NP with PB2 and PB1, two subunits of the viral polymerase complex is crucial for efficient viral replication. To allow better spread among infected cells IV HA has to have a matching NA (Baigent & McCauley, 2001). The role of host cellular factors in IV replication is at least as important as all of the other components of the virus (Karlas *et al.*, 2010; Konig *et al.*, 2010).

In summary, the avian-like reassortant H1N2 SIV variant appears to be gaining a stronger foothold among Swedish pig populations, and producing more clinical disease. The molecular genomic differences found here indicate that the virus population is steadily evolving. In order to determine the effect of this virus evolution on the swine industry and on influenza ecology, further surveillances and detailed analyses are needed.

5 Implications for future research, concluding remarks

Swine IVs should be systematically followed, because of the potential of swine to host rearrangement of the viral gene segments, so called reassortment, of porcine, avian and human origin. European avian-like SIV appears to be one of the ancestors of the H1N1pdm influenza virus that appeared in 2009. Since these unique reassortant variants of swine influenza virus may have a global importance as possible factors in the development of new pandemics, it would be of great benefit to further investigate these viruses regarding their replication characteristics, genetic variability, virulence factors and immune modulation capacity in both swine and human cells. The association of truncated and full forms of PB1-F2 and their biological implications for viral pathogenicity will be investigated in the future by using RG system. The advances of RG techniques have been of great value for the study of structure/function of different influenza virus genes and their proteins. Improved knowledge on all of these aspects of swine IVs will strongly benefit agribusiness and science and be of great importance for public health as well.

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