Swine Influenza A virus subtype H1N2 in Sweden

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2014

Acta Universitatis agriculturae Sueciae
2013:93

ISSN 1652-6880
ISBN (print version) 978-91-576-7924-6
ISBN (electronic version) 978-91-576- 7925-3

Print: SLU Service/Repro, Uppsala 2014

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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 for new human influenza pandemics, which have been suggested to evolve in pigs from viral genes of avian, human and porcine origin. Therefore, it is of major importance to record the evolution of swine influenza viruses in pigs, and in particular monitor hallmarks of adaptation to humans. The scope of this thesis was to increase the understanding of the genetics of swine influenza virus (SIV), and to investigate the importance of different viral gene markers in association with differences in pathogenicity of two viruses of H1N2 subtype in pigs. The results from this study demonstrate, for the first time, natural reassortment in H1N2 viruses in the pig populations of Sweden. These H1N2 viruses have an avian-like SIV H1N1 haemagglutinin (HA) and a European H3N2 SIV-like neuraminidase (NA). Nucleotide sequence comparison revealed significant differences between the two consecutive H1N2 isolates. To be able to understand the genotypic differences observed in the genomes of these H1N2s, and to identify the genetic markers responsible for the differences, a reverse genetic system was developed. Four recombinant SIV H1N2 viruses were constructed that displayed differences in virulence in mice, r1021 (more virulent) and r9706 (less virulent), as well as the same viruses with swapped PB1 segments. Interestingly, the current findings showed that the replacement of the PB1 segment of r9706 by that of r1021 increases the virulence of the virus that replicate with higher titer in mice lungs, while the opposite is true when PB1 r9706 is introduced into r1021. This study demonstrates that differences in virulence of swine influenza virus subtype H1N2 are attributed at least in part to the PB1 segment. The findings presented in this thesis support the observations concerning the continuous reassortment processes of SIVs in pigs, resulting in repeated and independent emergence of certain HA/NA combinations. This may lead to emergence of new viral variants of severe pathogenicity of pigs. Continuous and efficient surveillance and further detailed genetic and phenotypic analysis can help to identify such novel viral variants, having more potential to cross species barriers and to pose health risks even to humans and to other host species.

Keywords: Influenza A, swine influenza virus SIV, PB1 segment, reverse genetics

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Dedication

To my family

ვუძღვნი ჩემს ოჯახს და მეგობრებს რომლებიც, გვერდში მიდგანან და ჩემს წარმატებებს იზიარებენ.
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.

III **Metreveli G.** et al (2013) The origin of the PB1 segment of swine influenza A virus subtype H1N2 determines viral pathogenicity in mice (submitted).

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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	Matrix protein 2, 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 viral infection that appears to have caused serious disease in humans since ancient times. It is a viral disease of global importance, caused by various different influenza viruses. 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 infectious agents 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 killed more than 40 million people (Palese, 2004; Johnson & Mueller, 2002; Hoehling, 1961).

Influenza viruses have been isolated from various 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 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 the 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, on the other hand, is 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 into 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 (Sorensen *et al.*, 1981), and southern Sweden in 1983 (Abusugra *et al.*, 1987; Martinsson *et al.*, 1983), 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 to the pig population from wild ducks 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, as in Europe they have replaced the classical SI virus (Brown, 2000; Campitelli *et al.*, 1997).

1.1.3 Human-like H3N2

In the early 1970s, H3N2 viruses resembling the H3N2 strain, which was 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, as 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. H1N2 viruses found in Denmark are reassortants of "avian-like" H1gene and a European "swine-like" N2 gene (Trebbien *et al*., 2013). In 2009 and 2010 there were the first reported isolations and demonstrations of natural reassortants of H1N2 viruses in pigs in Sweden (Bálint *et al.*, 2009). The

characterized Swedish isolates possessed avian-like SIV H1N1 HA and European H3N2 SIV-like NA (Metreveli *et al.*, 2011).

After introduction of the H1N1pdm virus into the European pig population, some novel H1N2 swine influenza reassortant strains have been characterized in Europe. In Italy, a novel H1N2 reassortant was characterized which obtained seven genes from the H1N1pdm and NA closely related to two "avian-like" H1N2 swine influenza viruses, previously isolated in Sweden and Italy (Moreno *et al*., 2011). Also in the United Kingdom, a novel H1N2 reassortant comprised of HA and NA genes of the "human-like" swine influenza H1N2 and the rest six internal genes of H1N1pdm, was detected (Howard *et al*., 2011). In 2012 in Germany, novel H1N2 strains have been characterized that have seven genes of H1N1pdm and the NA gene of the European human H3N2 lineage (Lange *et al*., 2013).

Later in autumn 2013 a reassortment between H1N1pdm and Swedish "avian-like" H1N2 strains was detected, resulting in a novel reassortant H1N2 strain. The characterized novel Swedish H1N2 reassortant strain showed that seven genes belong to the H1N1pdm virus and the NA gene is similar to A/swine/Sweden/1021/2009(H1N2) and A/swine/Sweden/9706/2010(H1N2) (personal communication Siamak Zohari, SVA 2013).

1.1.5 Epidemiology

Continuous circulation of influenza A viruses in pigs can result in the production of new reassortant viruses. 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 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 populations. 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 this animal species 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 an "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. 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.

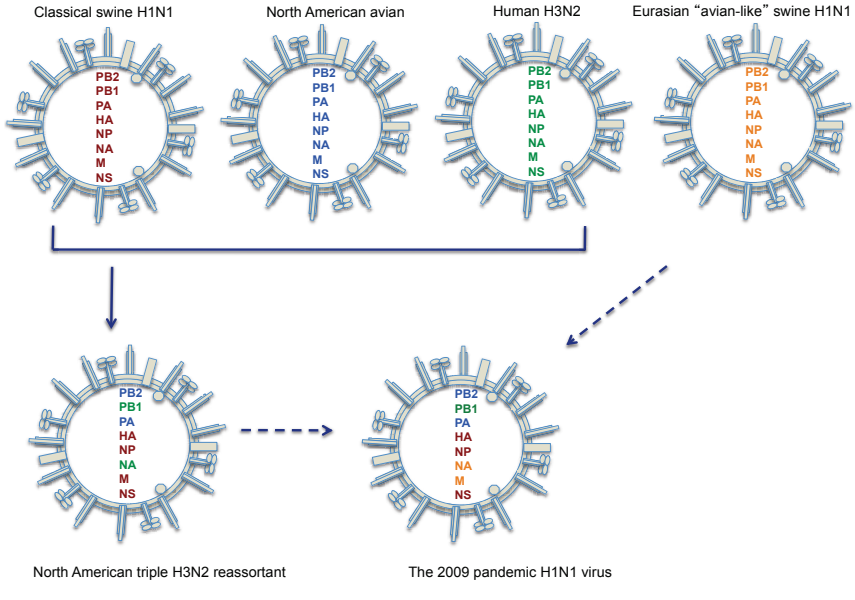

Figure 1. Emergence of the 2009 pandemic H1N1 virus. This 2009 H1N1pdm virus has PB2 and PA segments from North American avian viruses; the PB1 segment is from the human H3N2 viruses, (from the 1968 pandemic whose PB1 segment is of avian origin); the HA (of the H1 subtype), NP and NS are segments are 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 result 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 the 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. Influenza types 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). It is widely accepted that wild aquatic birds like ducks, gulls, and other seabirds are the

the subtype of the virus (Fouchier *et al.*, 2005; Alexander, 2000). It is widely accepted that wild aquatic birds like ducks, gulls, and other seabirds are the most important reservoirs 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. Recently, two novel influenza A viruses were discovered in bats from Guatemala (HA17NA10) and Peru (HA18NA11) suggesting that these species may constitute another reservoir with even greater genetic diversity (Tong *et al.*, 2013; Tong *et al.*, 2012). Influenza virus strains are given names according to their type (A, B or C), host (from which 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 has been observed especially in fresh clinical isolates (Chu *et al.*, 1949). The influenza A virus genome consists of eight single stranded, negative-sense RNAs that encode for up to twelve proteins. These eight segments that comprise the influenza virus genome form viral RNPs, and are packaged into the virus particle (McGeoch *et al.*, 1976). 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).

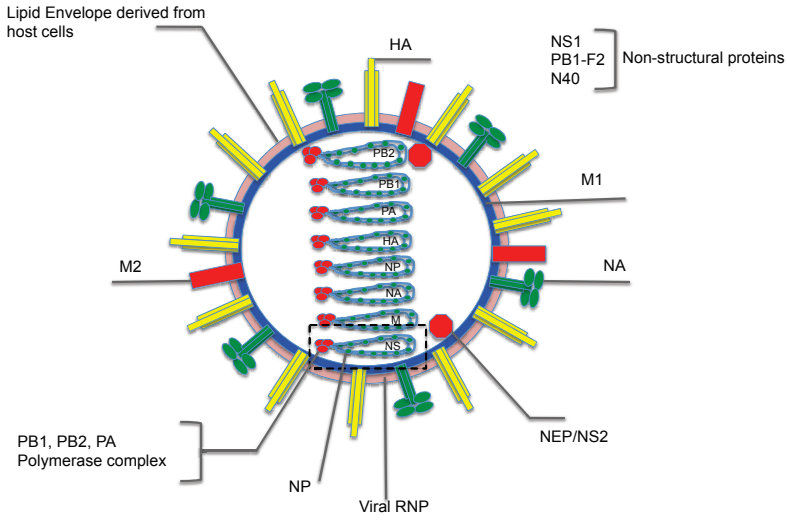

Figure 2. Schematic graph of an influenza A virus particle. The genome consists of eight single-stranded RNAs that interact with the nucleoprotein (NP) and components of the polymerase complex (PB2, PB1, PA). See text for details.

The HA protein exists as a homotrimer, and it plays important roles in the influenza virus life cycle by mediating 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 virion with encoded proteins, number of nucleotides and amino acids and function of virus protein*

Genome Segments	Virus encoded	Nucleotides	Amino acids	Functions
1	PB2	2341	759	Polymerase, host cell capped mRNA recognition and binding
2	PB1, PB1-F2,N40	2341	757 90	Transcriptase, capped mRNA endonuclease activity, induction of apoptosis
3	PA	2233	716	Required for replication, possible role in transcription
4	HA	1778	566	Major antigenic determinant, functions in virus binding to cell surface receptors and fusion
5	NP	1565	498	Associated with RNA segments to form ribonucleoprotein
6	NA	1413	454	Functions in virus release; targets of neuraminidase inhibitors
7	M1 M2	1027	252 97	Major virion component; involved in RNP transport out of nucleus, ion channel activity
8	NS1 NEP	890	230 121	Inhibits mRNA transport from nucleus; viral nuclear export protein

1.3 Replication of influenza A viruses

The replication processes 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 of both human and avian origin (Webster *et al.*, 1992).

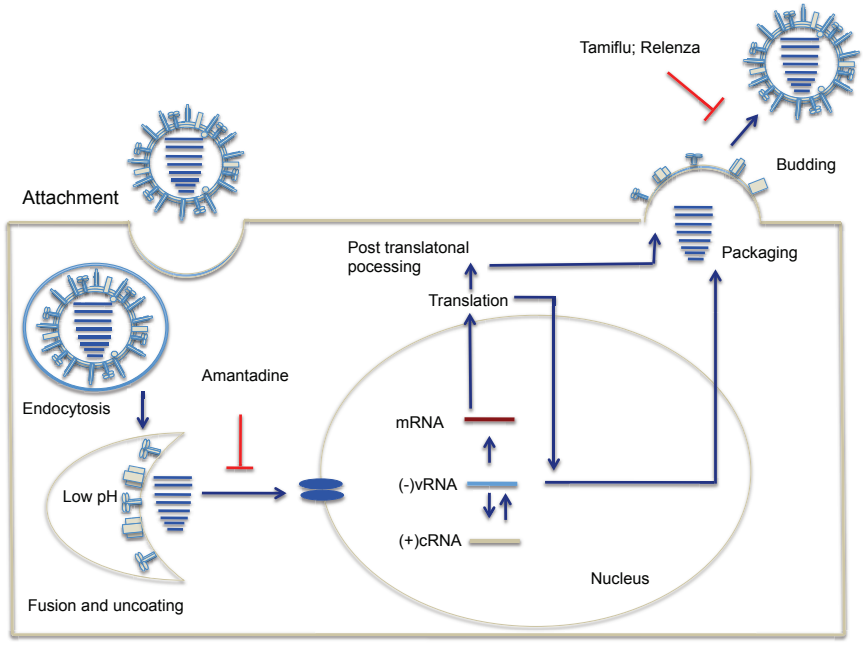

Figure 3. To initiate infection and replication, the HA protein on the virus surface attaches to the host cell receptors. After cell attachment, the virus enters via receptor-mediated endocytosis, the viral RNPs (vRNP) are released into the cytoplasm and transported to the nucleus where viral RNA (vRNA) 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 progeny virus are released from the cell by the activity of NA that 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 that have been cleaved into HA1 and HA2 and 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 they 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 viral mRNAs are transported from the nucleus into the cytoplasm and it’s translated into proteins. The translated viral proteins that are needed in

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 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 new pandemic influenza viruses. This process is able to introduce new proteins, which can drastically change the biology of the virion. For example, it 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).

1.4.3 History of the Swedish swine influenza virus isolates used in this study

Influenza H1N1 infected the Swedish pig population for the first time during the winter of 1982-1983, causing severe clinical signs in pigs of all ages, (Martinsson *et al.*, 1983). It has circulated without obvious symptoms since then, although very few studies have addressed this issue. In 2002, there was another acute outbreak of respiratory disease in pigs related to SIV A in central Sweden. This virus was not fully characterised, but was later shown to be SIV H1N1, similar to other SIV H1N1 strains circulating in Europe at that time (Kiss et al., 2010). This situation changed in 2009, when the A/swine/Sweden/1021/2009(H1N2) virus was isolated as part of the diagnostic investigation of a multisite pig herd (sow pool) with 4,000 sows affected with severe clinical signs of respiratory disease among growers during the winter of 2008-2009. The herd was centrally located in Sweden, 400 km from the southern coastline and 200 km from the western coastline. This was the first demonstration of H1N2 in pigs in Sweden (Wallgren *et al.*, 2009). According to a national serologic screening carried out in 2006, pigs in Sweden were free from H1N2 at that time. During early 2010 an outbreak of the H1N2 type, caused severe respiratory illness in fatteners in a farrow to finish herd in southern Sweden, whereas sows and piglets remained healthy. This virus was isolated and designated as A/swine/Sweden/9706/2010(H1N2). Since these are the first two reported isolations and demonstrations of natural reassortants of H1N2 viruses in pigs in Sweden, it was important to understand how these viruses were related to eachother.

However, H1N1pdm was also diagnosed and isolated in Sweden in 2013. As anticipated (Metreveli, 2012), later in autumn 2013 a reassortment between H1N1pdm and Swedish "avian-like" H1N2 strains was detected, and found to be a novel reassortant H1N2 strain. The characterization of the strain showed that PB2, PB1, PA, HA, NP, M and NS belongs to the H1N1pdm virus and the NA gene is similar to A/swine/Sweden/1021/2009(H1N2) and A/swine/Sweden/9706/2010(H1N2) (personal communication Siamak Zohari, SVA 2013).

1.5 Reverse Genetics

Influenza A viruses are negative sense-single strand RNA viruses, and therefore the introduction of genomic viral RNAs into cells does not result in the formation of infectious viral particles. This is in contrast to positive strand RNA viruses, because their genomic RNA resembles mRNA and can be translated into protein directly upon entering the cell. Previous experiments

using genetic engineering of influenza viruses have involved the reconstitution of functional RNP complexes in vitro and transfection of functional RNPs into cells (Parvin *et al.*, 1989; Honda *et al.*, 1987). Reverse genetics (RG) for negative-strand RNA viruses, developed for influenza viruses (Enami *et al.*, 1990; Luytjes *et al.*, 1989), has fundamentally changed our understanding of their replication cycles. This methodology has allowed genetic manipulation of viral genomes in order to generate new viruses and clarify their biological properties (Fodor *et al.*, 1999). The exact role of a gene or a single amino acid can be investigated by introducing appropriate mutations into the viral genome and then analyzing the phenotype of the rescued virus. RG is 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, a live attenuated H1N1pdm 2009 vaccine, made from a plasmid-generated strain with HA gene mutations, gave high yields without changing the antigenicity of the strain (Chen *et al.*, 2010).

2 Aims of the study

The main aim of this thesis was to investigate the importance of different viral genes in the pathogenicity of swine influenza A virus subtype H1N2 isolated in Sweden.

The specific aims were:

- To study and characterize SIV from the outbreak in 2009 (Paper I);
- To compare the two consecutive SIV isolates of 2009 and 2010 regarding their genetic and biological characteristics (Paper II); and
- To determine the contribution of the PB1 gene products of these two SIV to virus replication and pathogenicity, by using a reverse genetics system (Paper III).

3 Materials and Methods

This section gives a brief description of the materials and methods used in the studies of this thesis. More detailed descriptions are provided within each individual paper.

3.1 Clinical samples

Lung or nasal swab samples from infected pigs were received by the National Veterinary Institute (SVA) for diagnostic investigation (Papers 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 50 µl 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 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$ ml^{-1}. Haemagglutination (HA) titrations were performed using 0.5% suspensions of 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. Avian influenza strains, that 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 Sciences, section of Infectious Diseases (Paper II).

3.7 Cells and viruses

293T and PK1 cells were maintained in Dulbecco's modified Eagle's medium with 10 % fetal calf serum (FCS). MDCK cells were grown in Eagle's minimal essential medium with 10 % FCS. Viruses were grown in 10-day-old specific-pathogen-free chicken embryos at 37°C (Charles River Laboratories, SPAFAS). All animal studies were performed according to institutional guidelines (Paper III).

3.8 Rescue of recombinant influenza A virus

The H1N2 SIV used in these studies included reverse genetics derived A/swine/Sweden/1021/2009 (r1021) A/swine/Sweden/9706/2010 (r9706), r1021 (9706 PB1), encoding the PB1 gene segment from the r9706 virus strain, and r9706 (1021 PB1), encoding the PB1 gene segment from the r1021 virus strain. Recombinant viruses were rescued as described previously (Gao *et al.*, 2008). Briefly, 293T cells were transfected with eight ambisense pDZ vectors expressing viral genomic RNA and viral mRNAs. The transected cells were harvested by pipetting and 10-day-old Specific Pathogen Free (SPF) eggs were inoculated by injecting of cell mixture. The inoculated SPF eggs were incubated at 33 or 37°C for 3 days. Allantoic fluids were harvested from infected chicken embryonated eggs after putting eggs at 4°C overnight (O/N). Hemagglutination assay (HA) was performed to confirm the rescue of recombinant influenza viruses, and the titers of the rescued viruses were further determined by plaque assay in MDCK cells. The nucleotide sequence of the swapped PB1 segments was sequence confirmed.

3.9 Growth curves of recombinant and chimeric viruses

To analyze the recombinant viruses, PK1 cells were seeded at 10^6 per well in 6 well plates (duplicates for each virus) and were inoculated at a multiplicity of infection (MOI) of 0.01 PFU/cell, and incubated for 1 h at 37 °C (plates were

rocked every 10 min). At the indicated time points, 0.2 ml of supernatant was collected for virus titration by plaque assay using MDCK cells.

3.10 Infection of mice

Six to eight week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine. 50µl of infectious virus diluted in phosphate-buffered saline (PBS) and was inoculated intranasally (i.n). To determine virus pathogenicity, five animals per group were infected i.n. with different doses ($1X10^4$, $1X10^5$, $1X10^6$ PFU) and were monitored daily for weight loss for up to 14 days post infection (p.i). Mice showing more than 25% of body weight loss were considered to have reached predefined humane endpoints and were euthanized according to the institutional guidelines. To determine viral pulmonary replication kinetics, and to examine pulmonary pathology by histology, groups of 12 mice were inoculated with the indicated virus (1×10^5 PFU per mouse). Mice inoculated with PBS served as controls. At days 2 and day 4 p.i., three mice per experimental group per day were euthanized. For determination of virus titers, lungs were homogenized in 1 ml of PBS and viral titers were determined by plaque assays on MDCK cells. On days 2 and 4 p.i. (D2, D4), three mice per group were sacrificed for the histologic examination.

3.11 Histopathological examination

For the histopathological examination, the lungs from the sacrificed mice were removed and were infused with 10% neutral buffered formalin for >48 hours fixation, and then paraffin-embedded for sectioning. Lung sections were examined using light microscopy and images were obtained using an Olympus DP21 digital camera system.

4 Results and discussion

4.1 Papers I & II

In this thesis the whole genome sequences from two SIV isolates collected from Sweden in 2009 and 2010 were determined (Metreveli *et al.*, 2011; Bálint *et al*., 2009). 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 isolate and a full-length coding region in the more recent isolate. 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. A minority of the European SIVs have stop codons after 11, 25 and 43 amino acids (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 for 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.*, 2012) 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 provided 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 of 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. 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.

4.2 Paper III

Previous pandemic viruses have been postulated to evolve in swine from a mix of viral genes derived from viruses in birds, humans and swine. Studies by Khiabanian *et al.* (2009) have shown that, together with the glycoprotein coding segments HA and NA, the PB1 segment appears frequently in reassortment events among swine influenza viruses. Notably, the PB1 segment seems to be consistently involved in the reassortment events, particularly with 1957 (from avian species) and 1968 (from another avian virus) pandemic viruses as well as into swine viruses circulating since 1998 (Richt *et al.*, 2003; Zhou *et al.*, 1999; Kawaoka *et al.*, 1989). However, a clear mechanism of the preferential reassortment of this is still not fully understood.

The possible role in viral replication and virulence of the PB1 gene segments present in two swine H1N2 influenza A viruses was investigated here. In order to determine the contribution to virus replication and

pathogenicity of the PB1 gene products of these two SIVs four recombinant swine H1N2 influenza viruses were rescued by reverse genetics. These recombinant viruses included the parental sw1021 and sw9706 strains, and the chimeric viruses r1021 (9706 PB1), encoding the PB1 gene segment from the sw 9706 virus strain, and r9706 (1021 PB1), encoding the PB1 gene segment from the sw 1021 virus strain (Fig 4).

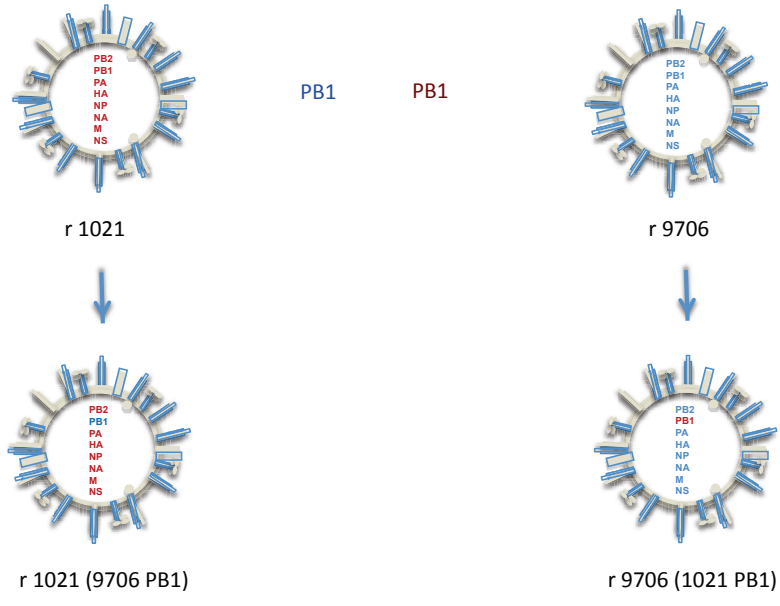

Figure 4. Schematics of the generated recombinant viruses r1021 and r9706 viruses and exchange of PB1 segment between the recombinants r1021 (9706 PB1) and r9706 (1021 PB1)

The replication kinetics of the recombinant viruses (r1021 and r9706) were compared to the replication of the PB1 exchanged recombinants r1021(9706 PB1) and r9706 (1021 PB1) in immortalized swine kidney cells (PK1 cells). Wild type sw 1021 and sw 9706 viruses were used as controls. PK1 cells were infected at an MOI of 0.01. Although there were slight differences in viral titers between 24 and 36 hours p.i. of PK1 cells, all virus titers became equal by 48 hours p.i. Plaque assays conducted in MDCK cells revealed no differences in plaque morphology, suggesting that there were no differences in replication phenotypes among the different viruses.

To determine whether the parental viruses differ in pathogenesis, and whether the PB1 segment could contribute to any observed differences, the C57 Bl/6 mouse model of influenza virus pathogenesis was utilized. Mice were infected with increasing doses ($1X10^4$, $1X10^5$, $1X10^6$ PFU) of the

indicated viruses, and were monitored daily for weight loss for up to 14 days p.i. At the highest inoculum tested, r1021 was more virulent than the r9706 virus, as evidenced by significant weight loss resulting in mice achieving the predefined endpoint, consistent with the differences seen for the wild type viruses. In the case of the chimeric viruses r1021(9706 PB1) and r9706(1021 PB1), mice infected with $1X10^6$ PFU of r9706(1021 PB1) showed a strong reduction in mouse weight loss (weight loss $\geq$ 21%). In contrast, only a mild reduction in weight loss (weight loss $\leq$ 10%) was observed for the mouse group inoculated with r1021 (9706 PB1) virus. The switch in virulence following the PB1 segment exchange showed that the PB1 gene of the r1021 virus increased the pathogenicity in the backbone of r9706 virus (Fig 5).

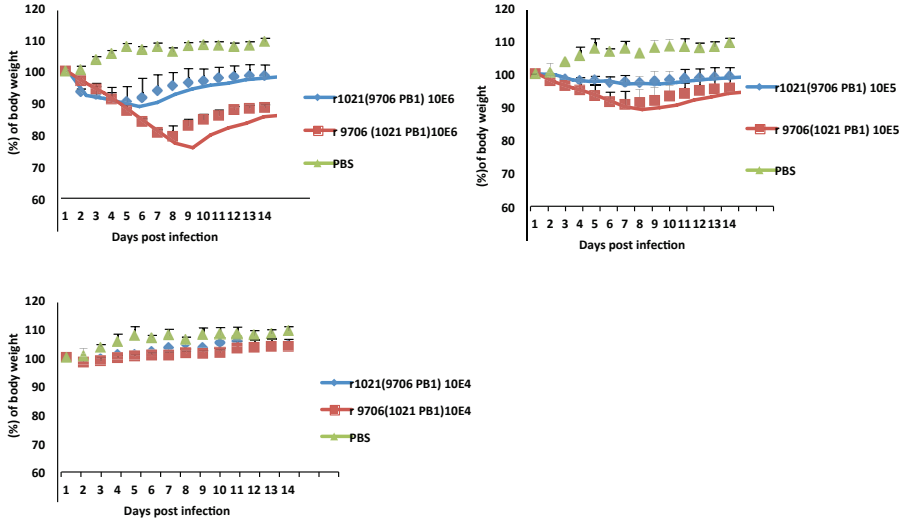

Figure 5. Pathogenesis of the chimeric r9706 (1021 PB1) virus in mice. Six to eight week-old female C57BL/6 were infected with increasing doses (1X104, 1X105, 1X106 PFU) of the chimeric r1021 (9706 PB1) and r9706 (1021 PB1) viruses and were monitored daily for weight loss for up to 14 days p.i. The chimeric virus r9706 (1021PB1), carrying PB1 gene from the r1021, induced significantly higher weight loss than the chimeric virus r1021 (9706 PB1).

In order to understand the differences in the virulence caused by infection of these recombinant and chimeric viruses, the virus replication were determined in the lungs of mice. Mice were infected i.n. with $1X10^5$ PFU of theses viruses. On days 2, and 4 p.i, three mice per group were sacrificed in order to determine viral titers in lung homogenates. The r1021 virus replicates with higher titer in mice lungs compared to r9706 at both days 2 and 4. However, after the PB1

segment was swapped, the virus lung titers were also changed accordingly, with the titer of r9706 (1021 PB1) higher than the r1021 (9706 PB1) (Fig 6).

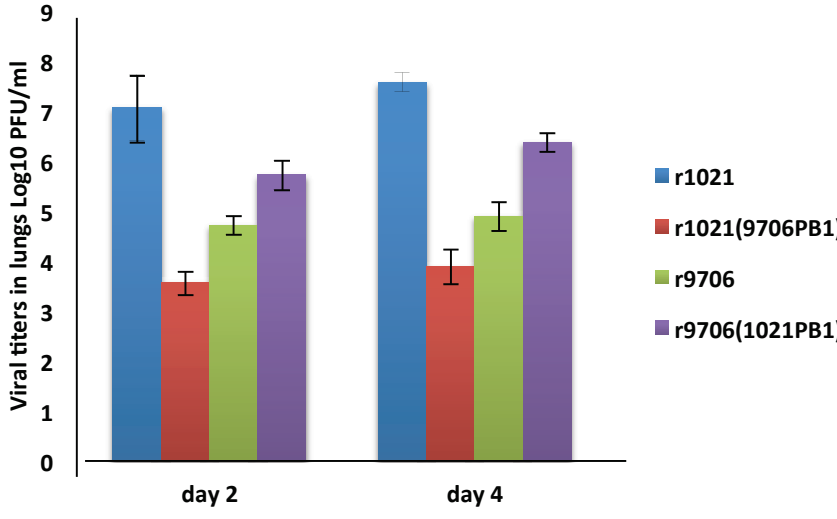

Figure 6. Mouse lung virus titers. Six mice per virus group for r1021, r9706, r1021 (9706 PB1) and r9706 (1021 PB1) were infected with 1X 10^5 PFU of each virus. Three mice from each group were sacrificed on days 2 and 4. Lung virus titers were evaluated by infection of MDCK cells.

These results in mice suggested that the PB1 segment might contribute to the virus virulence in Swedish H1N2 swine isolates. Macroscopically it was observed that mice inoculated i.n. with the r1021 virus showed gross pathology in lung appearance, with prominent edema, on both day 2 and day 4. The chimeric virus r9706 (1021 PB1) also showed lesions in the lung of one infected mouse. But by day 4 this was seen in all infected mice lungs. In histological examination, particular changes were seen in the lungs infected with the r1021 and r9706 (1021 PB1) viruses, with various degrees of leucocytes infiltrates and focal or diffuse alveolar damage with edema on both day 2 and day 4 p.i.

The PB1 subunit is a main component of the viral RNA polymerase complex and has multiple active sites critical for the elongation of RNA chains and also for association with subunits PA and PB2 to form a heterotrimer (Perez & Donis, 2001; Biswas & Nayak, 1996). The PB1 gene segment is important in reassortment events, particularly noted in the 1957 and 1968

pandemic viruses as well as in swine viruses circulating since 1998 (Kawaoka *et al*., 1989; Richt *et al*., 2003; Zhou *et al*., 1999). From our previous genetic analysis (Metreveli *et al*., 2011) we saw that the PB1 genes differ at four amino acid positions (75, 566, 587, 618), and the 2009 isolate has a truncated coding region of the PB1-F2 protein where 2010 has a full length version, which together or individually could cause the observed phenotypic difference between the viruses. Whether the truncated form in the 2009 isolate can be expressed as protein is not known. Regarding replication kinetics in cell culture, we did not find big differences between any of the viruses tested, in similarity with our previous results for the wild type viruses(Paper II). However, the aa differences could alter the structure of the PB1 protein in such a way that it contributes to viral fitness of the polymerase complex. If this could explain the difference in viral titers of mouse lung, and the pathological differences, remains to be seen. Further studies will be needed to identify the amino acid changes responsible within the PB1-segment as well as for the truncated PB1-F2 and full-length PB1-F2 for these phenotypes.

Overall, the findings showed that the PB1 gene segment of SIV H1N2 virus is an important determinant of virulence and replication in mice. The selection of "virulent" highly replicative PB1s may be important when influenza viruses cross species barriers, or when new viral strains are generated by reassortment. The success of influenza virus interspecies transmission depends on the viral gene constellation. Successful transmission between species can follow genetic reassortment with a progeny virus that contains a specific gene combination with the ability to replicate more efficiently in the new host.

5 Implications for future research, concluding remarks

Based on the work presented in this thesis, we aim to implement the aforementioned reverse genetics systems in Sweden and investigate further the findings from our research by studying functional differences among several SIV isolates. These unique reassortant variants of swine influenza virus have the potential to be a factor in the development of new pandemics; therefore, it is important to further investigate these viruses, including their replication characteristics, pathogenicity, virulence factors and immune modulation capacity in both swine and human cells. The association of truncated and full length forms of PB1-F2 with their biological implications for viral pathogenicity should be investigated by using the present RG system in a mouse model/swine.

A program where swine influenza viruses are systematically monitored should be established because of the potential, in swine hosts, of reassortment of gene segments of porcine, avian and human origin. It is important to monitor both swine populations and humans in their vicinity. The improved knowledge on the biology of these H1N2 reassortant SIV types will lead to a better diagnostic capacity, since upon the evaluation of their antigenic potential, recommendations will be made on their usage as antigens in routine diagnostic settings. This work would increase our knowledge of the genetic structure of SIV, provide better understanding of the molecular details of influenza virus replication processes from virus entry to exit, and help to examine the role of viral genes as markers for pathogenicity. Continuous and further detailed genetic and phenotypic analyses can help to identify novel viral variants with greater potential to cross species barriers and those that pose health risks to not just swine but even to humans and other host species.

Ultimately, this new data, when added to IV databases, will contribute to the general understanding of SIV biology, and potentially aid in the

preparedness against influenza outbreaks. The outcome of this project directly contributed to the improved detection and identification of novel variants of influenza viruses. In turn this contributes to the prevention of new pandemics caused by influenza viruses of swine origin and to the development of more powerful and specific control measures in the event of influenza outbreaks. The knowledge on all of these aspects of SIVs will strongly benefit agribusiness and science and be of great importance for public health as well.

In conclusion, the presented findings support the observations concerning the continuous reassortment process of SIVs, resulting in repeated and independent emergence of certain HA/NA combinations in pigs. Continuous and efficient surveillance and further detailed genetic and phenotypic analysis can help to identify novel viruses with more potential to cross species and pose health risks to humans. Furthermore, it is of major importance to study SIV in relation to its potential to evolve into severely pathogenic viruses in pigs. Respiratory disease of pigs is a major concern to pig health, and SIV is one important factor in this disease complex.

6 Populärvetenskaplig sammanfattning

Influensa A virus av subtyperna H1N1, H1N2 och H3N2 finns i svin populationer över hela världen, och kan där orsaka allvarlig respiratorisk sjukdom. Dessutom anses svin vara viktig när nya humana influensapandemier uppstår, och alla tidigare pandemier har sannolikt uppkommit i svin från en blandning av virusgener från fåglar, människor och svin. Därför är det mycket värdefullt att systematiskt följa utvecklingen av influensa hos svin, med fokus på kännetecken för dessa virus anpassning till människor. Dessa kännetecken är inte helt klarlagda, men innefattar förmågan att replikera snabbt och effektivt i humana celler, undgå medfödd immunitet och eventuellt andra ännu okända markörer. Av speciellt intresse är att europeiska svininfluensavirus (SIV) H1N2 av så kallad "aviär typ" tycks vara en av föregångarna till den pandemiska H1N1 influensan (H1N1pdm). Om H1N1pdm anpassar sig till svin kan det innebära problem för svinindustrin, eftersom majoriteten av svenska grisar är känsliga för viruset. Evolutionen av dessa H1N2-varianter är därför viktig att studera eftersom liknande SIV-typer också har beskrivits i Italien och Danmark, och risken för införande av stammar som nu associeras till de västra delarna av Europa inte bör underskattas.

Syftet med denna studie var att klarlägga svininfluensavirusets genetik, samt att undersöka betydelsen av olika virala gener som markörer för patogenicitet, hos två SIV H1N2-stammar med olika sjukdomsalstrande förmåga hos grisar. De är båda av en ovanlig SIV-variant som nyligen verkar att ha fått starkare fotfäste hos svenska svinbesättningar och dessutom ge allvarligare symptom. Virus isolerades på cellkultur från kliniskt material under sjukdomsutbrott 2009 och 2010, och hela virusgenomen sekvensbestämdes. Resultaten visade, för första gången i svenska svinbesättningar, naturlig reassortment av SIV-H1N2 virus, med aviär-liknande SIV H1N1 HA, och europeisk H3N2 SIV-liknande NA. Vid jämförelse av sekvenserna sågs stora skillnader mellan de två genomen, exempelvis i PB1-

gensegmentet, som ofta har visat sig bytas ut vid reassortment. Aviära PB1-gensegment har introducerats vid influensapandemierna 1957 och 1968, samt i SIV som cirkulerar sedan 1998. Mekanismen bakom denna preferens är inte helt klarlagd. Så kallad omvänd genetik, eller ”Reverse genetics” användes för att vidare studera genetiken bakom de båda virusens? olika patogenicitet, med fokus på PB1-gensegmentet. För detta ändamål utvecklades fyra rekombinanta virus, de båda ursprungsvirusen sw1021 och sw9706, samt de chimära virusen r1021(9706 PB1) och r9706(1021PB1) där PB1 gensegmentet var utbytt mot det andra virusets variant. Ökad virulens och replikation kunde härröras till PB1-segmentet från r1021 i infektionsförsök på mus. Sammanfattningsvis visade resultaten att PB1-gensegmentet hos SIV H1N2-viruset är en viktig faktor för virulens och replikation i möss. Våra iakttagelser stödjer tidigare observationer av SIVs benägenhet till reassortment hos grisar, vilket resulterar i en upprepad och oberoende uppkomst av vissa HA / NA kombinationer, liksom av övriga gensegment. Detta kan leda till nya och mer sjukdomsframkallande varianter av influensavirus hos gris. En kontinuerlig och effektiv övervakning samt en mer detaljerad genetisk och fenotypisk analys kan bidra till att identifiera sådana nya virala varianter, som kan ha större potential att korsa artbarriärer och medföra hälsorisker för människor såväl som andra värddjur.

References

Abusugra, I.A., Linné, T. & Klingeborn, B. (1987). The provenance of the Swedish swine influenza H1N1 virus of 1983. *Zentralblatt für Veterinärmedizin B* 34(8), 566-72.

Alexander, D.J. (1982). Ecological aspects of influenza A viruses in animals and their relationship to human influenza: a review. *Journal of The Royal Society of Medicine* 75(10), 799-811.

Alexander, D.J. (2000). A review of avian influenza in different bird species. *Veterinary Microbiology* 74(1-2), 3-13.

Baigent, S.J. & McCauley, J.W. (2001). Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. *Virus Research* 79(1-2), 177-85.

Bálint, A., Metreveli, G., Widén, F., Zohari, S., Berg, M., Isaksson, M., Renström, L.H., 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.

Biswas, S.K. & Nayak, D.P. (1996). Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. *Journal of Virology* 70(10), 6716-22.

Blakemore, F. & Gledhill, A.W. (1941). *Swine Influenza in the British Isles: Section of Comparative Medicine*. *Proceedings of the Royal Society of Medicine* 34(9):611-8.

Both, G.W., Siegman, L.J., Bellamy, A.R. & Atkinson, P.H. (1983). Coding assignment and nucleotide sequence of simian rotavirus SA11 gene segment 10: location of glycosylation sites suggests that the signal peptide is not cleaved. *Journal of Virology* 48(2), 335-9.

Brown, I.H. (2000). The epidemiology and evolution of influenza viruses in pigs. *Veterinary Microbiology* 74(1-2), 29-46.

Brown, I.H., Chakraverty, P., Harris, P.A. & Alexander, D.J. (1995). Disease outbreaks in pigs in Great Britain due to an influenza A virus of H1N2 subtype. *The Veterinary Record* 136(13), 328-9.

Brown, I.H., Harris, P.A., McCauley, J.W. & 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. *Journal of General Virology* 79 (Pt 12), 2947-55.

Brown, I.H., Hill, M.L., Harris, P.A., Alexander, D.J. & McCauley, J.W. (1997). Genetic characterisation of an influenza A virus of unusual subtype (H1N7) isolated from pigs in England. *Archives of Virology* 142(5), 1045-50.

Campitelli, L., Donatelli, I., Foni, E., Castrucci, M.R., Fabiani, C., Kawaoka, Y., Krauss, S. & Webster, R.G. (1997). Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. *Virology* 232(2), 310-8.

Castrucci, M.R., Donatelli, I., Sidoli, L., Barigazzi, G., Kawaoka, Y. & Webster, R.G. (1993). Genetic reassortment between avian and human influenza A viruses in Italian pigs. *Virology* 193(1), 503-6.

Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J.R. & Yewdell, J.W. (2001). A novel influenza A virus mitochondrial protein that induces cell death. *Nature Medicine* 7(12), 1306-12.

Chen, Z., Wang, W., Zhou, H., Suguitan, A.L., Jr., Shambaugh, C., Kim, L., Zhao, J., Kemble, G. & Jin, H. (2010). Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. *Journal of Virology* 84(1), 44-51.

Cheung, T.K. & Poon, L.L. (2007). Biology of influenza a virus. *Annals of The New York Academy of Sciences* 1102, 1-25.

Chizhmakov, I.V., Geraghty, F.M., Ogden, D.C., Hayhurst, A., Antoniou, M. & Hay, A.J. (1996). Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells. *The Journal of Physiology* 494 (Pt 2), 329-36.

Chu, C.M., Dawson, I.M. & Elford, W.J. (1949). Filamentous forms associated with newly isolated influenza virus. *Lancet* 1(6554), 602.

Connor, R.J., Kawaoka, Y., Webster, R.G. & Paulson, J.C. (1994). Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 205(1), 17-23.

Cross, K.J., Burleigh, L.M. & Steinhauer, D.A. (2001). Mechanisms of cell entry by influenza virus. *Expert Reviews in Molecular Medicine* 3(21), 1-18.

Drake, J.W. (1993). Rates of spontaneous mutation among RNA viruses. *Proceedings of the National Academy of Sciences of the United States of America* 90(9), 4171-5.

Dunham, E.J., Dugan, V.G., Kaser, E.K., Perkins, S.E., Brown, I.H., Holmes, E.C. & Taubenberger, J.K. (2009). Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses. *Journal of Virology* 83(11), 5485-94.

Enami, M., Luytjes, W., Krystal, M. & Palese, P. (1990). Introduction of site-specific mutations into the genome of influenza virus. *Proceedings of the National Academy of Sciences of the United States of America* 87(10), 3802-5.

Fodor, E., Devenish, L., Engelhardt, O.G., Palese, P., Brownlee, G.G. & Garcia-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *Journal of Virology* 73(11), 9679-82.

Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B. & Osterhaus, A.D. (2005). Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of Virology* 79(5), 2814-22.

Gambotto, A., Barratt-Boyes, S.M., de Jong, M.D., Neumann, G. & Kawaoka, Y. (2008). Human infection with highly pathogenic H5N1 influenza virus. *Lancet* 371(9622), 1464-75.

Gao, Q., Brydon, E.W. & Palese, P. (2008). A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. *Journal of Virology* 82(13), 6419-26.

Ghedin, E., Sengamalay, N.A., Shumway, M., Zaborsky, J., Feldblyum, T., Subbu, V., Spiro, D.J., Sitz, J., Koo, H., Bolotov, P., Dernovoy, D., Tatusova, T., Bao, Y., St George, K., Taylor, J., Lipman, D.J., Fraser, C.M., Taubenberger, J.K. & Salzberg, S.L. (2005). Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* 437(7062), 1162-6.

Gourreau, J.M., Kaiser, C., Valette, M., Douglas, A.R., Labie, J. & Aymard, M. (1994). Isolation of two H1N2 influenza viruses from swine in France. *Archives of Virology* 135(3-4), 365-82.

Guan, Y., Shortridge, K.F., Krauss, S., Li, P.H., Kawaoka, Y. & Webster, R.G. (1996). Emergence of avian H1N1 influenza viruses in pigs in China. *Journal of Virology* 70(11), 8041-6.

Hansen, M.S., Pors, S.E., Jensen, H.E., Bille-Hansen, V., Bisgaard, M., Flachs, E.M. & Nielsen, O.L. (2010). An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. *Journal of Comparative Pathology* 143(2-3), 120-31.

Harkness, W., Schild, G.C., Lamont, P.H. & Brand, C.M. (1972). Studies on relationships between human and porcine influenza. 1. Serological evidence of infection in swine in Great Britain with an influenza A virus antigenically like human Hong Kong-68 virus. *Bulletin of the World Health Organization* 46(6), 709-19.

Helenius, A. (1992). Unpacking the incoming influenza virus. *Cell* 69(4), 577-8.

Hinshaw, V.S., Bean, W.J., Jr., Webster, R.G. & Easterday, B.C. (1978). The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine. *Virology* 84(1), 51-62.

Hoehling, A.A. (1961). *The Great Epidemic*. Boston Little, Brown and Company.

Holsinger, L.J., Nichani, D., Pinto, L.H. & Lamb, R.A. (1994). Influenza A virus M2 ion channel protein: a structure-function analysis. *Journal of Virology* 68(3), 1551-63.

Honda, A., Ueda, K., Nagata, K. & Ishihama, A. (1987). Identification of the RNA polymerase-binding site on genome RNA of influenza virus. *Journal of Biochemistry* 102(5), 1241-9.

Horimoto, T. & Kawaoka, Y. (2005). Influenza: lessons from past pandemics, warnings from current incidents. *Nature Reviews Microbiology* 3(8), 591-600.

Horimoto, T. & Kawaoka, Y. (2006). Strategies for developing vaccines against H5N1 influenza A viruses. *Trends in Molecular Medicine* 12(11), 506-14.

Howard, W.A., Essen, S.C., Strugnell, B.W., Russell, C., Barass, L., Reid, S.M. & Brown, I.H. (2011). Reassortant Pandemic (H1N1) 2009 virus in pigs, United Kingdom. *Emerging Infectious Diseases* 17(6), 1049-52.

Ito, T., Kawaoka, Y., Vines, A., Ishikawa, H., Asai, T. & Kida, H. (1998). Continued circulation of reassortant H1N2 influenza viruses in pigs in Japan. *Archives of Virology* 143(9), 1773-82.

Johnson, N.P. & Mueller, J. (2002). Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bulletin of The History of Medicine* 76(1), 105-15.

Karasin, A.I., Landgraf, J., Swenson, S., Erickson, G., Goyal, S., Woodruff, M., Scherba, G., Anderson, G. & Olsen, C.W. (2002). Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. *Journal of Clinical Microbiology* 40(3), 1073-9.

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Archiv für Experimentelle Pathologie und Pharmacologie* 162, 480-483.

Karlas, A., Machuy, N., Shin, Y., Pleissner, K.P., Artarini, A., Heuer, D., Becker, D., Khalil, H., Ogilvie, L.A., Hess, S., Maurer, A.P., Muller, E., Wolff, T., Rudel, T. & Meyer, T.F. (2010). Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature* 463(7282), 818-22.

Kawaoka, Y., Krauss, S. & Webster, R.G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *Journal of Virology* 63(11), 4603-8.

Khiabanian, H., Trifonov, V. & Rabadan, R. (2009). Reassortment patterns in Swine influenza viruses. *PLoS Currents* 1, RRN1008.

Kiss, I., Bálint, A., Metreveli, G., Emmoth, E., Widén, F., Belák, S. & Wallgren, P. (2010). Swine influenza viruses isolated in 1983, 2002 and 2009 in Sweden exemplify different lineages. *Acta Veterinaria Scandinavica* 52, 65.

Kiss, I., Gyarmati, P., Zohari, S., Ramsay, K.W., Metreveli, G., Weiss, E., Brytting, M., Stivers, M., Lindstrom, S., Lundkvist, A., Nemirov, K., Thorén, P., Berg, M., Czifra, G. & Belák, S. (2008). Molecular characterization of highly pathogenic H5N1 avian influenza viruses isolated in Sweden in 2006. *Virology Journal* 5, 113.

Konig, R., Stertz, S., Zhou, Y., Inoue, A., Hoffmann, H.H., Bhattacharyya, S., Alamares, J.G., Tscherne, D.M., Ortigoza, M.B., Liang, Y., Gao, Q., Andrews, S.E., Bandyopadhyay, S., De Jesus, P., Tu, B.P., Pache, L., Shih, C., Orth, A., Bonamy, G., Miraglia, L., Ideker, T., Garcia-Sastre, A., Young, J.A., Palese, P., Shaw, M.L. & Chanda, S.K. (2010). Human host factors required for influenza virus replication. *Nature* 463(7282), 813-7.

Krug, R.M., Alonso-Caplen, F.V., Julkunen, I. & Katze, M.G. (1989). Expression and replication of the influenza virus genome In. pp. 89-152 Plenum Publishing Corp., New York.

Kuntz-Simon, G. & Madec, F. (2009). Genetic and Antigenic Evolution of Swine Influenza Viruses in Europe and Evaluation of Their Zoonotic Potential. *Zoonoses Public Health* 56(6-7):310-25.

Lamb, R.A. & Choppin, P.W. (1979). Segment 8 of the influenza virus genome is unique in coding for two polypeptides. *Proceedings of the National Academy of Sciences of the United States of America* 76(10):4908-12.

Lamb, R.A. & Choppin, P.W. (1981). Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. *Virology* 112(2), 729-37.

Lamb, R.A. & Choppin, P.W. (1983). The gene structure and replication of influenza virus. *Annual Review of Biochemistry* 52, 467-506.

Lamont, H.G. (1938). The problemns of the practitioner in connection with the differential diagnosis and traetment of diseases of young pigs. *Veterinary Record.* 50, 1377.

Lange, J., Groth, M., Schlegel, M., Krumbholz, A., Wieczorek, K., Ulrich, R., Koppen, S., Schulz, K., Appl, D., Selbitz, H.J., Sauerbrei, A., Platzer, M., Zell, R. & Durrwald, R. (2013). Reassortants of the pandemic (H1N1) 2009 virus and establishment of a novel porcine H1N2 influenza virus, lineage in Germany. *Veterinary Microbiology* 167(3-4), 345-56.

Luytjes, W., Krystal, M., Enami, M., Parvin, J.D. & Palese, P. (1989). Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* 59(6), 1107-13.

Marozin, S., Gregory, V., Cameron, K., Bennett, M., Valette, M., Aymard, M., Foni, E., Barigazzi, G., Lin, Y. & Hay, A. (2002). Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. *Journal of General Virology* 83(Pt 4), 735-45.

Martin, K. & Helenius, A. (1991). Transport of incoming influenza virus nucleocapsids into the nucleus. *Journal of Virology* 65(1), 232-44.

Martinsson, K., Klingeborn, B. & Rockborn, G. (1983). Utbrott av influenza suis i Sverige (in Swedish). *Svensk Veterinärtidning* 35:537.

Masurel, N., de Boer, G.F., Anker, W.J. & Huffels, A.D. (1983). Prevalence of influenza viruses A-H1N1 and A-H3N2 in swine in the Netherlands. *Comparative Immunology, Microbiology and Infectious Diseases* 6(2), 141-9.

Comparative Immunology, Microbiology and Infectious Diseases 6(2), 141-9.

McGeoch, D., Fellner, P. & Newton, C. (1976). Influenza virus genome consists of eight distinct RNA species. *Proceedings of the National Academy of Sciences of the United States of America* 73(9), 3045-9.

Metreveli, G. (2012). *Swine Influenza A virus subtype H1N2 in Sweden*. Diss. Uppsala: Swedish University of Agricultural Sciences.

Metreveli, G., Emmoth, E., Zohari, S., Bálint, A., 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(2):236-44.

Moreno, A., Barbieri, I., Sozzi, E., Luppi, A., Lelli, D., Lombardi, G., Zanoni, M.G. & Cordioli, P. (2009). Novel swine influenza virus subtype H3N1 in Italy. *Veterinary Microbiology* 138(3-4), 361-7.

Moreno, A., Chiapponi, C., Boniotti, M.B., Sozzi, E., Foni, E., Barbieri, I., Zanoni, M.G., Faccini, S., Lelli, D. & Cordioli, P. (2012). Genomic characterization of H1N2 swine influenza viruses in Italy. *Veterinary Microbiology* 156(3-4), 265-76.

Moreno, A., Di Trani, L., Faccini, S., Vaccari, G., Nigrelli, D., Boniotti, M.B., Falcone, E., Boni, A., Chiapponi, C., Sozzi, E. & Cordioli, P. (2011). Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. *Veterinary Microbiology* 149(3-4), 472-7.

Nardelli, L., Pascucci, S., Gualandi, G.L. & Loda, P. (1978). Outbreaks of classical swine influenza in Italy in 1976. *Zentralblatt für Veterinärmedizin B* 25(10), 853-7.

Neumann, G., Noda, T. & Kawaoka, Y. (2009). Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 459(7249), 931-9.

Neumeier, E., Meier-Ewert, H. & Cox, N. (1994). Genetic relatedness between influenza A (H1N1) viruses isolated from humans and pigs. *The Journal of General Virology* 75 (Pt 8):2103-7.

O'Neill, R.E., Talon, J. & Palese, P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *The EMBO Journal* 17(1), 288-96.

Olsen, C., Brown, I., Easterday, B. & al, e. (2005). Swine influenza. In: Straw B*, et al.* (Eds.) *Diseases of Swine*. Iowa: State University Press, Iowa.

Olsen, C.W., Brown, I.H., Easterday, B., and & K. van Reeth (2006). Swine influenza. In: E, S.B*., et al.* (Eds.) *Disease of Swine*. pp. pp. 469–482 Blackwell Publishing, Oxford.

Osterhaus, A.D., Rimmelzwaan, G.F., Martina, B.E., Bestebroer, T.M. & Fouchier, R.A. (2000). Influenza B virus in seals. *Science* 288(5468), 1051-3.

Palese, P. (2004). Influenza: old and new threats. *Nature Medicine* 10(12 Suppl), S82-7.

Palese, P. & Shaw, M. (2006). Orthomyxoviridae: the viruses and their replication. In: Knipe, D.M., *et al.* (Eds.) *Fields Virology. 5th ed*. p. 1647e1689. Philadelphia: Lippincott Williams &Wilkins.

Parvin, J.D., Palese, P., Honda, A., Ishihama, A. & Krystal, M. (1989). Promoter analysis of influenza virus RNA polymerase. *Journal of Virology* 63(12), 5142-52.

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

Perez, D., Rimstad, E., Smith, G. & al., e. (2011). Orthomyxoviridae. In: King AMQ, *et al.* (Eds.) *Virus Taxonomy*. pp. 749 - 762 Oxford: Elsevier.

Perez, D.R. & Donis, R.O. (2001). Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. *Journal of Virology* 75(17), 8127-36.

Pinto, L.H., Holsinger, L.J. & Lamb, R.A. (1992). Influenza virus M2 protein has ion channel activity. *Cell* 69(3), 517-28.

Plotch, S.J., Bouloy, M., Ulmanen, I. & Krug, R.M. (1981). A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* 23(3), 847-58.

Potier, M., Mameli, L., Belisle, M., Dallaire, L. & Melancon, S.B. (1979). Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. *Analytical Biochemistry* 94(2), 287-96.

Richt, J.A., Lager, K.M., Janke, B.H., Woods, R.D., Webster, R.G. & Webby, R.J. (2003). Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *Journal of Clinical Microbiology* 41(7), 3198-205.

Roberts, D.H., Cartwright, S.F. & Wibberley, G. (1987). Outbreaks of classical swine influenza in pigs in England in 1986. *The Veterinary Record* 121(3), 53-5.

Ruigrok, R.W., Calder, L.J. & Wharton, S.A. (1989). Electron microscopy of the influenza virus submembranal structure. *Virology* 173(1), 311-6.

Scholtissek, C. (1994). Source for influenza pandemics. *European Journal of Epidemiology* 10(4), 455-8.

Scholtissek, C., Rohde, W., Von Hoyningen, V. & Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* 87(1), 13-20.

Shaw, M.L. & Palese, P. (2008). Orthomyxoviruses: Molecular biology. In: Mahy, B., *et al.* (Eds.) *Encyclopedia of Virology*. pp. 483-489 Oxford, U.K.: Elsevier.

Shih, S.R. & Krug, R.M. (1996). Surprising function of the three influenza viral polymerase proteins: selective protection of viral mRNAs against the cap-

snatching reaction catalyzed by the same polymerase proteins. *Virology* 226(2), 430-5.

Shope, R.E. (1931). The Etiology of Swine Influenza. *Science* 73(1886), 214-5.

Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A. & Wiley, D.C. (1982). Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proceedings of the National Academy of Sciences of the United States of America* 79(4), 968-72.

Skehel, J.J. & Wiley, D.C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual Review of Biochemistry* 69, 531-69.

Sorensen, K.J., Have, P.D., Ronsholt, L., Hage, J.M., Westergaard, and & Pedersen, K.B. (1981). Influenza hos svin I Danmark. *Dansk Veterinærtidsskrift* 64, 826–829.

Stallknecht, D.E., Shane, S.M., Kearney, M.T. & Zwank, P.J. (1990). Persistence of avian influenza viruses in water. *Avian Diseases* 34(2), 406-11.

Sugrue, R.J. & Hay, A.J. (1991). Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology* 180(2), 617-24.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24(8), 1596-9.

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., Chen, L.M., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., McBride, R., Carney, P.J., Gilbert, A.T., Chang, J., Guo, Z., Davis, C.T., Paulson, J.C., Stevens, J., Rupprecht, C.E., Holmes, E.C., Wilson, I.A. & Donis, R.O. (2013). New world bats harbor diverse influenza a viruses. *PLoS Pathogens* 9(10), e1003657.

Tong, S.X., Li, Y., Rivailler, P., Conrardy, C., Castillo, D.A.A., Chen, L.M., Recuenco, S., Ellison, J.A., Davis, C.T., York, I.A., Turmelle, A.S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M.R., Tang, K., Rowe, L.A., Sammons, S., Xu, X.Y., Frace, M., Lindblade, K.A., Cox, N.J., Anderson, L.J., Rupprecht, C.E. & Donis, R.O. (2012). A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences of the United States of America* 109(11), 4269-4274.

Trebbien, R., Bragstad, K., Larsen, L.E., Nielsen, J., Botner, A., Heegaard, P.M., Fomsgaard, A., Viuff, B. & 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. *Virology Journal* 10(1), 290.

Van Reeth, K., Brown, I.H. & Pensaert, M. (2000). Isolations of H1N2 influenza A virus from pigs in Belgium. *The Veterinary Record* 146(20), 588-9.

Wallgren, P., Gerth Löfstedt, M. & Paulsson, M. (2009). New strain of influenza virus, H1N2, demonstrated in pigs in Sweden (in Swedish). *Svensk Veterinärtidning*.

Wang, C., Lamb, R.A. & Pinto, L.H. (1994). Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells. *Virology* 205(1), 133-40.

Wang, C., Takeuchi, K., Pinto, L.H. & Lamb, R.A. (1993). Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. *Journal of Virology* 67(9), 5585-94.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiological Reviews* 56(1), 152-79.

Wise, H.M., Foeglein, A., Sun, J., Dalton, R.M., Patel, S., Howard, W., Anderson, E.C., Barclay, W.S. & Digard, P. (2009). A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *Journal of Virology* 83(16), 8021-31.

Yuanji, G. & Desselberger, U. (1984). Genome analysis of influenza C viruses isolated in 1981/82 from pigs in China. *Journal of General Virology* (Pt 11), 1857-72.

Zell, R., Krumbholz, A., Eitner, A., Krieg, R., Halbhuber, K.J. & Wutzler, P. (2007). Prevalence of PB1-F2 of influenza A viruses. *Journal of General Virology* 88(Pt 2), 536-46.

Zhou, N.N., Senne, D.A., Landgraf, J.S., Swenson, S.L., Erickson, G., Rossow, K., Liu, L., Yoon, K., Krauss, S. & Webster, R.G. (1999). Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *Journal of Virology* 73(10), 8851-6.

Acknowledgements

The studies presented in this thesis were performed at the Section of Virology, Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Science, SLU, Uppsala, in collaboration with Department of Virology, Immunology and Parasitology, The National Veterinary Institute (SVA) and the Department of Microbiology Icahn School of Medicine at Mount Sinai, New York City, USA.

Financial support was provided by the "stimulansmedel" of Professor Mikael Berg, Award of Excellence of Professor Sándor Belák, and The Swedish Research Council's (Formas) "Strong Research Environments" project, "Next Generation Microfluidic Pathogen Detection Platforms", acronym BioBridges (no. 2011-1692).

This work was partially supported by CRIP (Center for Research on Influenza Pathogenesis) a NIAID funded Center of Excellence for Influenza Research and Surveillance (CEIRS, contract number HHSN266200700010C) to Professor Adolfo García-Sastre. This work was also supported by additional grants from The Swedish Research Council (Formas) and The Royal Swedish Academy of Agriculture and Forestry (KSLA).

I would like to express my appreciation and sincere gratitude to my work colleagues:

Former and present heads of the Department of Biomedical Sciences and Veterinary Public Health: Professor Martin Wierup and Professor Leif Norrgren and the head of Division Associate Professor Gunilla Trowald-Wigh for your encouragement and support during my research.

Former and present heads of the Department of Virology, Immunology and Parasitology at SVA, Professor Berndt Klingeborn, Dr. Jens Mattsson and Dr. Jean-Francois Valarcher, for creating a comfortable working environment.

Professor Mikael Berg, my main supervisor, for your continuous guidance and support throughout my thesis project, and for your invaluable suggestions, encouragement and scientific discussions.

Professor Sándor Belák, my co-supervisor, for giving me the opportunity to start and carry out this work and providing invaluable and continuous support, for granting my research, and for your inspiring ideas, encouragement and help.

Professor Adolfo García-Sastre, I am truly thankful for giving me the opportunity to perform my experiments in your laboratory, sharing your knowledge and showing great interest in my work. Thank you also for your financial and scientific support, which allowed me to generate and motivate new ideas. Without your help and guidance I would not have completed my study.

István Kiss, my former co-supervisor, for initiating and carrying out the swine influenza project work, and for your support, suggestions and contributions to the work.

Mikael Leijon, my co-supervisor, for providing support and for all your guidance and help in my work.

Neil LeBlanc, my co-supervisor, for your support, research suggestions and encouragement throughout my thesis.

I would like to thank all my co-authors for your valuable contributions, discussion and comments on manuscript.

I would like to thank many current or former colleagues at SLU/SVA VIP, Department of BVF and at other Departments for all of your company both in the lab as well as during different social events: Anna M. T., Ákos, Amin, Anna-Malin, Attila, Babu, Balaje, Bilal, Christina, Gordana, Fredrik G., Krister B., Linda, Miklós, Martí, Mehdi, Peter, Rajiv, Valeria, Vinay, Zoltán, Sandra, Shahid, Åsa, Anna R., Jolanta, Annie P. W., Anne-Sofie O., Gunilla B.,

Erik H., Olov C., Eva M., Sofia H., Sofia S., Malin H. G., Andrea M., Karin, Marlene A., Camilla G., Annie E., Kim R., with special thanks to:

Mikhayil Hakhverdyan, for sharing your deep knowledge in real time PCR and being always kind and helpful in science and outside of science, for your friendship and having always an open door for me, and for providing me great feedback, reading my papers and giving me the best suggestions, I am thankful for all your support and help.

Alia Yacoub, for your friendship and support, for being always helpful, for your encouragement and all interesting discussions on many different subjects, and for your delicious meals and always saying the right things.

Claudia Baule, for sharing your knowledge, excellent advices, always having answers on my questions, and for being friendly and helpful.

Jonas J. Wensman, my first office mate and very good friend, for our great scientific conversations, and for all good times at work and outside of work.

Anne-Lie Blomström, for being such a very good friend. I could always share my thoughts with you. Thank you for your support, being a great office mate and travel-partner.

Johanna Lindahl, my “half-time” office mate, sharing the desk with you was always fun as well as working in the lab, for organizing various dinners and barbeques, and for our discussions about other important matters in life.

Siamak Zohari, co-author, and friend, thank you for sharing your knowledge and for all our various conferences, workshops and lab courses with veterinary students.

Jay Lin, for your friendship and support, for our chats and for being such a good person and always sharing information about upcoming courses.

Jenna Anderson, for giving me great feedback, reading my papers and giving good suggestions, and being a very good friend, former office mate, colleague and training mate.

Muhammad Munir, for being a great office mate, co-author and friend, and for your constructive comments, support, encouragement and discussions about science and life.

Jörgen Fidjeland, for our interesting discussions about science and life at lunches and coffee breaks, and for helping me understand the Norwegian subway system.

Harindranath Cholleti, my office mate and friend, for our discussions about science and life, and for your support and encouragement.

Maja Malmberg, my recent office mate, for our chats and nice company on after works.

Oskar Karlsson, for your friendship, all our interesting chats and your company during the lab course.

Lihong Liu, for being always friendly and giving me a helping hand.

Hongyan Xia, for your skilful help in my protein work.

Tommy Linné, for your expert help in western blot experiments

Karl Ståhl, for our interesting scientific discussions, for being a good friend, for various workshops even in "my" Georgia☺, and for good memories working with Tajik colleagues.

Ádám Bálint, co-author, for your valuable contribution to the work, discussions and comments on manuscript.

Thomas Segall, co-author, for your expert pathological help with influenza samples.

Frederik Widén, co-author, for your constructive advice on manuscripts, and for giving me the opportunity to work on Hepatitis E.

Lena Renström, co-author, for your support and help in veterinary issues and in getting detailed information from SVALA.

Per Wallgren, co-author, for your constructive support sharing your expert knowledge in swine medicine. I appreciate your encouragement,
our discussions and your comments on my work.

Eva Emmoth, co-author, for sharing your great knowledge, for your critical thoughts, constructive advice on manuscript, and for always being supportive.

Shaman Muradrasoli, co-author, for your help with performing the “munana” assay.

Mats Isaksson, co-author, for your generous help and for sharing various PCR protocols.

Louise Treiberg Berndtsson, for always giving me answers to my questions.

Jan Chirico, for nice company in Saint-Malo, and for all our interesting discussions.

Cecilia Hultén, for very interesting work in the decontamination project.

Ulrika Allard Bengtsson, for introducing me the BSL3 laboratory and the decontamination project.

Anna Lindhe, for nice company in Budapest and for all our chats.

Eva Liljekvist, for your help with ELISA work with veterinary students, and for always being a good friend.

Anna-Britta Pettersson, for your expert help with influenza isolation work and for all our nice chats.

Fakhri Shahi, for your help with HI test for the veterinary student course, and for always being nice and helpful.

Anna Lundén, for your kind help and for answering back patiently to all my questions.

Karin Ullman, for always being helpful and for nice company at after works.

Pia Fällgren, for nice cooperation and our chats about running.

Anna Rothman, for your excellent administrative help and for all kinds of practical matters.

David Morrison, for prompt answers on my emails and for language revision of my thesis.

Jonas Tallkvist, for your enjoyable course about Principles of molecular biology and its application in veterinary pathobiology, and for your help with siRNA work.

Anne-Sofie Lundquist, Karin Rudolfsson, Madeleine Johansson and Annkristin Andersson for various administrative help.

Sten-Olof Fredriksson, Johnny Ericsson and Mikael Pettersson, for your help and support with technical computer problems.

Sven-Åke Bergqvist, "Berka", for your excellent technical assistance, for our nice chats and for always helping with various matters.

Sevinc Ferrari, I am thankful for all your help and support. You were always very kind and nice, for your supportive talks, for our good times and for being a great friend.

Anthony Österdahl, thank you for introducing me to the running world and for always being a great person and friend. I remember our trip to Turku, my first marathon, I thought I would never come to the finish because of the heat, but we made it.

Stefan Widgren, for your friendship and nice company at lunches and afterwork.

Alkwin Wanders, for your expert pathological help, and for your friendship and morning rowing sessions.

Helena Back, colleague and training mate, for your friendship and for your excellent tips in rowing.

Malik Merza, for your support, inspiration and all our interesting discussions about science and many other aspects of life, for always being friendly and showing great hospitality.

Paulo Eduardo Brandão, thank you for your friendship and encouragement, for all your stimulating e-mails and for your great hospitality during my stay in Brazil.

Dimitris Papageorgiou, for your help whenever I needed printing my posters over these years and for all our chats and your friendship.

I would also like to thank all of Adolfo García-Sastre's laboratory members for creating a calm and wonderful working environment.

Thanks to all my friends at the rowing club, Uppsala Akademiska Roddarsällskap (UARS), for lots of great trainings, competitions and social events.

Albert Mihranyan, my very good friend, for your warm support, help, for all our great times, and for our discussions about science and life. I am thankful to you for introducing me into the nanoworld and for interesting and harmonized work.

My brilliant friends outside work: David Khoshtaria, Zura Chemia, Jonas Berggren, Carl Olsson, Yury Dorofeev, Lisa Ekstrand, Asta Sarkki, Stefanie Haese, Teemu Mönkkönen, for your supportive talks, dinners, discussions about this and that, for our good times and for being great friends. Thank you for all our adventures since we met, for your company, for encouragement and support and for helping me get through difficult times.

Sarah McGill, for your support and encouragement, for all our interesting discussions about many other aspects of life, I am glad to know you.

I have many friends and colleagues worldwide. I did not forget about you, my dear friends. You are all important people to me and I am thankful for your friendship and support!

My dear parents, you have done so much for me. Thank you for your understanding and encouragement, for the many years' of hard work to support my studies and life. My brother Alexander with his wonderful family, wife

Nino and their little daughter Barbara, you have always been encouraging. My beautiful sister Magdalena, thank you for your continuous support, understanding and inspiration. Thank you, for always being there whenever I need it. Without your help I could not have reached so far.