LIVE ATTENUATED SWINE INFLUENZA VACCINE BY REVERSE GENETICS

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ABSTRACT

Swine influenza (SI) is an acute, highly contagious, respiratory disease of swine. The causative agent of SI infections is swine influenza virus (SIV). SIV is a type A influenza virus classified into the *Orthomyxoviridae* family and is an enveloped particle with a genome composed of eight negative-orientated RNA segments.

The mortality rate of influenza disease in pigs is generally low but morbidity can reach up to 100%. SI infections considerably contribute to respiratory disease in post-weaning pigs, causing significant economic losses due to an increase in the number of days pigs need to reach market weight. In addition, SI infections possess significant human public health concerns.

Vaccination is the primary method for the prevention of SI. Currently available vaccines against SI are a combination of two inactivated antigenically distinct SIVs with oil adjuvant. The application of these vaccines induce mainly humoral immune responses. In contrast, application of live attenuated influenza vaccines (LAIV) mimics natural infection and induce strong, long-lived cell-mediated and humoral immunity. Furthermore, LAIV induces cross-protective immunity against different subtypes of influenza A viruses. LAIVs are developed for human and equine influenza viruses but at present no LAIV is available for SIVs.

The critical step in influenza virus infection is an initial interaction between virus and cell surface carbohydrates followed by receptor-mediated endocytosis and fusion of the viral and endosomal membranes. Influenza virus entry into cells is mediated by the viral surface glycoprotein hemagglutinin (HA). HA is primary synthesized as a polypeptide in HA0 form. In order to be infectious, HA0 must be cleaved by host proteases into HA1 and HA2 subunits. Therefore, this process is crucial determinant for virus pathogenicity.

Our objective was to generate a live attenuated SIVs, particularly a viruses with a modified HA cleavage site resistant to activation during natural infection but which can be activated *in vitro* by an exogenous protease. Using the reverse genetics technique, we generated two mutant SIVs of strain A/SW/SK/18789/02 (H1N1) containing a modified cleavage site within their HA. Mutant A/SW/SK-R345V (R345V) contained a mutation within HA segment at amino acid (AA) position 345 from Arginine (Arg) to Valine (Val) while the second mutant, A/SW/SK-R345A (R345A) encoded Alanine (Ala) instead of Arginine (Arg) at position AA345 on HA. We showed that HA cleavage in both mutants was strictly dependent on the presence of human

neutrophil elastase in tissue culture. These tissue-culture grown mutant SIVs showed similar growth properties in terms of plaque size and growth kinetics, compared to the wild type virus. Both mutant SIVs were able to preserve introduced mutations after multiple passages in tissue culture suggesting that AA substitution within HA cleavage site did not alter genetic stability in the presence of appropriate protease. Furthermore, these mutant SIVs were highly attenuated in pigs but capable of inducing significant cell-mediated and humoral immune responses after two vaccinations via intratracheal (IT) and intranasal (IN) routes. Immune responses induced by vaccination with elastase dependent SIV were sufficient to confer full protection against parental homologous and antigenic variant of H1N1 SIVs and partial protection from heterologous subtypic H3N2 after the challenge. Therefore, elastase-dependent mutant SIV could serve as live vaccine against antigenically distinct swine influenza viruses in pigs.

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DEDICATION

For my wife Ivona and son Stefan,

You are my inspiration and meaning of life …

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ABBREVIATIONS USED

AA	Amino acid
Ala	Alanine
ANOVA	One-way analysis of variance
APC	Antigen presenting cell
ASC	Antibody secreting cell
AI	Avian influenza
BCIP/NBT	5-Bromo-4-chro-3-indolyl phosphate/nitroblue tetrazolium
BALF	Broncho alveolar lavage fluid
BSA	Bovine serum albumin
CAT	Chloramphenicol-acetyl-transferase
CFIA	Canadian Food Inspection Agency
CMI	Cell mediated immunity
cpm	Counts per minute
CTLs	Cytotoxic lymphocites
cH1N1	Classical H1N1
cRNA	Complementary RNA
cDNA	Complementary deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreiol
d.p.i	Days post infection
dsRNA	Double stranded RNA
ELISPOT	Enzyme-Linked Immuno Spot Assay
ELISA	Enzyme-Linked Immuno Sorbent Assay
EM	Electron microscopy
EID_{50}	Egg Infectious dose 50%
FBS	Fetal bovine serum
Gly	Glycine
HA	Hemagglutinin
HI	Hemagglutination inhibition

h.p.i	Hours post infection
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IN	Intranasally
IM	Intramuscularly
IT	Intratracheally
IFN	Interferon
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
Ikβ	Inhibitor of NF-κB
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IRF-3/7	Interferon regulatory factor 3/7
ISGs	Interferon stimulated genes
ISREs	Interferon stimulated response elements
JAK	Janus-activated kinase-signal transducer interferon regulatory factor 1
kDa	Kilodalton
LAIV	Live attenuated influenza vaccine
LPR	Lymphocyte proliferative response
LNC	Lymph node cells
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II

MEM	Minimal essential medium
MOI	Multiplicity of infection
M1	Matrix protein 1
M2	Matrix protein 2
NA	Neuraminidase
NCR	Non-coding region
NCFAD	National Centre for Foreign Animal Diseases
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NP	Nucleoprotein
NLS	Nuclear localization signal
NLR	Nucleotide-binding domain and leucine-rich-repeat
NS1	Non-structural protein 1
NS2/NEP	Non-structural protein 2/Nuclear export protein
OAS	2’5’-oligoadenylate synthetase
OD	Optical density
ORF	Open reading frame
OIE	Office International des Epizooties
PAMPs	Pathogen associated molecular patterns
PA	Polymerase acid
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing 0.05% Tween 20
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PI3K	Phosphatidylinositol 3-kinase
PKR	Protein kinase R
PMN	Polymorphonuclear cells
PNPP	Diethanolamine phosphate
PNP	Proliferative and necrotizing pneumonia

PRRs	Pathogen recognition receptors
Pro	Proline
PRCV	Porcine respiratory corona virus
PRRS	Porcine reproductive and respiratory syndrome virus
RBC	Red blood cell
RNA	Ribonucleic acid
RNP	Ribonucleprotein complex
RIG-I	Retionic acid inducible helicase I
rRNA	Ribosomal RNA
rtPCR	Real time PCR
RT-PCR	Reverse transcriptase polymerase chain reaction
SA α2,3Gal	N-acetylneuraminic acid attached to the galactose sugar by an α2,3 linkage
SA α2,6Gal	N-acetylneuraminic acid attached to the galactose sugar by an α2,6 linkage
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SIV	Swine Influenza virus
SI	Swine Influenza
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
$TCID_{50}$	Tissue culture infectious dose 50%
Th1	T-helper cell type 1
TLR-3	Toll-like receptor 3
TLR-7	Toll-like receptor 7
TLR-8	Toll-like receptor 8
TNF-α	Tumor necrosis factor alpha
TR	Triple reassortant
U.S	United States
UV	Ultraviolet
Val	Valine

VIDO	Vaccine and Infectious Disease Organization
VN	Virus neutralization
WT	Wild type

1. LITERATURE REVIEW

1.1 INFLUENZA A VIRUS

1.1.1 Classification and nomenclature

Influenza viruses are classified as members of the *Orthomyxoviridae* family. The viruses within the *Orthomyxoviridae* family have a genome composed of negative-sense, segmented, single stranded, ribonucleic acids (RNAs). The family of *Orthomyxoviridae* contains five different genera: the influenza A, B and C viruses, *Thogotovirus* and *Isovirus*. Influenza viruses type A, B and C are distinguished according to antigenic differences between their internal nucleocapsid (NP) and matrix (M) proteins (Peter Palese 2007). Further, all influenza A viruses are classified into subtypes based on the antigenic characteristics of their surface glycoproteins HA and NA. So far, 16 different HA (1-16) subtypes and 9 different NA (1-9) subtypes of influenza A viruses have been identified (Fouchier, Munster et al. 2005).

Influenza A, B and C viruses could be also distinguished according to the species they infect and numbers of RNA segments. Influenza A viruses naturally infect a variety of avian species, humans, swine, horses and rarely some other mammalian species. Influenza B viruses infect only humans while influenza C viruses have been isolated mainly from humans and from swine populations in China (Yuanji and Desselberger 1984; Webster, Bean et al. 1992). Influenza viruses types A and B each contain eight distinct RNA segments (Ritchey, Palese et al. 1976; Peter Palese 2007), whereas the influenza C virus genome is composed of only seven segments (Herrler and Klenk 1991).

Nomenclature system for different influenza virus strains includes their genus, the species from which the virus was isolated (except for humans), location of isolate, the number of the isolate, the year of isolation, and the HA and NA subtypes (for influenza A viruses only). For example, the nomenclature: A/SW/SK/18789/02 (H1N1) represents the type A influenza virus isolated from swine in Saskatchewan as a virus strain 18789 isolated in 2002, and according to the HA and NA characteristics this virus is assigned as H1N1 subtype.

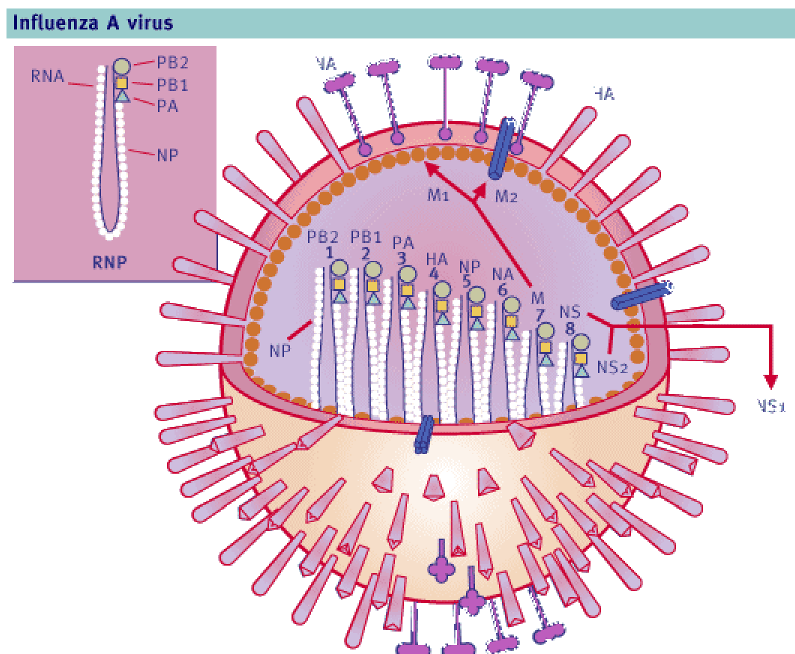
1.1.2 Virion structure of influenza A virus

The virions of the influenza A viruses possess a complex structure and have a lipid envelope derived from the plasma membrane of the host cell in which the virus is grown. HA,

NA and matrix 2 (M2) proteins are embedded in the viral envelope and project from the influenza A virus surface. The viral matrix protein (M1) is the most abundant virial protein that underlies the lipid bilayer and associates with the ribonucleoprotein complex (RNP) (Nayak, Hui et al. 2004). The RNP complex is the core of the virion and it consists of RNA, nucleoprotein (NP) and three polymerase subunits [polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA)] (Compans, Meier-Ewert et al. 1974; Peter Palese 2007). The nuclear export protein/non-structural protein 2 (NEP/NS2) was also found in influenza A virions after purification (Richardson and Akkina 1991). In general, the influenza A virion is composed of about 1% RNA, 5-8% carbohydrates, 20% lipid and 70% protein (Frommhagen, Knight et al. 1959).

Morphologically, influenza A virus particles are characterized by distinctive rod-shaped spikes of HA and mushroom-shaped spikes of NA seen under electron microscopy (EM). These spikes are usually 10-14 nm long and estimated ratio of HA to NA is 4:1. The influenza A viruses exhibit pleomorphism and could be visualized as spherical or filamentous formations. The spherical virus particles are characteristic for egg or tissue culture grown viruses (diameter of 80-120 nm) while filamentous particles are mainly characteristic for fresh clinical isolates (diameter ~300nm) (Chu, Dawson et al. 1949; Peter Palese 2007).

The internal structure of influenza A viruses is less defined. Recent reports suggest that individual influenza A virus particles each package eight RNA segments into fully functional virion (Noda, Sagara et al. 2006; Hutchinson, von Kirchbach et al. 2010) (Figure 1.1).



http://www.pasteur.ac.ir/researchDepartment/flu/images/flu_structure.gif

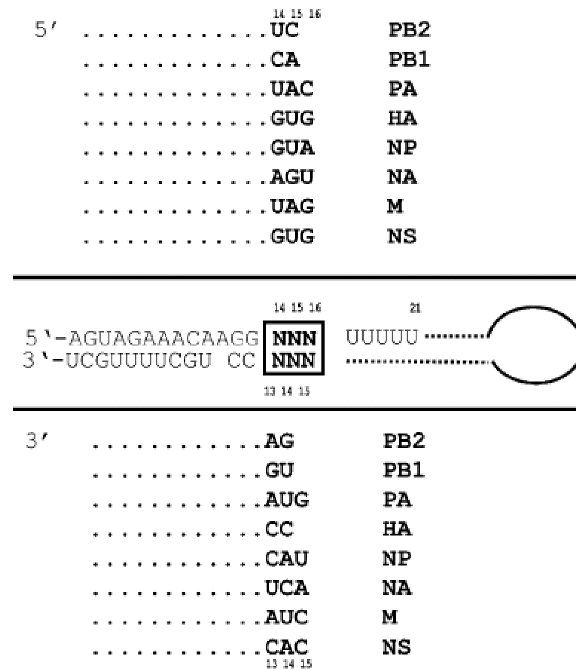
Figure 1.1. Influenza A virus genome structure and organization. A schematic diagram of the structure of the influenza A virus particle. Eight single stranded RNAs of negative polarity compose the influenza A genome. RNA is associated with polymerase complex proteins (PB1, PB2 and PA) and NP in RNP complex (top left). Three types of integral membrane proteins HA, NA and M2 are inserted through the lipid bilayer of the viral membrane. The virion matrix protein M1 underlies the lipid bilayer and interacts with the helical RNP complexes. NS1 is a nonstructural protein found only in infected cells while NS2 could be found in purified virions.

1.1.3 Genome structure and organization of influenza A viruses

Electrophoresis of isolated viral RNAs on polyacrylamide gel revealed that all influenza A viruses possess eight single stranded RNA (ssRNA) segments (McGeoch, Fellner et al. 1976; Palese and Schulman 1976; Ritchey, Palese et al. 1976). Influenza virus genome was mapped by using the hybridization properties and protein characteristics of different reassortant viruses (Scholtissek, Harms et al. 1976) as well as hybrid arrest of translation of individual mRNAs by isolated RNA segments (Lamb and Choppin 1979).

Each of the eight influenza A virus RNA segments contain non-coding regions (NCR) at both 3’ and 5’ ends that flank the coding sequence. The farthest ends of 3’ (12 nucleotides) and 5’ (13 nucleotides) of NCR are conserved among all influenza A RNA segments, and they are usually followed by a segment-specific non-coding region (Robertson 1979; Desselberger,

Racaniello et al. 1980) (Figure 1.2). These conserved regions at 3' and 5' terminal ends are considered as the core vRNA promoter (Flick, Neumann et al. 1996). The influenza vRNA promoter is defined as double-stranded element formed by the conserved sequences at 3' and 5' terminal ends of vRNA molecule. Conserved sequences at 3' and 5' terminal ends exhibit partial and inverted complementarity to each other, and may form secondary panhandle structure (Hsu, Parvin et al. 1987; Pritlove, Fodor et al. 1995). Another theory proposes corkscrew model for vRNA promoter secondary structure where single-stranded regions base pair within themselves to form 5' and 3' stem-loops (Flick, Neumann et al. 1996). These proposed secondary structures have been critical for influenza vRNA promoter activity, endonuclease activity of PB1 and polyadenilation (Pritlove, Poon et al. 1999; Leahy, Dobbyn et al. 2001; Leahy, Pritlove et al. 2001).



(Hoffmann, Stech et al. 2001)

Figure 1.2. Schematic representation of the conserved terminal regions of the eight influenza A vRNA segments. The non-coding regions of the vRNA segments differ in length and sequence, but are characteristic for each of the eight segments. The 5' terminus of each influenza A vRNA segment has 13 conserved nucleotides, and the 3' terminus has 12 conserved nucleotides. These conserved regions are followed by segment specific nucleotides.

The eight viral RNA segments of influenza A viruses encode 11 recognized gene products. These are PB1, PB1-F2, PB2 and PA, HA, NP, NA, M1 and M2, NS1 and NEP/NS2 proteins.

The PB2 protein is encoded by RNA segment 1 and plays an essential role in the initiation of transcription for viral mRNA. It is responsible for recognition and binding to the 5' methylated cap structures of host cell pre-mRNAs (Blaas, Patzelt et al. 1982) which are used as primers for viral mRNA synthesis (Braam, Ulmanen et al. 1983; Ulmanen, Broni et al. 1983; Shi, Summers et al. 1995; Neumann, Brownlee et al. 2004). The PB2 also plays an important role in influenza virus replication. Gastaminza and colleagues showed that a single amino acid mutation in the N-terminal region of PB2 specifically affected replication but not transcription (Gastaminza, Perales et al. 2003). Furthermore, it was shown that AA at position 627 of PB2 plays a role in influenza virus pathogenicity (Hatta, Gao et al. 2001) and host specificity (Subbarao, London et al. 1993).

The PB1 subunit of the RNA polymerase complex is encoded by RNA segment 2. It serves as the backbone of the viral polymerase complex, containing binding sites for PB2 and PA (Digard, Blok et al. 1989). The influenza virus protein PB1 is responsible for elongation of the primed nascent viral mRNA cRNA and vRNA synthesis (Braam, Ulmanen et al. 1983; Gonzalez and Ortin 1999). In addition, PB1 possess RNA endonuclease activity and it is responsible for the generation of capped primer required for mRNA synthesis (Li, Rao et al. 2001).

An additional open reading frame (ORF) near the 5' end of the PB1 gene encodes the 87-AA long PB1-F2 polypeptide (Chen, Calvo et al. 2001). The PB1-F2 protein plays a role in influenza virus-induced apoptosis by interacting with mitochondrial proteins ANT3 and VDAC 1 thus altering the mitochondrial membrane potential and leading to the release of cytochrome C (Chanturiya, Basanez et al. 2004). Most likely, this protein is an accessory protein because several influenza A virus strains isolated from humans and animals lack this ORF.

RNA segment 3 encodes the PA subunit which is a third member of the RNA-dependent RNA polymerase complex along with PB1 and PB2. The PA is the least characterized of the polymerase proteins in terms of function (Neumann, Brownlee et al. 2004). However, mutations within PA affected both transcription and replication of influenza virus, indicating its role in both processes (Fodor, Mingay et al. 2003). In addition, PA might have proteolytic activity (Sanz-Ezquerro, de la Luna et al. 1995) but the level of proteolysis does not match polymerase activity (Naffakh, Massin et al. 2001).

Influenza A virus RNA segment 4 encodes an integral membrane glycoprotein HA. HA binds to sialic acid receptors on the cell surface providing the attachment of a virus particle to the cell. Amino acids at positions 226 and 228 in H3/H2 and positions 190 and 225 in H1 subtypes are crucial deteminat for receptor specificity and interspecies transmission. (Mikhail N. Matrosovich 2006). The HA mediates the fusion of the viral envelope and the endosomal membrane, which results in the release of the viral genome into the cytoplasm. In addition, HA is the major surface antigen against which neutralization antibodies are produced (Robert A. Lamb 2001). In infected cells HA is initially synthesized as a single polypeptide in a precursor form (HA0) (Copeland, Doms et al. 1986; Robert A. Lamb 2001). Depending on the virus strain, host cell type, and growth conditions, HA may exist either in an uncleaved HA0 or in a cleaved form HA1 and HA2 connected by disulfide linkages (Steinhauer 1999; Robert A. Lamb 2001). The cleavage of HA0 is accomplished by host-produced trypsin-like proteases. Cleavage of HA0 to HA1 and HA2 is a prerequisite for virus infectivity (Lazarowitz and Choppin 1975) and a crucial determinant in pathogenicity and in the spread of infection (Steinhauer 1999). HA molecules form homotrimers and each molecule consists of a globular head on a stalk (Copeland, Doms et al. 1986). The globular head is made up solely of HA1 and contains the receptor-binding domain as well as antigenic sites. The stalk is composed of HA2 and partially of HA1. HA2 contains the transmembrane hydrophobic sequence which is highly conserved among HAs of different influenza A virus strains, and is an essential participant in HA fusion activity (Schoch and Blumenthal 1993; Cross, Burleigh et al. 2001).

NP is encoded by RNA segment 5. It is a major structural protein which binds to and encapsidates newly synthesized cRNA and vRNA. It has been proposed that the availability of new soluble NP controls the switch between mRNA and cRNA synthesis (Hay, Lomniczi et al. 1977; Vreede, Jung et al. 2004). NP also plays a role in the transport of RNPs from cytoplasm to nucleus by interacting with cellular cargo protein karyopherin α (Melen, Fagerlund et al. 2003). Furthermore, NP is one of the type-specific antigens and a major target of the host cell-mediated immune responses (Yewdell, Bennink et al. 1985).

Influenza RNA segment 6 encodes NA protein which is also an integral membrane glycoprotein and a second major surface antigen of the influenza virion. The NA has a role in releasing the virus from the infected cell and in removing the sialic acid receptors from the cell to permit virus spread (Palese, Tobita et al. 1974; Palese and Compans 1976). Furthermore, it has

been shown that the influenza A NA could play a role in an early stage of infection, probably facilitating entry of the virus (Matrosovich, Matrosovich et al. 2004) and/or enhancing late endosome/lysosome trafficking (Suzuki, Takahashi et al. 2005). Like HA, NA contains highly variable regions considered as antigenic sites targeted by the host immune defences (Air, Els et al. 1985; Gulati, Hwang et al. 2002).

RNA segment 7 of influenza A is bicistronic, encoding both M1 and M2 proteins. Collinear transcription of segment 7 yields mRNA for the matrix protein M1. The M1 is the most abundant protein and lies just beneath the lipid envelope. M1 interacts with both RNPs and NEP/NS2 mediating the export of new RNPs from nucleus to cytoplasm (O'Neill, Talon et al. 1998; Ma, Roy et al. 2001). In addition, M1 makes contact with the cytoplasmatic tails of the HA and NA glycoproteins as well as with RNPs, thus linking the inner core components and the membrane proteins (Schmitt and Lamb 2005). Therefore, it is proposed that M1 might play a critical role in assembly by recruiting the viral components to the site of assembly at the plasma membrane. The M1 is necessary and sufficient for the formation of virus like particles, suggesting that M1 plays an essential role in the influenza virus budding process (Gomez-Puertas, Albo et al. 2000; Latham and Galarza 2001).

The mRNA for M2 is also transcribed from RNA segment 7. It is derived from the collinear M1 transcript by splicing. The M2 protein is the third integral protein of influenza A viruses consisting of a short ectodomain, a transmembrane domain and endodomain (Holsinger, Nichani et al. 1994; Peter Palese 2007). M2 acts as ion channel permitting the influx of protons from acidified endosomes into the interior of the virion to dissociate the RNP complex from the rest of the viral components, thus finishing the uncoating process (Bron, Kendal et al. 1993; Wharton, Belshe et al. 1994; Schnell and Chou 2008). A further function appears to be a role for M2 in assembly and influenza A virus budding (Hughey, Roberts et al. 1995; Schroeder, Heider et al. 2005). In addition, the external portion of M2 has been targeted for universal influenza vaccine development because the M2 protein preserves a highly conserved sequence (Fiers, De Filette et al. 2004).

RNA segment 8 encodes two non-structural proteins NS1 and NS2/NEP. The NS1 protein of influenza A viruses. is encoded from vRNA segment 8 and translated from unspliced mRNA (Lamb and Choppin 1979). The NS1 protein is expressed at significant levels in infected cells and it is considered as the key component by which all influenza A viruses suppress host innate

immune defences (Kochs, Koerner et al. 2007; Hale, Randall et al. 2008). NS1 functions as an efficient IFN-α/ß antagonist that allows continuous virus replication in *in vivo* models (Garcia-Sastre 2001; Katze, He et al. 2002). The influenza NS1 is also involved in a variety of cellular processes during viral infection, which may contribute to efficient virus replication and virulence. These include: a) regulation of viral RNA synthesis, b) control of viral mRNA splicing, c) enhancement of viral mRNA translation, d) regulation of virus particle morphogenesis, e) activation of phosphoinositide 3-kinase (PI3K), f) role in strain-dependent pathogenesis (Hale, Randall et al. 2008).

The NEP/NS2 protein forms an association with the M1 protein (Richardson and Akkina 1991; Yasuda, Nakada et al. 1993). This interaction is essential in the virus life cycle for the export of the new RNP complex from the nucleus (O'Neill, Talon et al. 1998).

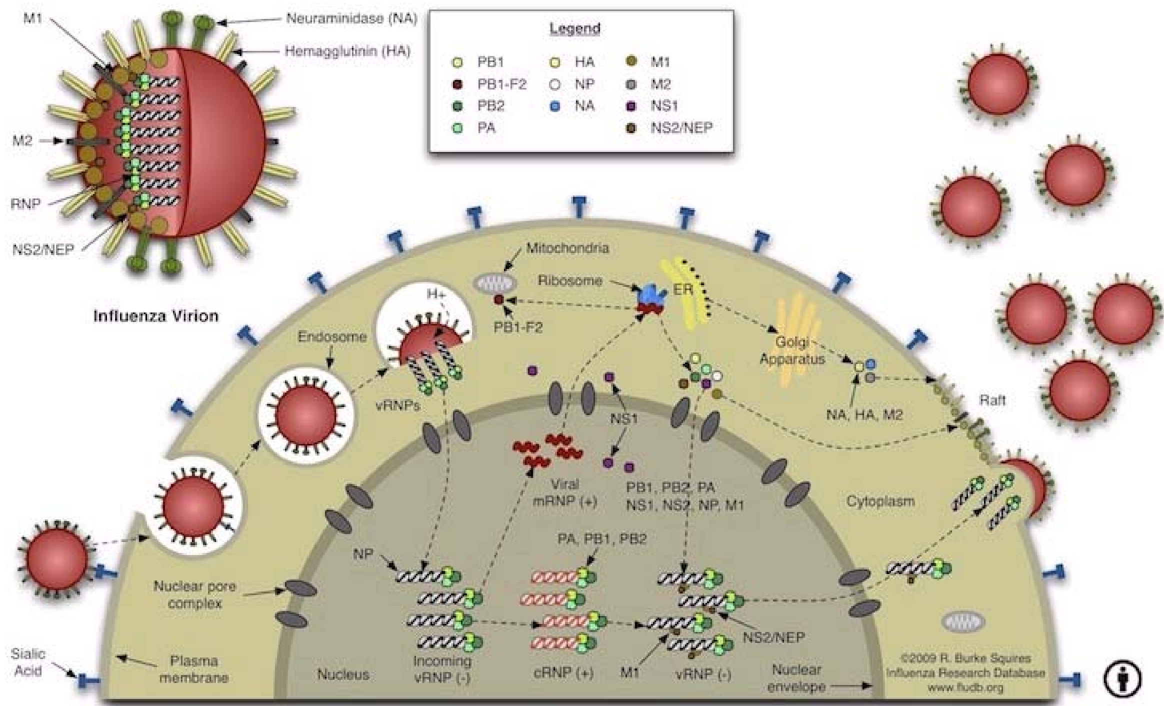
1.1.4 Influenza A virus replication

The first step in influenza A virus replication cycle is the virus attachment by HA to neuraminic acids on the cellular surface (Skehel and Wiley 2000). The interaction of influenza A viruses with sialic acids is restricted by the fact that the HA of viruses that replicate in different species have affinity for sialic acids with different linkages. Human viruses preferentially bind to N-acetylneuraminic acid attached to the galactose sugar by an α2,6 linkage (SA α2,6Gal) while avian viruses mostly bind to sialic acid with an α2,3 linkage (SA α2,3Gal) (Connor, Kawaoka et al. 1994; Ito, Suzuki et al. 1997; Gambaryan, Yamnikova et al. 2005). However, this influenza A virus specificity is not absolute and it is reported that both avian and human cells can contain both neuraminic acid linkages (Shinya, Ebina et al. 2006). In addition, swine influenza viruses can recognize both SA α2, 6 Gal and SA α2, 3 Gal types of sialic acid receptors (Gambaryan, Karasin et al. 2005).

Following attachment, influenza A virus enters the cell by receptor-mediated endocytosis (Robert A. Lamb 2001; Lakadamyali, Rust et al. 2004) (Figure 1.3). Clathrin-mediated endocytosis has been the most common model for influenza virus entry (Matlin, Reggio et al. 1981). The low pH within the endosome triggers a conformational change in cleaved HA, which results in exposure of fusion peptide at the N-terminus of the HA2 subunit. The hydrophobic free N-terminus of HA2 is inserted into the vesicular membrane, resulting in the fusion of the viral and vesicular membranes (Stegmann 2000). In addition, successful uncoating depends on the

presence of the M2 protein, which has ion channel activity (Pinto, Holsinger et al. 1992). M2 protein permits the influx of protons from the endosome into the virion (Shimbo, Brassard et al. 1996; Lear 2003). As a result protein-protein interactions are disrupted and RNPs are dissociated from the M1 protein (Matlin, Reggio et al. 1981; Peter Palese 2007). The HA mediated fusion of the viral envelope with the endosomal membrane and the M2-mediated release of the RNPs result in the appearance of free RNPs complexes in the cytoplasm which completes the uncoating process (Martin and Helenius 1991).

The released RNP complexes migrate into the host cell nucleus which is the major site for the influenza virus transcription and replication (Herz, Stavnezer et al. 1981). Viral RNA serves as a template for synthesis of mRNA and cRNA (Mikulasova, Vareckova et al. 2000). In the early stage of infection, the primary mRNA transcripts are predominantly used for translation of NP and NS1 proteins (Hay, Lomniczi et al. 1977). Newly synthesised NP and NS1 migrate to the nucleus where increased concentration of free NP triggers the shift from the synthesis of mRNA to synthesis of cRNA and vRNA. Early synthesized NS1 protein in the nucleus interferes with the host innate defense mechanisms by blocking synthesis of antiviral cytokines and proteins in order to provide continuous virus replication (Hay, Skehel et al. 1982; Shapiro and Krug 1988; Hale, Randall et al. 2008). Newly synthesised vRNAs are encapsidated in NP and serve as templates for secondary transcription of viral mRNAs and synthesis of the remaining proteins. In the late stages of the virus life cycle, newly synthesized vRNAs associated with the NP and polymerase complex proteins are exported from the nucleus. This event is carried out by the activity of viral NS2/NEP and M1 proteins (O'Neill, Talon et al. 1998; Whittaker and Helenius 1998). Newly synthesized HA and NA proteins are posttranslationally processed and transported to the cell surface where they integrate into the cell membrane (Figure 1.3). Interactions between M1 coupled with RNPs and the cytoplasmic domains of HA, NA or M2 activate the signals for budding which is the last stage in influenza A virus replication cycle (Compans, Meier-Ewert et al. 1974).



Influenza Research Database www.flubd.org

Figure 1. 3. Influenza A virus life cycle The influenza virus initially associates with a host cell by binding to sialic acid-containing receptors on the host cell surface. The bound virus is endocytosed. The low endosomal pH sets in action a number of steps that lead to viral membrane fusion mediated by the viral HA protein. Upon fusion the viral RNP complex is released into the cytosol of the host cell. The RNP complex is transported through the nuclear pore into the nucleus. Once in the nucleus, the vRNA is transcribed into messenger RNA (mRNA) by a primer-dependent mechanism. Replication occurs via a two step process. A full-length complementary RNA (cRNA), a positive-sense copy of the vRNA, is first made and this in turn is used as a template for a synthesis of new vRNA. The viral proteins are expressed, processed and assembled with vRNAs at budding sites within the host cell membrane. The viral protein complexes and RNPs are assembled into viral particles. Progeny viruses bud from the host cell, enveloped in the host cell's membrane

1.1.5 Evolution of influenza A viruses

Virus evolution is the constant change of a viral population over time in response to selection pressures (S.J. Flint 2004; Peter F. Wright 2007). There are three general theories of the origin of viruses: (i) the regressive theory suggests that viruses are derived from intracellular parasites that have lost many essential functions required for replication and parasitic lifestyle. (ii) The cellular origin theory proposes that viruses arose from cellular components that gained the ability to replicate independently within the host cell. (iii) The third theory postulates that viruses coevolved with cells from the origin of life itself (S.J. Flint 2004).

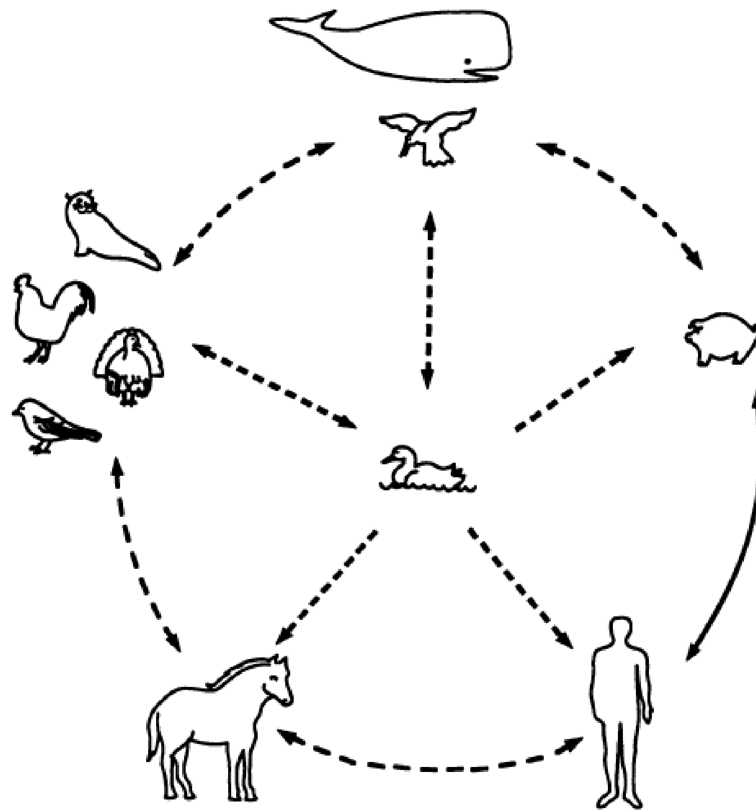
Continuing evolution occurs in each of the eight influenza viral gene segments (Webster, Bean et al. 1992). The irregularity in influenza virus genetic information is the result of the accumulation of molecular changes in all of the eight RNA segments but is the most documented in the surface HA and NA glycoproteins (Peter F. Wright 2007).

Phylogenetic analysis accompanied with the findings that influenza A viruses of all known 16 HA and 9 NA subtypes are maintained in avian species, led to the existing hypothesis that all mammalian influenza viruses are derived from avian influenza reservoirs (Webster, Bean et al. 1992; Peter F. Wright 2007) (Figure 1.4). In aquatic wild birds, influenza viruses do not cause disease which suggests that these viruses are fully adapted to their hosts (Webster, Bean et al. 1992). Avian influenza viruses unlike mammalian strains show low evolutionary rates at the nucleotide and amino acid levels (Gorman, Bean et al. 1990). Nucleotide changes occur at a similar rate in both avian and mammalian influenza viruses, however, these changes do not result in amino acid substitution in the avian viruses in contrast to mammalian and land-based poultry viruses in which continuous amino acid substitution has been documented in all viral genes (Webster, Bean et al. 1992).

The phylogenetic analyses have revealed host-specific virus lineages for several viral proteins. The phylogenetic studies conducted for the NP genes identified seven host-specific lineages: (i) human viruses, (ii) classic swine viruses, (iii) old H7N7 equine viruses, (iv) recent equine viruses, (v) H13 gull viruses, (vi) North American avian viruses and (vii) Euroasian avian viruses. Host-specific lineages have been identified for other influenza genes, except for the HA and NA (Webster, Bean et al. 1992). In addition, there is a difference in phylogenetic tree of the PB1 and other influenza virus genes. Phylogenetic analyses sort the PB1 genes of human H1N1 viruses in the same group with H1N1 classic swine viruses. Furthermore, the PB1 genes of

human H2N2 and H3N2 viruses were grouped in another sublineage that is in correlation with the introduction of avian PB1 genes in human viruses during 1957 and 1968 pandemics (Kawaoka, Krauss et al. 1989). The phylogenetic tree of the influenza NS gene is divided into two alleles A and B (Treanor, Snyder et al. 1989). The allele A contains all mammalian while allele B includes avian influenza NS genes.

Overall, phylogenetic analysis indicates common ancestry for human H1N1 and classic swine influenza viruses (Gorman, Bean et al. 1991). In contrast, two sublineages, an American and Euroasian can be defined for all eight segments of avian influenza viruses (Donis, Bean et al. 1989).



(Webster, Bean et al. 1992)

Figure 1.4. Reservoir of influenza A viruses. The long-time hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission of influenza A virus has been demonstrated between pigs and humans (solid line). There is extensive evidence for transmission between wild ducks and other species.

1.1.6 Genetics of influenza viruses

Despite a minimal set of genes, influenza virus populations display impressive diversity, which enables them to evolve continuously. The diversity within genes can occur by a number of different mechanisms including: a) gene recombination, b) point mutations or antigenic drift and c) reassortmentt or genetic shift (Webster, Bean et al. 1992). Each of these mechanisms represents a powerful tool in the genetics of influenza viruses.

a) Recombination; New influenza virus variants can emerge when genetic information is exchanged by the process of recombination. Mechanism of recombination creates new combinations of many mutations that may be essential for survival under selective pressures. In influenza A viruses, recombination occurs when polymerase changes templates during replication or when nucleic acid segments are broken and rejoined. For example, recombination by template switching lead to increased biological fitness of the avian influenza virus. Insertion of 54 nucleotides of 28S ribosomal RNA increased the HA cleavability of avian influenza A/Oregon/71 (Khatchikian, Orlich et al. 1989; Peter F. Wright 2007). Similarly, two low pathogenic avian viruses reverted into high pathogenic after the insertion of 21 nucleotides of the M segment (Bowes, Ritchie et al. 2004; Hirst, Astell et al. 2004; Pasick, Handel et al. 2005) or 30 nucleotides of the NP segment (Suarez, Senne et al. 2004) into the HA segment. Classic recombination in which influenza RNA segments contain genetic material from two different parental viruses is uncommon among influenza viruses (Rohde and Scholtissek 1980; Lamb A. 2000). The increased detection of recombinant viruses may indicate that recombination occurs more often than previously believed. In general, recombination is masked by the low biological fitness of the recombinant viruses, whereas under selective pressure, recombination may provide a selective advantage.

b) Mutations including substitutions, insertions and deletions are central mechanisms for producing variation in influenza viruses. The lack of proofreading ability among RNA polymerases leads to replication errors on the order of 1 in 10^4 bases (Holland, Spindler et al. 1982; Steinhauer and Holland 1987). Consequently, each round of influenza virus replication results in mixed population with many variants. Most new viruses are not viable, but some might have potentially advantageous mutations that can become dominant under the right selective pressure (Webster, Bean et al. 1992). *Antigenic drift* is a result of accumulation of a series of point mutations in mammalian influenza A and B viruses (Peter F. Wright 2007). These

mutations lead to amino acid substitutions and minor, gradual, antigenic changes in the influenza HA and NA proteins (Peter F. Wright 2007). In addition, changes in HA and NA antigenic sites prevent virus neutralization by antibodies induced during previous infection or vaccination (Webster, Bean et al. 1992). Consequently, new influenza A virus drift variants arise as a result of the positive selection by neutralizing antibodies. To a lesser extent than in human population, antigenic drift was observed among influenza viruses in land based poultry (Peter F. Wright 2007). Mutations within the amino acid sequence of HA and NA in human influenza viruses occur at a frequency of less than 1% per year. However antigenic drift variants cause epidemics and normally exist for 2 to 5 years before being replaced by different variant (Peter F. Wright 2007) (Figure 1.5).). Interestingly, genetic drift in the HA segment of SIV cH1N1 viruses is limited to regions unrelated to antigenic sites (Luoh, McGregor et al. 1992; Brown, Ludwig et al. 1997) which is in contrast to genetic drift observed in the HA segment of human H1N1 viruses (Xu, Rocha et al. 1993). This limited antigenic variation in the HA segment of cH1N1 SIVs is most likely due to the lack of considerable immune selection in pigs because of the constant introduction of non-immune naïve pigs.

c) Reassortment or genetic shift is the exchange of viral genetic information in a cell infected with two different influenza viruses. Reassortment occurs for Influenza A, B and C viruses but has not been observed among different types of influenza viruses. Reassortment involves major antigenic changes in which a new HA or NA subtype is introduced into the human population (Peter F. Wright 2007) The newly introduced influenza viruses possess proteins that are immunologically distinct from previously circulating strains. Thus genetic shift results in high infection rates in the immunologically naïve population leading to pandemics (Peter F. Wright 2007).

The importance of reassortment in genetics and evolution of influenza viruses is highlighted by the past and current pandemics. The new H1N1"quadruple" reassortant viruses emerged from the reassortment of recent triple reassortant (TR) H3N2 and H1N2 North American swine viruses with Eurasian avian-like swine influenza viruses (Neumann, Noda et al. 2009). Consequently, these viruses possess PB2 and PA genes of North American avian virus origin, a PB1 gene of human H3N2 virus origin, HA (H1), NP, and NS genes of classical swine virus origin, and NA (N1) and M genes of Eurasian avian-like swine virus origin (Neumann, Noda et al. 2009). In addition, pandemics of 1957 and 1968 were caused by reassortant viruses

that contained HA, PB1, and NA or HA and PB1 segments of avian virus origin in a human genetic background (Scholtissek, Rohde et al. 1978; Kawaoka, Krauss et al. 1989).

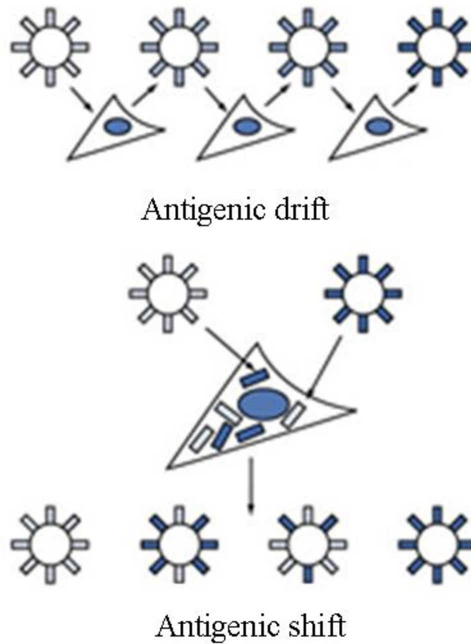


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Figure 1.5. Antigenic drift and genetic shift (reassortment). (Top) Antigenic drift-accumulation of point mutatuins within viral gene segments. (Bottom) Reassortment-exchange of genetic material between two or more viruses

1.2 REVERSE GENETICS SYSTEM

Reverse genetics is a method used in molecular virology for the generation of negative sense RNA viruses entirely from cloned cDNA (Neumann 2004). The genomes of negative sense RNA viruses are complementary to mRNA in their orientation so they are not infectious by themselves (Neumann, Whitt et al. 2002). Negative sense RNA viruses require the presence of vRNA(s) and virion packaged RNA-dependent polymerase to initiate the viral replication cycle (Neumann 2004). The generation of segmented negative sense RNA viruses from cloned cDNAs had been extremely challenging because of the technical difficulties to provide several vRNPs, NP and polymerase proteins at the same time in one cell.

1.2.1 Cell culture system to study influenza virus replication

Cell culture system for influenza virus requires in vitro reconstitution of RNPs from in vitro synthesized virus-like RNA and purified polymerase and NP proteins (Huang, Palese et al. 1990). The crucial step for artificial generation of influenza virus was the reconstitution of functional RNP complexes (RNPs) (Neumann and Kawaoka 2002). Fully functional RNPs could be isolated from detergent-treated virus (Honda, Ueda et al. 1987), *in vitro* assembled from purified NP and polymerase proteins (Szewczyk, Laver et al. 1988) or from proteins expressed in insect cells (Kobayashi, Tuchiya et al. 1992).

For the intracellular reconstitution of vRNP complexes, the polymerase and NP proteins could be provided by several protein expression systems. recombinant vaccinia virus (Huang, Palese et al. 1990), recombinant simian virus 40 expression systems (de la Luna, Martin et al. 1993), cell lines stably expressing all four viral proteins (Kimura, Nishida et al. 1992) or from protein expression plasmids under the control of T7 RNA polymerase promoter (Mena, de la Luna et al. 1994). In general, these systems enable *in vitro* RNA transcription and fusion with purified polymerase and NP proteins in order to form influenza vRNPs. Newly formed vRNPs were then transfected into cells expressing the polymerase and NP proteins.

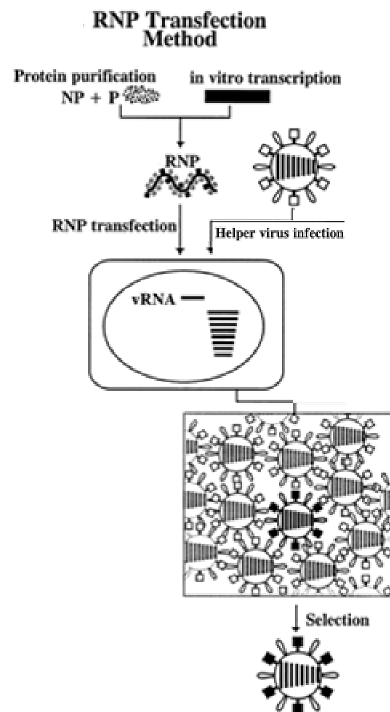
The cell culture systems allow the modification of vRNAs non-coding regions thus allowing identification of viral promoter elements and regulatory signals (Neumann and Kawaoka 2002). Furthermore, they permitted mutagenesis of the NP and polymerase proteins which in turn provide insight on their roles in virus replication and transcription (Perales, Sanz-Ezquerro et al. 2000). The major drawback of these cell culture systems is that the ratios of the polymerase to NP proteins may not be identical to those in infected cells, which may affect viral transcription and/or replication (Neumann and Kawaoka 2002).

1.2.2 Ribonucleoprotein transfection method for generation of influenza virus

Ribonucleoprotein transfection method is the first system for the generation of influenza virus that contained vRNA derived from cloned cDNA (Figure 1.6). A cDNA encoding the reporter protein CAT replaced the coding region of the influenza NS gene (Luytjes, Krystal et al. 1989). The 5’ and 3’ non-coding regions of NS gene containing the influenza virus promoter sequence remained unchanged. A T7 RNA polymerase promoter sequence and a recognition site for a restriction endonuclease that allowed the formation of viral 3’ ends flanked this construct

(Luytjes, Krystal et al. 1989). The constructed plasmid was transcribed *in vitro* and mixed with polymerase and NP proteins to reconstitute RNP complexes. These artificially generated RNPs were transfected into eukaryotic cells infected with helper influenza virus to provide the remaining vRNPs. The rescued viruses contained the virus-like RNA encoding CAT in addition to the eight influenza vRNAs (Luytjes, Krystal et al. 1989). CAT expression in cell lysates from vRNP-transfected and helper virus infected cells confirmed the presence of the NS-CAT-NS vRNA. (Luytjes, Krystal et al. 1989). However, CAT expression was significantly reduced after three passages suggesting that the segment encoding CAT was not stably preserved (Luytjes, Krystal et al. 1989).

Using this system, the majority of progeny viruses were helper viruses. To distinguish the modified virus from the wild type helper virus, strong selection systems based on antibody-mediated restriction (Barclay and Palese 1995), temperature sensitivity (Enami, Sharma et al. 1991), host-range restriction (Enami, Luytjes et al. 1990) or drug resistance (Castrucci and Kawaoka 1995) were required.



(Neumann, Watanabe et al. 1999)

Figure 1.6 RNP transfection method – In vitro sysnthesised viral RNA is mixed with purified polymerase and NP proteins to reconstitute vRNP complexes. Artificially assembled vRNP complexes are transfected into eukryotic cells that are infected with influenza helper virus to provide the remaining seven vRNPs. Selection process required.

1.2.3 RNA polymerase I system

The RNP transfection method is technically demanding requiring *in vitro* RNA transcription, protein purification and *in vitro* reconstitution of RNP complexes. Therefore, a new system that could circumvent these steps was highly desirable.

One hallmark of the influenza virus life cycle is that replication takes place in the nucleus of infected cells. Therefore, the intracellular synthesis of viral RNA requires the generation of full-length transcripts with authentic virus 5' and 3' ends and their nuclear localization. The goal to generate viral RNA *in vivo* from cloned cDNA was achieved in 1993 when the RNA polymerase I system for influenza vRNA synthesis was established by Hobom's group (Zobel, Neumann et al. 1993; Neumann, Zobel et al. 1994; Neumann 2004).

RNA polymerase I is an abundant nuclear enzyme that transcribes ribosomal RNA (rRNA), which like influenza vRNA does not contain a cap structure on the 5' or poly (A) structures on the 3' ends (Neumann and Kawaoka 2001). In addition, RNA polymerase I initiates and terminates transcription at defined promoter and terminator sequences that do not extend into the transcribed region (Neumann and Kawaoka 2001). Therefore, RNA polymerase I transcription yields transcripts that do not contain additional nucleotides at their 5' or 3' ends and it seemed feasible to use this enzyme for the nuclear synthesis of non-capped and non-polyadenylated influenza viral RNAs (Neumann and Kawaoka 2001).

To test if RNA polymerase I can be used for the accurate and efficient synthesis of influenza virus transcripts Neumann and colleagues generated plasmid based RNA polymerase I transcription units (Zobel, Neumann et al. 1993; Neumann, Zobel et al. 1994). Sequences around the RNA polymerase I core promoter and the upstream control element as well as two of the eight terminator elements were cloned into a vector backbone (Neumann and Kawaoka 2002). Further, a cDNA that contained the coding sequence for HA or CAT in antisense orientation flanked by the 5' and 3' noncoding regions of HA were inserted between RNA polymerase I promoter and terminator sequences (Zobel, Neumann et al. 1993; Neumann, Zobel et al. 1994; Neumann 2004). Transfection of the resulting construct into eukaryotic cells demonstrated the RNA polymerase I driven synthesis of full-length viral transcripts encoding HA or CAT. The RNA polymerase I derived influenza virus-like transcripts were not only replicated and transcribed by the viral polymerase complex but they were packaged into virions after infection with influenza helper virus (Neumann 2004). These results demonstrate that for RNA

polymerase I initiation and termination, the core promoter up to the transcription initiation site and the highly conserved terminator element are sufficient(Neumann and Kawaoka 2002).

Pleschka et al modified the original RNA polymerase I system by replacing the RNA polymerase I terminator with a ribozyme sequence for the 3' end formation of virus-like transcripts (Palese, Zheng et al. 1996; Pleschka, Jaskunas et al. 1996; Neumann and Kawaoka 2002). Transfection of a plasmid containing the RNA polymerase I promoter, the influenza viral NA gene in negative sense-orientation, and a ribosome segment together with infection with influenza helper virus resulted in the generation of recombinant virus. However, a rigorous selection system was required to select the recombinant virus that contained a NA gene derived from cloned cDNA (Neumann and Kawaoka 2002).

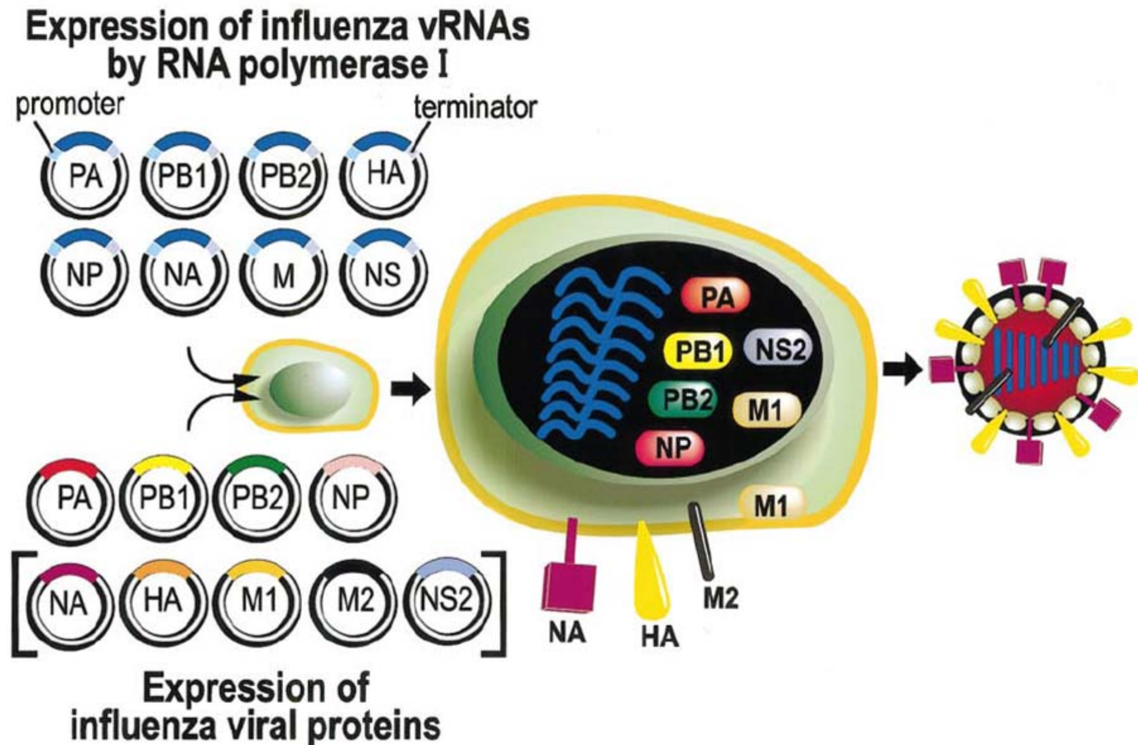
Both RNP and polymerase I systems are helper-virus dependent systems with a low virus generation efficiencies.

1.2.4 Generation of influenza A virus entirely from plasmids

Numerous technical obstacles in generating influenza virus entirely from cloned cDNA were overcome in 1999 when Neumann and colleagues used an established RNA polymerase I system to successfully rescue A/WSN/33 influenza A virus from cloned cDNA (Neumann, Watanabe et al. 1999). Kawaoka's research group cloned cDNAs encoding all eight A/WSN/33 influenza virus segments in negative orientation between human RNA polymerase I promoter and mouse RNA polymerase I terminator sequences (Neumann 1999). Generated constructs were transfected into eukaryotic human embryonic kidney cells (293T) in which transcription by cellular RNA polymerase I yielded all eight vRNAs. In addition, 293T cells were co-transfected with plasmids expressing all nine viral structural proteins (i.e PB2, PB1, PA, HA, NP, NA, M1, M2 and NS2) which made a total number of 17 plasmids used in the initial experiment (Figure 1.7). In an alternative approach, 293T cells were co-transfected with plasmids expressing proteins required for transcription and replication of the influenza vRNAs (i.e. NP and PB2, PB1, PA). Consequently, this strategy required co-transfection of 12 plasmids in total. Both transfection experiments (with 17 and 12 plasmids) yielded more than $1x10^7$ infectious particles per millilitre of the supernatant from cells transfected with the plasmids (Neumann, Watanabe et al. 1999). Fodor et al(Fodor, Devenish et al. 1999) also reported the generation of influenza A virus using a slightly modified system where a hepatitis delta ribozyme was used instead of the

RNA polymerase I terminator to produce vRNA with the authentic 3' end sequence (Neumann 2004).

The RNA polymerase I system is undemanding and straightforward, because it requires only DNA cloning, DNA purification, and DNA transfection techniques, which are widely established in molecular biology and virology laboratories (Neumann 2004).



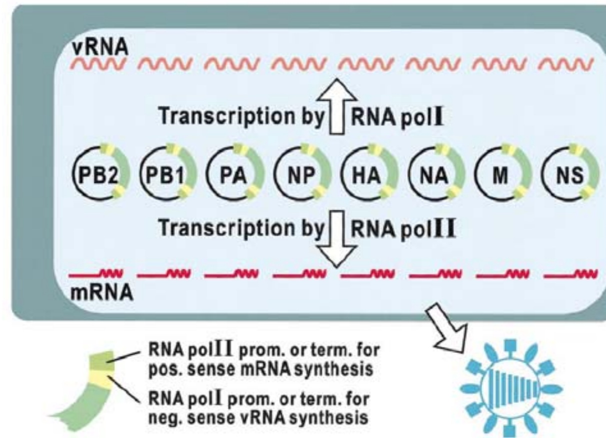
(Neumann and Kawaoka 2001)

Figure 1.7. Generation of influenza A virus entirely from plasmids (RNA polymerase I system). Viral cDNAs are cloned in negative sense orientation between RNA polymerase I promoter and terminator sequences. After transfection of the resulting constructs into eukaryotic cells, vRNAs are synthesised by the cellular enzyme RNA polymerase I. The polymerase and NP proteins are provided from protein expression plasmids.

1.2.5 The RNA polymerase I/II system for the generation of influenza A virus

The RNA polymerase I/II system represents a modification of the existing RNA polymerase I system (Hoffmann, Neumann et al. 2000). A cDNA encoding a viral segment was cloned in the negative–sense orientation between human RNA polymerase I promoter at the 5' end and the mouse RNA polymerase I terminator at the 3'end. This entire RNA polymerase I transcription unit was then cloned in the positive-sense orientation between an RNA polymerase II promoter and polyadenilation site. The orientation of the two transcription units allows the synthesis of negative sense vRNA by RNA polymerase I and positive-sense mRNA by RNA polymerase II from one cDNA template (Figure 1.8). This approach circumvented the need for protein expression constructs and as a result influenza virus could be generated from 8 instead of previously used 12 plasmids (Neumann, Watanabe et al. 1999). Reduction in number of plasmids required for the virus generation may be useful for cell lines that otherwise cannot be transfected with a high level of efficiency. Additional attempts were made to further reduce the number of plasmids required for the generation of influenza virus by reverse genetics (Neumann, Fujii et al. 2005). The most recent report suggests that a single plasmid containing the cDNAs of all eight RNAs can be used in the generation of infectious virus after transfection into cells (Neumann, Fujii et al. 2005).

Application of reverse genetics in the generation of influenza virus reached a level of sophistication where one can rescue virus entirely from cloned cDNA. The reverse genetics technique based on the RNA polymerase I and I/II systems allows one to introduce any mutation into the genome of influenza virus. Furthermore, the newly established system makes it feasible to study molecular mechanisms of influenza virus replication (viral regulatory sequences, structure-function relationships), identification of factors determining viral pathogenicity, and host cell tropism as well as to generate both killed and live attenuated vaccines and gene delivery vehicles (Neumann and Kawaoka 2001).



(Neumann and Kawaoka 2001)

Figure 1.8. The RNA polymerase I/II system for the generation of influenza A virus-Viral cDNAs are cloned in negative sense orientation between RNA polymerase I promoter and terminator sequences, and the entire sequence is flanked by an RNA polymerase II promoter and polyadenylation signal in positive-sense orientation. In eukaryotic cells, both vRNA and mRNA are synthesised from the same template, eliminating the need for protein expression plasmids.

1.3 SWINE INFLUENZA VIRUS (SIV)

1.3.1 Etiology

Swine influenza (SI) is an acute, contagious, respiratory disease of swine (Olsen C.W 2006). The first SI infections in North American pigs were documented in 1918 and coincided with the Spanish influenza pandemic (Olsen C.W 2006). SIV caused epizootic disease in pigs, having many clinical and pathologic similarities with human influenza. The precise location and date of initial SIV outbreak was unknown but the earliest reports described cases from farms in Western Illinois (Shope 1964). The etiology of SI was confirmed in 1931 when Shope presented experimental evidence that the disease in pigs was caused by a virus (Shope 1931; Shope 1931; Shope 1931).

The causative agent of SI is a type A influenza virus of the Orthomyxoviridae family. The genetic, structural and biologic characteristics of SIVs are identical to other type A influenza viruses characterized by negative-sense, segmented RNA genomes. Viruses of the classical H1N1 (cH1N1) lineage were the predominant subtype and leading cause of SI in North America, Europe, Asia and South America since 1918 (Brown 2000; Kanta Subbarao 2006). Recent reports suggested that the early SIVs and the human influenza viruses of 1918 were antigenically

and genetically closely related (Reid, Taubenberger et al. 2001; Reid, Fanning et al. 2003; Reid, Taubenberger et al. 2004). However, it remains to be determined whether an original virus was transmitted from pigs to human or from human to pigs (Kanta Subbarao 2006). The cH1N1 SIV infection capacity extends to wild pigs (Saliki, Rodgers et al. 1998) and to domestic turkeys (Hinshaw, Webster et al. 1983; Wright, Kawaoka et al. 1992). The genetic makeup of cH1N1 SIVs in North America remained antigenically and genetically highly conserved from 1965 through 1980s (Sheerar, Easterday et al. 1989; Luoh, McGregor et al. 1992; Noble, McGregor et al. 1993) and first antigenic and genetic variants were isolated during the 1990s (Olsen, McGregor et al. 1993; Rekik, Arora et al. 1994; Olsen, Carey et al. 2000).

Influenza A viruses of three different subtypes: H1N1, H1N2 and H3N2 are currently circulating in swine populations worldwide (Olsen C.W 2006). Unlike human influenza viruses the origin and genetic makeup of SIV in swine populations differs in different continents. For instance, in Europe the predominant the SIV H1N1 subtype is of avian origin and it was introduced into pigs from wild ducks in 1979 (Olsen C.W 2006). In contrast, two genetically unrelated types of H1N1 SIVs circulate in North American pigs. Classical H1N1 (cH1N1) has been present for more than 80 years and these viruses are genetically and antigenically similar to first SIV isolates from 1930 (Olsen C.W 2006). The second H1N1 SIV subtype that is present in North American swine population is a novel reassortant virus with the mixture of human, avian and swine virus genes.

1.3.2 Epidemiology

Outbreaks of SI in North America and Western Europe occur most frequently during the late summer through early winter and are associated with the sudden drop in outdoor temperatures and start of cold autumn rains (B. C. Easterday 1999; Olsen C.W 2006). Protection of susceptible pigs from environmental fluctuations by confinement housing could reduce the seasonal pattern of disease (Olsen C.W 2006). However, studies have shown that SIVs are constantly circulating throughout the year (Hinshaw, Bean et al. 1978; Olsen, Carey et al. 2000).

The introduction of SIVs into the swine populations is usually associated with the movement of animals, especially with introduction of breeding stock or feeder pigs and return of show stock to the farm (B. C. Easterday 1999). Outbreaks of SI are rapid and explosive with all pigs within the herd infected at the same time. Direct pig to pig contact via nasopharyngeal route

is the primary course of virus transmission (Olsen C.W 2006). During the acute febrile stage of infection (usually 2-5 days after initial exposure) SIV localizes in nasal secretions and may reach titers of $\geq 10^7$ infectious particles per millilitre, (Landolt, Karasin et al. 2003; Van Reeth, Gregory et al. 2003). Once within the herd, SIV may continue to circulate as long as susceptible pigs are available. In all-in/all-out systems, the SIV quickly disappears from heard after an outbreak because of the cycle of depopulation and disinfection of the housing facilities. Depending on their prevalence in a particular region, the SIVs may be reintroduced at a later time, causing infections in the seronegative breeding and fattening stock (Olsen C.W 2006).

For a long time the existence of an SIV carrier has been hypothesized to account for interepidemic persistence of virus. Maintenance of SIVs on premises was proposed to occur via lungworms and earthworms (Shope 1941). However, there is no clear evidence for this mechanisms and it is more likely that virus is maintained through continual availability of susceptible pigs (Olsen C.W 2006).

The respiratory tract of pigs contains both the SAα2,6Gal and SAα2,3Gal receptors which are preferentially recognized by human and avian influenza A viruses respectively. Therefore, pigs are susceptible to infection with both human and avian influenza A viruses (Ito and Kawaoka 2000), thus serve as intermediate host or “mixing vessel” by exchanging RNA segments derived from two or more influenza A viruses during the replication cycle. In addition, pigs may act as hosts for adaptation of avian viruses to replication in mammals.

The reassortment or genetic recomposition could give rise to novel influenza viruses containing a mixture of swine and human and/or avian virus genes. Moreover, genetic reassortment of influenza A viruses in pigs raises concern for generation of pandemic human viruses, and creation of novel viruses of high importance to the pigs themselves. In general, there are three SIV subtypes H3N2, H1N1 and H1N2 that are a result of multiple reassortant events and have established themselves in the swine population; and two SIV reassortants H3N1 and H1N7 that have been recovered occasionally.

Reassortant H3N2 viruses with a mixture of human and classical swine virus genes have been isolated from pigs throughout Asia and North America (Kanta Subbarao 2006; Olsen C.W 2006). Additionally, H3N2 SIVs containing human HA and NA segments and avian internal protein genes have been isolated from pigs in Europe (Campitelli, Donatelli et al. 1997) and Asia (Peiris, Guan et al. 2001). Since 1998, “triple reassortant” (TR) H3N2 viruses have been isolated

all over North America (Kanta Subbarao 2006). The HA, NA and PB1 polymerase genes of these TR viruses are of human origin, while NP, M and NS genes of cH1N1 virus origin and PB2 and PA polymerase genes are of North American avian virus origin (Kanta Subbarao 2006). Infection of pigs with TR H3N2 viruses results in not only respiratory disease but also spontaneous abortion in sows and death of adult pigs (Karasin, Schutten et al. 2000; Kanta Subbarao 2006). The fatal outcomes are not common for cH1N1 SIVs and there is no evidence that SIV directly targets the reproductive tract of pigs. Consequently, it remains to be determined whether the TR SIV associated abortions were due to direct viral effects or the result of high fever in the infected sows (Kanta Subbarao 2006).

Shortly after initial isolation of TR H3N2 SIVs, reproductive disorders and respiratory disease similar to influenza were attributed to infection with H1N2 virus. According to the phylogenetic analysis, H1N2 virus emerged through reassortment between TR H3N2 SIV and cH1N1 SIV (Karasin, Landgraf et al. 2002; Kanta Subbarao 2006). The novel reassortant H1N2 virus retains the entire genetic backbone of the TR virus but adopted a classical swine H1 HA gene (Karasin, Olsen et al. 2000). This H1N2 lineage spread throughout North American swine populations as well as into domestic turkey populations (Suarez, Woolcock 2002) and in the wild waterfowl (Olsen, Karasin et al. 2003). In addition to the first H1N2 SIV isolates in North America, SIVs of H1N2 subtype were isolated from pigs in Japan (Ito, Kawaoka et al. 1998), France (Marozin, Gregory et al. 2002) and Taiwan (Tsai and Pan 2003). However, these H1N2 SIVs were the result of reassortment between human (or human-like swine) H3N2 and cH1N1 viruses. Another SIVs of H1N2 subtype containing human lineage genes for surface glycoproteins HA and NA and internal protein genes derived from avian-like European H1N1 SIVs, were identified in Western European countries (Marozin, Gregory et al. 2002).

Viruses of the third reassortant genotype have been isolated in North America since 1998. These are H1N1 subtypes containing the HA and NA genes derived from a cH1N1 SIV and the remaining internal genes derived from the TR H3N2 or H1N2 SIVs (Webby, Swenson et al. 2000). The latest surveillance activities suggest that this reassortant H1N1 (rH1N1) SIV has replaced cH1N1 and became the predominant genotype of H1N1 virus within North American pigs (Kanta Subbarao 2006).

SIVs of H3N1 and H1N7 subtypes were also derived through reassortment events but they have been recovered from pigs only on a limited basis in U.K and Taiwan. H3N1 viruses were

reassortants between human H3N2 and cH1N1 (Tsai and Pan 2003) while H1N7 contained NA and M genes of equine influenza A virus and remaining genes of human influenza origin (Brown, Hill et al. 1997).

1.3.3 Pathogenesis

SIVs cause acute respiratory tract infection in susceptible pigs. The virus efficiently replicates in epithelial cells of nasal mucosa, tonsils, trachea, lungs, and tracheobronchial lymph nodes (Brown, Done et al. 1993; Heinen, van Nieuwstadt et al. 2000). SIV almost never enters other tissues but short-term viremia of low titer might be detected (Brown, Done et al. 1993; Olsen C.W 2006). The lungs seem to be the major target organ and virus titres in the lungs may reach up to 10^8 egg infectious dose 50 (EID_{50}) per gram of tissue (Olsen C.W 2006). SIV has a highly specific tropism for bronchiolar epithelium and massive infection was observed in epithelial cells of the bronchi, bronchiole and alveoli (B. C. Easterday 1999; Olsen C.W 2006). Usually, upon infection with SIV, bronchi and bronchioles are filled with exudate composed of necrotic epithelial cells and neutrophils. Influx of inflammatory cells in the airways causes obstruction of the bronchi, bronchiole and significant lung damage due to release of their enzymes. The duration of infection and the disease are very short with extremely rapid virus clearance from the respiratory tract. Virus shedding from nostrils or virus replication in the lungs could not be detected on or after day 7 in neither experimental nor natural infection (Brown, Done et al. 1993; Van Reeth and Pensaert 1994).

In experimental conditions SIV infections can be reproduced by using an intratracheal (IT), intranasal (IN) or aerosol route of inoculation. However, only high virus doses of 10^7-$10^{7.5}$ EID_{50} administered via the IT route could produce clinical signs and pathology characteristic for SI (Olsen C.W 2006). Less invasive challenge models such as IN inoculation or aerosol may result only in mild or subclinical infection (Van Reeth and Pensaert 1994; Larsen, Karasin et al. 2000).

There is not enough information about SIV pathogenesis at the cellular level. Recent studies suggest that direct epithelial cell damage in the respiratory tract by influenza viruses could be attributed to apoptosis caused by NA and or PB1F2 proteins (Schultz-Cherry and Hinshaw 1996; Gibbs, Malide et al. 2003). However, proinflammatory cytokines produced during the acute stage of an infection most likely play a critical role in development of clinical

signs characteristic of SI. Experimental studies suggest that bronchoalveolar production of the early pro-inflammatory cytokines interferon alpha (IFN- α), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) contributes to the typical lung inflammatory changes following virus infection (Van Reeth 2000; van Reeth and Nauwynck 2000; Van Reeth, Van Gucht et al. 2002). These cytokines induce lung dysfunction and inflammation, fever, malaise and loss of appetite. These symptoms are associated with the peak of virus replication and cytokine levels and were usually seen within the first 18-24h after IT SIV inoculation (Van Reeth, Van Gucht et al. 2002; Van Reeth, Van Gucht et al. 2002).

1.3.4 Clinical signs

SI is a herd disease. Infection of pigs with any of the three most common SIV subtypes H1N1, H3N2 or H1N2 are clinically similar. After an incubation period of 24-72 h, the onset of disease is sudden with clinical signs appearing in many animals of all ages within a herd at the same time (Olsen C.W 2006). General signs include fever (40.5-41.7°C), apathy, inappetence, huddling and reluctance to move. These signs are accompanied by respiratory distress symptoms such as open-mouthed, labored, abdominal breathing, paroxysmal coughing, bronchial rales at auscultation, conjunctivitis, rhinitis, nasal discharge and sneezing (Olsen C.W 2006). Due to the loss of appetite and inactivity a significant drop in body weight was observed in all sick animals. Morbidity is high reaching almost 100% while mortality rate is usually low (1%-4%) unless there are concurrent secondary infections or pigs are very young. In addition, SIV infections have effects on herd fertility, including abortions in late pregnancy. Recovery from SI begins 5 to 7 days after beginning of disease and it is rapid as the onset. Acute outbreaks of clinically characteristic SI are usually limited to fully susceptible, seronegative pigs. However, some pigs may become chronically affected.

Multiple factors, including immune status, age, infection pressure, ongoing infections, climatic conditions and housing, may determine the clinical outcome of infection with SIV. Intercurrent secondary bacterial infections especially with *Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parasuis* and *Streptococcus suis type 2* complicate the severity and course of an infection with SIV. In addition, dual or triple infections with SIVs and either porcine respiratory coronavirus (PRCV) or porcine reproductive and respiratory syndrome virus (PRRS) extend the course of disease and increase the severity.

The common findings at necropsy or during the histopathological evaluation of lungs are the presence of gross macroscopic and microscopic lesions. The gross lesions in uncomplicated SI infections are limited to the chest cavity and are characteristic for viral pneumonia. The changes are most dominant in the apical and cardiac lung lobes while diaphragmatic and accessory lobes are less affected. The total percentage of lung tissue containing gross visible consolidated areas varies between different SIV strains and/or subtypes as well as between type of infection (natural or experimental).

In general, affected pneumonic areas are sharply demarcated, firm, collapsed, and purplish-red while non-pneumonic areas are pale and emphysematous (Figure 1.9). Severe pulmonary edema, especially of interlobular septae, or a serous or serofibrinous pleuritis is a common finding at necropsy. In addition, the airways may be filled with fibrinous to mucopurulent exudate, and the bronchial and mediastinal lymph nodes are edematous but rarely congested (B. C. Easterday 1999). These lesions and changes in the chest cavity during naturally occurring SIV infections are often complicated or masked by intercurrent infections.

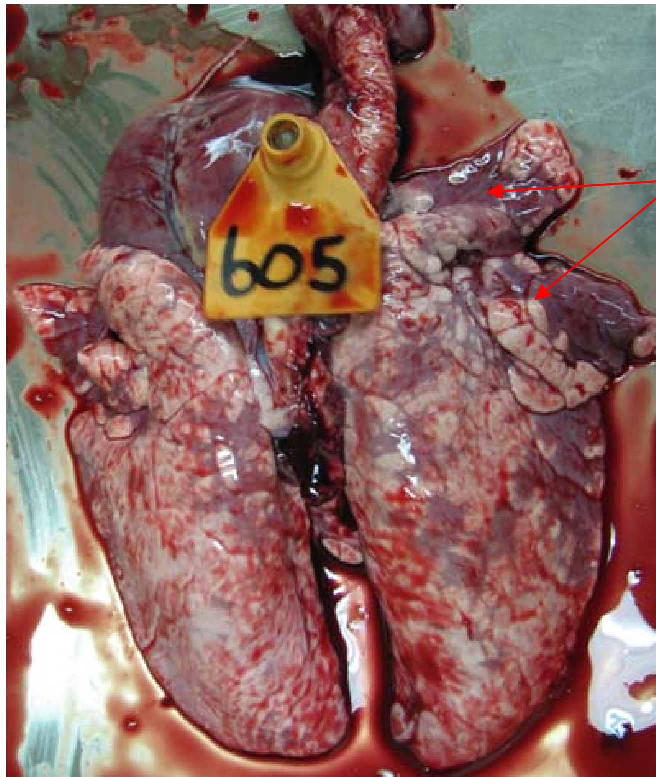


Figure 1.9. Lung gross lesions characteristic for SIV infection. Sharply demarcated purple-plum coloured, firm consolidated areas are most dominant in apical and cardiac lung lobes of SIV infected pigs (Zhou lab).

At the microscopic level, lung injury during SIV infection is characterized by the desquamation and necrosis of the epithelium in bronchi and bronchiole (Figure 1.10). The lumen of bronchi, bronchioli and alveoli are filled with necrotic epithelial and inflammatory cells within first 24h post infection (Van Reeth, Nauwynck et al. 1998). Peribronchial and perivascular cellular infiltration occurs after a few days and is a common finding at histopathology (Richt, Lager et al. 2003).

A proliferative and necrotizing pneumonia (PNP) has been associated with strains of H1N1 and H3N2 SIV (Dea, Bilodeau et al. 1992). PNP is characterized by alveoli filled with protein rich edema and large macrophages, necrotizing bronchiolitis and proliferation of type II pneumocytes and coagulates of necrotic cells in the alveoli. Like clinical signs, both macroscopic and microscopic lung lesions can also be mild or insignificant.

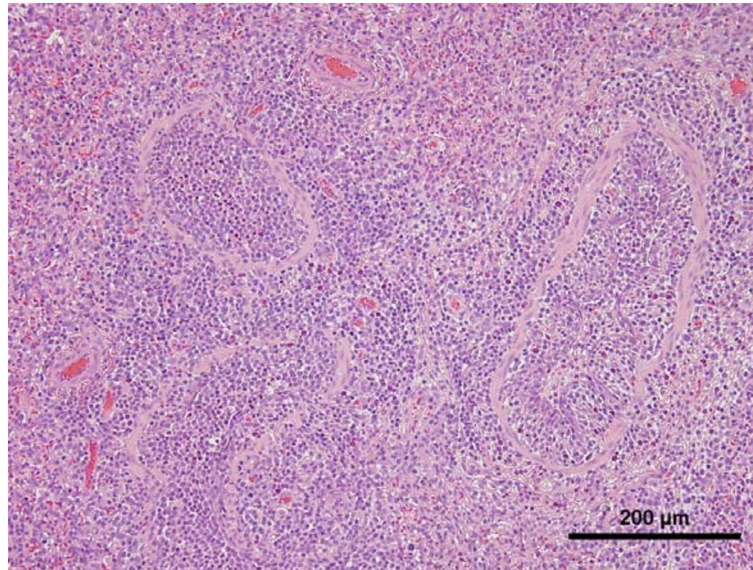


Figure 1.10. Microscopic lung lesions (histopathology) characteristic for SIV infections. Histopathology changes typical for viral pneumoniae characterized by severe peribronchial infiltration with PMN cells, necrosis of bronchial epithelia, epithelial desqamation and neutrophil infiltration in the lumen of bronchi, bronchiole and alveoli (Zhou lab).

1.3.5 Diagnosis

According to clinical and pathologic findings only a presumptive diagnosis can be made (Olsen C.W 2006). Absence of SI pathognomonic signs and need to differentiate SI from other respiratory diseases in pigs require laboratory testing in order to determine final diagnosis. SI can be diagnosed either through virus isolation, detection of viral RNA and/or proteins, or by presence of virus specific antibodies.

SIVs can be isolated from mucus samples obtained by swabbing the nasal passages and/or pharynx of live animals in the febrile phase. Swabs should be placed in tubes with antibiotic/antimycotic containing media and maintained at 4°C if the samples are going to be tested within 48h after collection. SIVs are not stable at temperatures of -20 °C and higher, therefore for long-term storage samples should be kept at temperatures of -70°C to -80°C (OIE 2004). Tissue samples from trachea and lungs are used for virus isolation from the animals that are euthanized or die during the acute stage of the disease. Handling and storage of tissue

samples should be the same as for the swabs until ready for processing. Processing of tissue samples requires fine mincing by scissors and homogenization in a sterile, antibiotic rich media. Detailed methods for SIV isolation have been described in the Manual of Diagnostic Tests and vaccines for Terrestrial Animals by OIE (OIE 2004). Generally, inoculum prepared from nasal swabs or tissue samples is administrated in to the allantoic cavity of 10 to 11 day old embryonated chicken or fowl's eggs and eggs are incubated for 72 to 96 h at 35 °C. Usually, SIVs do not kill embryos, thus the hemagglutination assay (HA) using chicken or turkey erythrocytes is used to give a presumptive evidence for SIV presence. In addition, cell culture systems also have been used for SIV isolation. SIV HA and NA subtypes are determined by hemagglutination inhibition (HI) or neuraminidase inhibition (NI) assays.

Recently, molecular techniques that identify viral genomic material, such as reverse transcription polymerase chain reaction (RT-PCR) and real time PCR (rtPCR) found their application in virus identification and disease confirmation (Hall, Peacey et al. 2009). These molecular methods enable testing of a large numbers of samples at the same time, higher sensitivity and final results within several hours.

Presence of SIV specific antibodies is determined by serological assays such as HI, virus neutralization (VN) test, ELISA and single radial hemolysis test (Ogawa, Sugimura et al. 1978; Heinen 2003). Serologic diagnosis of SI requires the use of paired serum samples. First sample should be obtained during the acute phase of disease and the second 3-4 weeks later, in order to demonstrate an increase in the antibody titer when tested against specific antigens (B. C. Easterday 1999).

Other methods used for the detection of SIV or its antigens are direct and indirect immunoflouresence applied to lung tissue, nasal epithelial cells or bronchoalveolar lavage contents. Immunohistochemistry (IHC) detection in fixed tissues, enzyme-linked immunosorbent assay (ELISA), rapid cell culture assay using immunoperoxidase staining for typing and subtyping and commercially available enzyme immunoassay membrane test (Directigen FLU-A) are also widely used in SIV diagnostics (B. C. Easterday 1999; Olsen C.W 2006).

Existence of maternal antibodies in suckling or weanling pigs could complicate or lead to false positive SI diagnosis by serological or virological methods. Studies have shown that suckling and weanling pigs can be infected and shed virus in the presence of maternal antibodies.

The time of virus recovery and severity of SI disease signs are inversely related to levels of maternal antibodies (Renshaw 1975; Olsen C.W 2006).

1.4 SIV AND IMMUNITY

1.4.1 Innate immune response to SIV

The components of innate immunity against respiratory pathogens include mucin layer, ciliary action, protease inhibitors and a wide range of cytokines and immune cells. These nonspecific protective mechanisms prevent virus attachment and entry into the cells or interfere with virus replication.

Influenza virus attachment to the epithelial cells of respiratory tract triggers a cascade of intracellular signals and events. Activation of cellular signals in response to influenza A virus infection results in the production of cytokines, particularly interferons (IFNs), activation of natural killer cells (NK) and accumulation of neutrophils and alveolar macrophages. A molecular mechanism by which SIV activates innate immune responses at molecular level is still unclear, but it is assumed that it is similar to one described in humans and rodents.

Influenza A virus pathogen associated molecular patterns (PAMPs) are recognized by members of cellular pattern recognition receptors (PRRs). Three classes of PRRs were considered to play an important role in recognizing influenza virus infections. These are : Toll-like receptors 3, 7 and 8 (TLR-3, TLR-7, TLR-8)(Akira 2006), the retinoic acid inducible helicase RIG-I (Yoneyama and Fujita 2007; Yoneyama and Fujita 2007) and the nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRs)(Allen, Scull et al. 2009). These PRRs are expressed in myeloid–derived APCs and are located on the cell surface or membranes of cytoplasmic vesicles, such as endosomes and the endoplasmic reticulum (Ehrhardt, Seyer et al.).TLR-3 recognizes viral replicative intermediate double stranded RNA (dsRNA) while TLR-7 and TLR-8 recognize uridine-rich sequences of single stranded RNA (ssRNA)(Kato, Sato et al. 2005). RIG-I activation in cytoplasm is mediated by the presence of triphosphate groups at 5' terminal ends of vRNA (Kato, Sato et al. 2005) while NLRs could be activated with either dsRNA, ssRNA.or M2 (Ichinohe, Pang et al.). Activation of PRRs leads to induction of interferon (IFN) signalling pathways (mostly of IRF-3/IRF-7 and NF-κB) and production of inflammatory cytokines.

IFNs are a group of secreted cytokines considered as the most important component of innate immunity (Randall and Goodbourn 2008). IFNs exhibit diverse antiviral effects capable of controlling viral replication and virus spread to neighbouring cells. According to their amino acid sequence, IFNs are classified into three classes: type I, type II and type III. IFN-α/β are the most studied members of type I IFN class, and they are induced directly in response to viral infection. In general, IFNs act through a ubiquitously expressed heterodimeric receptor and activate a signal-transduction pathway, which triggers the transcription of IFN-stimulated genes (ISGs) and establish an antiviral response in infected cell. ISGs can also be induced directly by viral infection in an IFN- independent manner (Randall and Goodbourn 2008).

The initial IFN-α/ß induction is at the transcription level and does not require synthesis of new cellular or viral proteins (Randall and Goodbourn 2008). Activation of cytoplasmic nuclear factor kappa B (NF-κB) and interferon regulatory factor 3 and/or 7 (IRF-3/IRF7) is required for IFN-α/ß induction (Honda and Taniguchi 2006). The C-terminus of IRF-3 is phosporylated upon initial activation of TLRs. Phosporylation of IRF-3 triggers the conformational changes leading to dimerization and exposure of nuclear localization signal (NLS) of IRF-3 (Dragan, Hargreaves et al. 2007; Panne, Maniatis et al. 2007). Translocated IRF-3 is retained in the nucleus until it is dephosphorylated (Kumar, McBride et al. 2000). Translocation of NF-κB from cytoplasm to nucleus is prevented by its association with the NF-κB inhibitor molecule (IκB). Molecular signals created during viral infection lead to phosphorylation of IκB, its subsequent ubiquitination and degradation by proteosomes. Once released from the complex with IκB the NLS of the p65 subunit direct NF-κB transport to the nucleus (Hayden and Ghosh 2004; Wullaert, Heyninck et al. 2006; Randall and Goodbourn 2008). Furthermore, induction of IFN-α/ß genes requires binding of c-jun/ATF-2 heterodimer to the promoter. The IRF-3, NF-κB and c-jun/ATF-2 complexes assemble on the promoter to form complex-enhanceasome (Merika and Thanos 2001; Randall and Goodbourn 2008). The enhanceasome supports the recruitment of transcriptional co-activators p300 and CREB-binding protein that in turn promotes the assembly of the transcriptional machinery and RNA polymerase II (Randall and Goodbourn 2008). A main characteristic of this proposed enhanceasome model is that each of the transcription factors binds the IFN-α/ß promoter with limited affinity and that tight cooperation between factors is required for optimal induction (Merika and Thanos 2001; Randall and Goodbourn 2008).

Once the IFN-α/ß molecules are synthesized and secreted from virus infected cells they play a role in establishing the antiviral state in neighbouring cells. Key players in IFN-α/ß induced signaling pathway are the Janus/just another kinase (JAK) and the signal transducers and activators of transcription (STATs) (Murray 2007). JAK kinases are cytoplasmic tyrosine kinases that are recruited to a range of receptors in response to ligand binding while STATs are members of a family of transcription factors that are phosphorylated by JAKs and bind to DNA (Murray 2007).

IFN-α/ß binding to the type I IFN receptors lead to the activation of receptor-associated JAK kinasies (JAK1 and Tyk2) (Tang, Gao et al. 2007). This activation results in phosphorylation of STAT members (STAT1 and STAT2) and further provides their association. STAT1-STAT2 heterodimer is translocated into the nucleus where it interacts with the DNA binding protein IRF-9 (Tang, Gao et al. 2007; Randall and Goodbourn 2008). A newly formed protein complex STAT1-STAT2-IRF-9 is called ISGF3 (Tang, Gao et al. 2007). ISGF3 binds to all IFN stimulated response elements (ISREs) of cellular genes, thereby inducing transcription and synthesis of many proteins with antiviral properties, such as dsRNA dependent protein kinase R (PKR), Mx proteins and 2'5'-oligoadenylate synthetase (OAS)/RNase L.

In general, all cytokines released during SIV infection could be classified into "early" and "late" cytokines. Early cytokines are produced by non-immune cells at the site of infection and they are responsible for local inflammatory reactions and some systemic effects. Early cytokines produced in SIV infection are: interferon alpha (IFN-α), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), as well as chemotactic cytokines such as interleukin-8 (IL-8) and macrophage inflammatory proteins (van Reeth and Nauwynck 2000; La Gruta, Kedzierska et al. 2007). Late cytokines are mainly produced by T-cells after recognition of antigens in association with major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC). These cytokines are important modulators of the specific immune response to infection (van Reeth and Nauwynck 2000; La Gruta, Kedzierska et al. 2007). During SIV infections early cytokines initiate the cascade of inflammatory reactions, which in turn results in rapid infiltration of phagocytic cells. Neutrophil infiltration of the lungs is the first and most important event in pulmonary inflammation. Both TNF-α and IL-1 induce rapid neutrophil infiltration in intraalveolar space (Ulich, Watson et al. 1991). Neutrophil infiltration peaks within 12-24 h after cytokine release and returns to baseline after 48h. The

process of neutrophil infiltration is a complex series of steps which include: expression of endothelial cell adhesion molecules, neutrophil-endothelial cell adhesion, neutrophil activation and expression of neutrophil derived adhesion molecules, neutrophil dipedesis and migration beyond the vascular barrier. Both TNF-α and IL-1 upregulate or induce the adhesion molecules ICAM-1, E and P selectin on the vascular endothelium (Strieter, Lukacs et al. 1993). In addition, these cytokines facilitate adhesion molecule expression on neutrophils and respiratory epithelium, and induction of chemoattractant cytokines such as IL-8 (La Gruta, Kedzierska et al. 2007). Besides neutrophil chemoattractants, TNF-α and IL-1 also induce chemokines with selective macrophage attracting properties (monocyte chemoattractant protein-1) (Standiford, Kunkel et al. 1991). Neutrophils and macrophages can produce several inflammatory cytokines and mediators, including platelet activating factor, leukotriens and arachidonic acids in response to TNF-α and IL-1, which also play a role in local pulmonary inflammation. Despite their beneficial role in initiating inflammation and recruiting the immune cells, early and late cytokines are more associated with lung pathology during SIV infection. The local release of neutrophil enzymes induced by TNF-α and IL-1 results in increased respiratory burst activity, which contributes to lung injury, increased vascular permeability, lung hemorrhages and edema (Ulich, Watson et al. 1991; Patton, Saggart et al. 1995).

1.4.2 Adaptive immune response to SIV

The immune response to SIV infection is extremely rapid and efficient resulting in complete virus clearance from the respiratory tract within one week after initial infection. The adaptive immune response to SIV infection includes humoral and cell-mediated immunity (CMI). The humoral immune system possesses two, mucosal and systemic arms, both playing a major role in resistance to influenza virus infection. The CMI response is predominantly involved in clearance of virus-infected cells and recovery from disease.

The humoral immune system produces antibodies mainly against HA, NA, M and NP viral proteins (Cox, Brokstad et al. 2004). However, only antibodies to the globular head region of the HA can prevent virus attachment and consequently, neutralize virus infectivity. The NA, M and NP specific antibodies are less effective in preventing infection, but they may reduce the release of virus from infected cells and mediate killing of infected cells by other antibody-dependent mechanisms (Olsen C.W 2006).

In primary SIV infection all three major immunoglobulin (Ig) classes IgA, IgG and IgM, can be detected in serum, nasal and lung washings (Heinen, van Nieuwstadt et al. 2000; Larsen, Karasin et al. 2000). In response to infection, antibodies of the IgM isotype are produced first followed by IgA and IgG antibodies (Heinen 2003). A sharp rise of IgM antibodies in serum and mucosal secretions could be detected between 4 and 7 d.p.i. Interestingly, SIV specific IgM was approximately 8 to 4 times higher in BALF and nasal secretions then in serum (Heinen, van Nieuwstadt et al. 2000). After reaching a peak on day 7 p.i, the IgM antibody titer start to decline, being detectable in serum and BALF only until day 25 p.i. (Heinen, van Nieuwstadt et al. 2000). IgM antibodies are highly efficient in aggregating virions and in mediating lysis of infected cells by complement via the classical pathway (Heinen 2003). The rapid appearance of influenza specific IgM antibodies between 4 and 7 d.p.i corresponds with the virus clearance from the oropharyngeal tract by day 5 p.i. which could suggest that IgM plays a role in viral clearance after a primary infection (Heinen, van Nieuwstadt et al. 2000). However, in contrast to IgA, IgM probably has a minor role in long lasting immunity to a secondary infection (Heinen, van Nieuwstadt et al. 2000). In young pigs, IgM was shown to in part take over the role of IgA at the respiratory mucosa (Bianchi, Zwart et al. 1992; Heinen, van Nieuwstadt et al. 2000). Moreover, it was demonstrated that like IgA, IgM behaves more like a mucosal isotype in pigs than in other species (Bianchi, Scholten et al. 1999; Heinen, van Nieuwstadt et al. 2000).

First detection of SIV specific IgA antibodies in serum and mucosal secretions was on day 7 p.i reaching its maximum titer around 14-17 d.p.i.(Heinen, van Nieuwstadt et al. 2000). The kinetics of IgA antibody response in nasal secretions and BALF is different from that in serum. Serum IgA titer start to decline soon after reaching a peak 17 d.p.i while the mucosal SIV specific IgA remained at constant level until 44 d.p.i. (Heinen, van Nieuwstadt et al. 2000). In addition, the concentration of SIV specific IgA antibodies was much higher in nasal secretions and BALF than in serum. On day 44 p.i, SIV specific IgA antibody titer was 280 times higher in nasal secretions and 570 higher in BALF than in serum (Heinen, van Nieuwstadt et al. 2000). These findings indicate a local production of IgA and suggest a predominant role of IgA in mucosal defence against SIV infection. Mucosal IgA is the major and most dominant neutralizing antibodies directed against SIV. IgA antibodies function mainly by binding to released virions (Daniele 1990) and aggregated IgA support phagocytosis by binding to polymorphonuclear cells (PMN) (Heinen 2003). In addition, IgA can mediate lysis of virus

infected cells by complement activation through the alternative pathway thus preventing virus replication (Cox, Brokstad et al. 2004).

The temporary character of serum SIV specific IgM and IgA could be used as an indicator for recent infection with SIV. Monitoring serum IgM and IgA could provide an alternative for examining paired sera as a diagnosis of recent SIV infections. Moreover, the ELISA for IgM and IgA could possibly distinguish SIV infected pigs from pigs with maternal antibodies, since these antibodies are mainly of the IgG isotype (Heinen, van Nieuwstadt et al. 2000).

SIV specific IgG antibodies (IgG_1 subclass) could be detected in serum and BALF as early as 10 d.p.i. The IgG titer reaches its maximum 15 d.p.i and remain at this level until day 50 under experimental conditions (Heinen, van Nieuwstadt et al. 2000). Interestingly, Heinen et al. (Heinen, van Nieuwstadt et al. 2000) reported that even though the kinetics of the IgG antibody responses were similar in serum, BALF, and nasal secretions, the specific activity (ratio of SIV antibody titer to Ig concentration) of IgG (IgG_1 subclass) was approximately 14 times higher in BALF than in serum. In a similar study Larsen et al.(Larsen, Karasin et al. 2000) demonstrated that SIV specific IgG subclass predominates in serum and that the titer of SIV specific IgG antibodies in BALF and nasal mucosa was 10 times lower than in serum. However, both studies agreed that titers of IgG antibodies specific for SIV were low in nasal secretions. The high specific activity of IgG_1 in BALF also suggested a local production of this antibody isotype in the lungs (Heinen, van Nieuwstadt et al. 2000). This is confirmed by Larsen et al. who demonstrated the presence of antibody secreting cells in tracheobronchial lymph nodes and nasal mucosa (Larsen, Karasin et al. 2000). However, it is still believed that the majority of IgG antibody class in lungs is derived as a transudate from serum (Larsen, Karasin et al. 2000; Heinen, de Boer-Luijtze et al. 2001). Activity of IgG antibodies is based on binding to released virions, thus promoting phagocytosis by PMN and macrophages. Furthermore, IgG can mediate cellular lysis by complement via classical pathway and antibody dependent cellular cytotoxicity by NK cells.

Serum HI antibodies are the most commonly measured to determine the level of protection against influenza. These serum HI antibodies are directed in particular to SIV HA and NA (Van Reeth, Gregory et al. 2003; Cox, Brokstad et al. 2004). Serum HI antibodies play a role in both resistance to and recovery from influenza infection (Olsen C.W 2006). HI antibodies in pig sera can be detected from 7 to 10 days post infection. Peak HI titers of 1:160 to 1:320 are

usually seen between 14 and 21 days post infection (Heinen, van Nieuwstadt et al. 2000; Larsen, Karasin et al. 2000). Serum HI antibody titers remain high for several weeks and start to decline around 8 to 10 weeks after infection (Reeth, Brown et al. 2004).

CMI plays a role in recovery from influenza infection and may prevent influenza-related complications. However, CMI does not seem to contribute significantly in preventing infection. SIV specific cellular lymphocytes have been detected in the blood, local respiratory lymph nodes, pharyngeal and nasal mucosal tissues and spleen, of experimentally infected pigs (Larsen, Karasin et al. 2000). These SIV specific lymphocytes contribute to the clearance of the infection by stimulating antibody and cytokine production and proliferation of T-helper (Th) and cytotoxic T lymphocytes (CTLs) (Cox, Brokstad et al. 2004). T-cell responses could been detected from 7 d.p.i (Larsen, Karasin et al. 2000; Heinen, de Boer-Luijtze et al. 2001) reaching a peak levels at day 14 and return to baseline after 6 months p.i (Ennis, Rook et al. 1981; Cox, Brokstad et al. 2004). It should be noted that SIV specific Th cells specific for internal NP and M proteins can also stimulate B cells specific for HA (Heinen 2003). Cytolysis of SIV infected cells is mediated by CTLs together with SIV specific antibodies and complement activation (Cox, Brokstad et al. 2004; McMurry, Johansson et al. 2008). CTLs destroy influenza virus infected cells upon recognition of the viral pepides presented via MHC class I receptors (MHC I). In general, CTL epitopes are less abundant than epitopes on B and Th cells and are mostly concentrated within the highly conserved regions in NP and M influenza proteins (Heinen 2003). CTLs directed against conserved antigens have been shown to confer protection against heterosubtypic influenza A viruses (Heinen 2003). The level of memory CTLs (during the secondary response) to influenza A does not correlate with susceptibility to infection, illness or long-lived immunity in experimental conditions. However, it does correlate with the speed of viral clearance from the respiratory tract (McMichael, Michie et al. 1986; Heinen 2003).

1.5 PREVENTION AND CONTROL OF SIV

1.5.1 Public health aspects

A SIV infection in pigs poses two very important human public health aspects. SIVs have zoonotic potential and cases of human infection with SIV are well documented (Thacker and Janke 2008; Neumann, Noda et al. 2009). Secondly, pigs could serve as an intermediate host for the generation of novel viruses with pandemic potential for the human population.

Zoonotic infections with SIVs have been reported in North America, Europe and Asia (Alexander and Brown 2000; Gregory, Bennett et al. 2003). The majority of these infections were by cH1N1 SIVs. However, the infections with wholy avian H1N1 SIV from European pigs, reassortant H3N2 SIV with avian internal genes from pigs in Europe and Asia, and a reassortant H1N1 virus from pigs in North America have also been recovered from humans (Olsen C.W 2006; Neumann, Noda et al. 2009). At present, there are no distinctive clinical features that could distinguish zoonotic SIV from human influenza virus infections.. The majority of cases in which humans have been infected with swine-origin influenza viruses have involved individuals in direct contact with pigs (Olsen C.W 2006). In addition, serologic studies in both North America and Europe have recorded increased rates of SIV exposure among persons in contact with pigs (Nowotny, Deutz et al. 1997). Nevertheless, there are examples of zoonotic SIV infections without apparent animal contact (Olsen C.W 2006; Neumann, Noda et al. 2009). These several cases suggested the possibility of human-to-human spread of SIVs after initial pig-to person transmission. Together with the Fort Dix incident (Top and Russell 1977), the current H1N1 pandemic provided evidence for efficient spread of swine viruses from one person to another and back from humans to animals (Neumann, Noda et al. 2009; Peiris, Poon et al. 2009). Sequencing and phylogenic analysis revealed genetic composition of 2009 H1N1 pandemic influenza virus and described it as a "quadruple" reassortant virus (qH1N1) (Neumann, Noda et al. 2009). The new qH1N1 viruses emerged from the reassortment of recent TR H3N2 or H1N2 North American swine with Eurasian avian-like swine viruses (Figure 2.) (Neumann, Noda et al. 2009). Consequently, these viruses possess PB2 and PA genes of North American avian virus origin, a PB1 gene of human H3N2 virus origin, HA (H1), NP, and NS genes of classical swine virus origin, and NA (N1) and M genes of Eurasian avian like swine virus origin (Neumann, Noda et al. 2009). These particular H1N1 viruses have not circulated previously in human populations. The emergence of the swine origin pandemic qH1N1 influenza A virus in humans showed that flow of genes and viruses between swine and humans goes in both directions.

Pigs can serve as intermediate hosts thus playing a significant role in human influenza epidemiology. Available data on genome composition of the 1957 H2N2 and the 1968 H3N2 pandemic strains suggests that both viruses arose through genetic reassortment between an avian influenza virus and pre-existing human viruses (Webster, Bean et al. 1992). In addition, the genomic sequences of 1918 pandemic virus were determined recently and revealed an avian like

H1N1 virus that contains human like amino acids markers in several proteins (Reid, Taubenberger et al. 2004). Due to the limited susceptibility of birds to infection with human influenza viruses and vice versa, it is assumed that the origin of pandemic viruses does not rest exclusively in either birds or humans (Kanta Subbarao 2006). However, the rise of pandemic viruses may be attributed to pigs and their capability to serve as intermediate hosts. Because of their susceptibility to infection with influenza viruses of both avian and human origins (Ito 2000) pigs have been suggested to be "mixing vessel" hosts in which pandemic human influenza viruses emerge through genetic reassortment or adaptation for new host species (Brown 2000). Still, there is no direct evidence that the 1918, 1957 and 1968 pandemic viruses arose in pigs, but there is growing evidence that influenza viruses can move across species barriers from birds to pigs, from people to pigs, and from pigs to people. These three steps could be necessary to create a pandemic virus in pigs and move it from pigs into the human population (Kanta Subbarao 2006).

1.5.2 Security measures and SIV vaccine

Biosecurity measures and vaccination are the primary means of preventing SI in swine populations. Segregation and partial depopulation of early weaned piglets, all-in/all-out system and rigorous hygiene, are considered essential steps to control the spread of the SIV within the farm and minimize the effects of the disease on the farm's economy (Kothalawala, Toussaint et al. 2006). Even though antiviral drugs for control of influenza A infections are available on the market, vaccines are still the first choice as the specific method for prevention and control of SI. In general, viral antigenic variation (shift and drift) and the presence of maternally derived antibodies in young pigs are the two primary obstacles for successful vaccination against SI in pigs. Mismatch between vaccine and field virus strains due to the genetic and antigenic variabilities as a result of viral drift and shift is the common cause for increased vaccination failure. In addition, lack of constant global SIV surveillance is a reason why SIV vaccines are not updated on a regular basis and therefore vaccination results are variable. In the swine industry, vaccination of sows is a common practice used to increase and prolong levels of maternally derived antibodies in piglets which can protect them against clinical disease. However, the existence of maternal antibodies reduces SI vaccine efficacy. Presence of maternal antibodies complicates the vaccination strategy and results in increased incidence of disease among pigs as

their maternal antibodies decay (Kitikoon, Nilubol et al. 2006). As a result, influenza viruses continue to circulate in swine population on a regular basis, due to the constant introduction of immunologically naïve animals into the herd (Thacker and Janke 2008).

Current SI vaccines are inactivated, adjuvanted, whole-virus or ‘split’ vaccines prepared typically by virus propagation in embryonated chicken eggs. These vaccines stimulate high titers of IgG in serum and lungs, which decrease the incidence and severity of clinical disease but they do not consistently provide complete protection from virus infection (Brown 1994; Kothalawala, Toussaint et al. 2006). Even though protection against infection is not complete, virus replication and shedding are greatly reduced.

Commercial inactivated and adjuvanted SIV vaccines are available both in Europe and North America. SIV vaccine strain composition differs between continents because of the antigenic and genetic differences between SIVs circulating in European and the North American pigs. In North America, monovalent H1N1 was first introduced in 1993 but after the emergency of H3N2 influenza viruses in the U.S swine population in 1998, monovalent and bivalent (H1N1/H3N2) SIV vaccines become available (Olsen C.W 2006). The current North American SIV vaccines primarily contain representatives of cH1N1 and TR H3N2 viruses although a trivalent vaccine also containing reassortant rH1N1 virus has recently become available (Olsen C.W 2006). Autogenous vaccines containing herd specific strains are also used in the U.S. Primary vaccination of susceptible herd is usually consists of two IM injections 2 to 4 weeks apart. Biannual booster vaccinations are recommended only for sows (Olsen C.W 2006).

Despite recent reports on NS1 deletion based SIV vaccines (Richt, Lekcharoensuk et al. 2006; Vincent, Ma et al. 2007) live attenuated influenza virus (LAIV) vaccines are not commercially available for pigs. The LAIV vaccines are capable of enhancing cell-mediated immune response, directed primarily against conserved NP and M, thus providing more heterosubtypic immunity. The major concern about LAIV would be possible reassortment between field viruses and the vaccine virus, generating new reassortant viruses (Thacker and Janke 2008).

DNA vaccines are an alternative to conventional vaccination strategies to SIV infections (Olsen 2000). DNA vaccines are naked DNA plasmids that have been genetically engineered to produce defined antigens within transfected cells. Theoretically, vaccination with these vaccines results in the production of intracellular antigens that can be presented by MHC I and MHC II

molecules, leading to induction of humoral and CMI responses. DNA vaccines could provide heterosubtypic immunity and the internalization of DNA inside host cells would minimize interference by maternal antibodies (Thacker and Janke 2008). However, experimental trials with DNA vaccines against SI infections showed that DNA vaccines could be used only as primer vaccines and need to be followed by more conventional inactivated vaccines (Heinen, Rijsewijk et al. 2002; Larsen and Olsen 2002). Safety concerns such as vaccine DNA integration into host genome which could increase risk of malignancy and production of auto-antibodies against DNA leading to autoimmune disease (Kim and Jacob 2009) are another drawback of DNA vaccination. Vectored vaccines against SI infections, using alphavirus (Vander Veen, Kamrud et al. 2009), adenovirus (Wesley, Tang et al. 2004) or pseudorabies virus (Tian, Zhou et al. 2006) also are being studied .

2. HYPOTHESIS AND OBJECTIVES

2.1 OVERALL GOALS AND RATIONALE

Influenza A viruses are highly contagious respiratory pathogens of mammals. Influenza virus causes significant morbidity in swine, resulting in a substantial economic burden. Vaccination is the primary method for the prevention of influenza disease. Influenza vaccines currently in use for pig protection are inactivated and disrupted ('split') virus vaccines resuspended in an oil adjuvant and intended for intramuscular (IM) application. Immunity induced by these vaccines is based on the induction of neutralizing serum antibody against the viral surface proteins HA and NA of the vaccine strain. However, use of these inactivated vaccines has a number of limitations. First, the serum antibodies induced by inactivated vaccine are strain specific and the antigenic differences between the vaccine and circulating strain may reduce the efficacy of the vaccine and fail to protect animals in the field. Second, the IM delivered inactivated vaccine leads to relatively poor cell-mediated response and low induction of mucosal IgA antibody response in the upper and lower respiratory tract which are thought to play an important role in protection from infection and in limiting transmission from infected animals. And third, because of the genetic composition, influenza viruses undergo antigenic variation by antigenic drift and antigenic shift. Consequently, inactivated vaccines need to be updated frequently in order to keep pace as the virus antigen changes. However, there is no annual monitoring of SIVs in the field so inactivated SIV vaccines have not been changed in the last ten years. Therefore*, there is an urgent need to develop safe, stable and cross-protective LAIVs.*

LAIVs, which are administered IN may have advantages over their inactivated counterparts. IN application of LAIV mimics the natural infection and is capable of inducing immunity similar to one after exposure to wild type virus in the nature. Live vaccines are thought to induce cross-reactive cell-mediated cytotoxicity as well as a humoral antibody response, providing superior immune protection than inactivated vaccines. In addition, protective immunity to influenza with LAIV after IN application is likely to involve a strong local mucosal IgA response which is not seen with traditional IM administered vaccines. Another advantage of IN given LAIVs over IM delivered inactivated vaccines is decreased possibility of the tissue reaction, swelling and muscle soreness that is occasionally associated with the IM administration

of inactivated, adjuvanted vaccines. Cold adapted LAIV have been developed and approved for human and equine species only. However, no live attenuated vaccines are available for SI.

The reverse genetics, technique that allows generation of RNA viruses entirely from cloned cDNA has reached a level of sophistication that enables the generation of virtually any influenza virus in a short amount of time. This technique allows not only rapid generation of vaccine strains but also provides the ability to manipulate virus genes for further attenuation and efficacy of vaccines. If we can develop an efficacious influenza vaccine by reverse genetics, we will be able to control the circulating strains and quickly respond to any new emerging influenza strains. This would be of significant economic benefit to the swine industry and to human society as well.

Influenza virus entry into cells is mediated by viral surface protein HA. HA is synthesized as a precursor HA0 which consists of HA1 and HA2. In order to be infectious, HA0 must be cleaved by a host protease into HA1 and HA2. Therefore, this process is a crucial determinant in virus pathogenicity.

Our overall goal is to generate a live attenuated SIV, specifically a SIV with an unusual HA cleavage site that is resistant to activation during natural infection but which can be activated in vitro by a protease. The rationale of this project is that a SIV with modified HA cleavage site cannot be activated *in vivo*, but which can be activated *in vitro* can serve as a live vaccine thus reducing the risk of generating the pathogenic reassortants and providing broad immune response similar to natural immunity.

2.2 HYPOTHESIS

Mutant viruses with a modified HA cleavage site that are resistant to activation during natural infection but that can be readily activated in vitro by a protease could serve as a LAIV providing strong broad immune response against antigenically different SIVs.

2.3 OBJECTIVES

Our main objective is to generate live attenuated SIVs that can be used as LAIV, thus minimizing the risk of generating the pathogenic reassortants and inducing highly effective cell-mediated and humoral systemic and mucosal immune responses.

In order to achieve this objective we set up three aims:

Aim 1. Generation of a mutant SIVs with their modified hemagglutinin (HA) segment

To generate a mutant SIVs with their HA gene manipulated so that the original trypsin-specific cleavage site (arginine-glycine; Arg-Gly) is replaced with the elastase-sensitive site (valine-glycine; Val-Gly or alanine-glycine Ala-Gly).

Aim 2. Characterization of the mutant viruses in vitro and in vivo

To characterize the mutant viruses in the aspects of growth, genetic stability in tissue culture and virulence in pigs.

Aim 3. Evaluation of the immune responses to LAIV after IT and IN administration and protection against challenge by antigenically related and unrelated SIVs.

To test the potential of the mutant virus to serve as a live vaccine.

3. REVERSE GENETICS GENERATED ELASTASE-DEPENDENT SWINE INFLUENZA VIRUSES ARE ATTENUATED IN PIGS

(As published in Journal of General Virology (2009), 90, 375-385)

3.1 INTRODUCTION

Swine influenza virus (SIV) is a member of the Orthomyxoviridae family, classified into the genus of influenza A (Lamb A. 2000). SIV is the causative pathogen for swine influenza, a highly contagious, acute viral disease of swine. SIV induces an acute respiratory tract infection and lung lesions. After an incubation period of 24 to 72 hours, the beginning of disease is abrupt, often appearing in many animals in the herd at the same time. Clinical signs are characterized by a high fever, sneezing, rhinitis with nasal discharge, labored abdominal breathing and bronchial rales at auscultation. In general, morbidity rates may approach 100% while mortality is usually less than 1%. SIV infections can be associated with secondary bacterial/viral infections and reproductive disorders that can result in abortions. Along with porcine reproductive and respiratory syndrome virus, SIV contributes significantly to post-weaning respiratory disease, causing economic losses due to decreased body condition and an increase in the number of days needed to reach market weight. Currently, H1N1, H3N2 and H1N2 are the dominant subtypes causing disease in the North American swine population (Olsen 2002).

SIV infection also poses very important human public health concerns. SIV naturally infect pigs and can be transmitted to humans (Wells, Hopfensperger et al. 1991). Since pigs are able to support replication of avian, human, and swine influenza viruses, it is very likely that genetic reassortments between these viruses could create novel influenza subtypes. Recently avian/swine virus reassortant H2N3 influenza A viruses were isolated from diseased swine in the United States. The H2N3 virus has undergone some adaptation to the mammalian host and is able to transmit among pigs and ferrets (Ma, Vincent et al. 2007). Data from SIV surveillance studies and characterization of influenza virus isolates from pigs are critical for understanding of long term evolutionary and epidemiological patterns of human influenza and pandemics (Rota *et al.*, 1989; Wells *et al.*, 1991).

The genome of influenza A viruses consists of eight segmented RNAs of negative polarity. The crucial step for infection by influenza A virus is an initial virus binding to the cells followed by receptor-mediated endocytosis and fusion of the viral envelope to endosomal membranes

(Cross *et al.*, 2001; Skehel & Wiley, 2000). Influenza A virus entry into cells is mediated by the viral surface glycoprotein hemagglutinin (HA). HA has three major roles during virus replication: (i) HA binds to sialic acid receptors on the cell surface; (ii) it provides penetration of the virus into the cytoplasm by mediating fusion between the viral and the endosomal membranes; (iii) it is the main viral antigen against which neutralizing antibodies are produced (Fleury *et al.*, 1999; Ha *et al.*, 2002; Lamb A., 2000). HA is synthesized as a precursor HA0 that consists of HA1 and HA2 (Skehel and Wiley 2000). In order to be infectious, HA0 must be cleaved by host proteases into HA1 and HA2. Therefore, this process is a crucial determinant in virus pathogenicity (Bosch *et al.*, 1981; Klenk *et al.*, 1975).

Multiple SIV subtypes continue to circulate in swine populations despite available vaccines. Current SIV vaccines are inactivated and their application does not provide the desired immune response and cross-protection against multiple antigenic SIV variants in the field. Application of cold-adapted, live attenuated influenza virus (LAIV) in humans and horses provided significantly higher and more efficient immune response than killed influenza vaccines (Paillot, Hannant et al. 2006). Although recent studies by Richt *et al.* showed mutant SIV with truncated NS1 protein was highly attenuated in pigs and conferred protection against swine influenza (Solorzano, Webby et al. 2005; Richt, Lekcharoensuk et al. 2006; Vincent, Ma et al. 2007), there is no commercially available LAIV for SIV in North America.

It has been shown that conversion of HA cleavage site from a trypsin sensitive motif to an elastase sensitive motif resulted in attenuated viruses *in vivo* (Stech, Garn et al. 2005; Gabriel, Garn et al. 2008). However, these studies were performed with mouse adapted influenza virus or avian influenza virus in mouse models. Application of this attenuation approach in a natural host has not been achieved yet. Here we report that using reverse genetics, we generated two mutant SIVs derived from strain A/SW/SK/18789/02 (H1N1) (SIV/SK) (Karasin, West et al. 2004). The mutant SIVs encode modified HA, as such the original trypsin-specific cleavage site of HA (arginine-glycin, Arg-Gly) (Garten *et al.*, 1981; Lazarowitz *et al.*, 1973) was replaced with the elastase-sensitive site (valine-glycine, Val-Gly or alanine-glycine, Ala-Gly) (Castillo *et al.*, 1979; Gertler & Hofmann, 1970). These mutations resulted in the generation of HA glycoproteins that are resistant to activation during the natural infection by trypsin-like proteases but can be readily activated *in vitro* by elastase. Furthermore, the mutant viruses are attenuated in pigs, suggesting that these genetically engineered SIVs have great potential to serve as LAIVs for SIV.

3.2 MATERIALS AND METHODS

3.2.1 Cells and viruses.

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). 293T (human embryonic kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The influenza A/Swine/Saskatchewan/18789/02 (H1N1) virus was obtained from the Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan, Canada. SIV/SK influenza virus was propagated at 37ºC in the allantoic cavities of 11-day-old embryonated chicken eggs. Virus titers were determined on MDCK cells by plaque assay as described previously (Shin, Liu et al. 2007).

3.2.2 Plasmids and primers.

Viral RNA of SIV/SK was isolated from 600µl of allantoic fluid using an RNeasy kit, (Qiagen). Viral RNA (0.04 µg) was reverse transcribed into cDNA using Uni12 primer (5 -AGC AAA AGC AGG- 3’) (Hoffmann, Stech et al. 2001) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. cDNAs were then amplified by polymerase chain reaction (PCR) using segment specific primers (Hoffmann, Stech et al. 2001). All eight cDNA segments were individually cloned into vector pHW2000 (Kindly provided by Dr. E. Hoffmann and R. Webster) (Hoffmann *et al.*, 2000), resulting in constructs pHW-SIV/SK-PB2, pHW-SIV/SK-PB1, pHW-SIV/SK-PA, pHW-SIV/SK-HA, pHW-SIV/SK-NP, pHW-SIV/SK-NA, pHW-SIV/SK-M and pHW-SIV/SK-NS. Mutations in the HA coding sequence were introduced into the plasmid pHW-SIV/SK-HA by site-directed mutagenesis as described previously (Shin, Li et al. 2007). Plasmid pHW-SIV/HA-R345V encoding mutant HA with an Arg replaced by Val at aa 345 was generated using the primers 5’ -GTC CCA TCC ATT CAA TCC GTA GGC CTG TTT GGA GCA ATT GCC- 3’ and 5’ -GGC AAT TGC TCC AAA CAG GCC TAC GGA TTG AAT GGA TGG GAC- 3’. Similarly, plasmid pHW-SIV/HA-R345A encoding mutant HA with Arg at 345 replaced by Ala was generated using primers 5’ -GTC CCA TCC ATT CAA TCC GCG GGA CTG TTT GGA GCA ATT G- 3’ and 5’ -CAA TTG CTC CAA ACA GTC CCG CGG ATT GAA TGG ATG GGA C- 3’. All of the above plasmids were DNA sequenced to ensure that additional mutations were not introduced during PCR.

3.2.3 Generation of viruses by reverse genetics.

WT and mutant viruses were generated using an 8-plasmid reverse genetics system described by Hoffmann et al (Hoffmann, Neumann et al. 2000). Briefly, 293T and MDCK cells were co-cultured at the same density (2.5×10^5 cells/well) in a 6-well plate and maintained in DMEM containing 10% FBS at 37 ºC, 5% CO_2 for 24 hrs. One hr prior to transfection, medium containing FBS was replaced with fresh Opti-MEM (Invitrogen). To rescue SIV/SK-WT, cells were transfected with eight plasmid constructs (pHW-SIV/SK-PB2, pHW-SIV/SK-PB1, pHW-SIV/SK-PA, pHW-SIV/SK-HA, pHW-SIV/SK-NP, pHW-SIV/SK-NA, pHW-SIV/SK-M and pHW-SIV/SK-NS) by Transit-LT1 transfection reagent (Mirus). The viruses (rgSIV/SK-R345V and rgSIV/SK-R345A) containing mutations within HA segment were generated in the same way but substituting pHW-SIV/HA with either pHW-SIV/HA-R345V or pHW-SIV/HA-R345A. Six hrs later, the transfection mixture was replaced with 1ml of fresh Opti-MEM. Twenty four hrs post transfection, one ml of Opti-MEM containing 0.4% BSA and 2µg/ml of TPCK-treated trypsin (for WT virus) , 1µg/ml human neutrophil elastase (for mutant viruses) or 10µg/ml porcine pancreatic elastase (for mutant viruses) (Serva Electrophoresis GmbH) was added to each well. Supernatants were collected 72 hrs post transfection.

3.2.4 Western blot analysis.

Western blotting was performed as described previously with minor modifications (Shin, Liu et al. 2007). MDCK cells (7×10^5) were plated into 35 mm dishes and were mock infected or infected with influenza viruses at a determined multiplicity of infection (MOI). At the indicated times, cell monolayers were lysed and 30 µg of total protein was resolved on sodium dodecyl sulfate – 10% polyacrylamide gels (SDS-PAGE), and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were probed with polyclonal antiserum against NP (1:2000) or M1 (1:2000) antibody (raised in our lab, (Shin, Liu et al. 2007)) followed by an incubation with AP-conjugated anti rabbit IgG (1:10,000) (Jackson ImmunoResearch Lab). The immunoblots were then visualized by incubating with BCIB/NBT premix solution (Sigma).

3.2.5 Virus purification.

To prepare virus stocks for animal experiments without any residues of proteases, we purified cell culture grown viruses (SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A). MDCK

cells grown in 10-cm dishes were infected with the viruses. Cells were incubated in the presence of either TPCK-treated trypsin or human neutrophil elastase for 36-48 hrs in MEM supplemented with 0.2% BSA. Supernatants were harvested and cell debris was removed by centrifugation for 25 min, at 1500 rpm (700 g) in a Beckman Coulter Allegra 6R centrifuge. Viruses were pelleted by ultracentrifugation at 25,000 rpm for 2.5 hrs at 10°C using a Beckman rotor SW28. Pelleted viruses were resuspended in 1 ml of TSE buffer (20mM Tris pH 7.8, 150mM NaCl pH 7.8, 2mM EDTA pH 7.8) and were then overlaid on a 30% - 60% sucrose cushion and further centrifuged at 25,000 rpm for 2.5 hrs at 10°C using a Beckman rotor SW41. The visible opalescent virus band on the boundary of 30% and 60% sucrose was harvested and stored at -80 °C. Virus titers were determined by plaque assay.

3.2.6 Infection of pigs with SIV.

Thirty-five four-week old SIV negative pigs were randomly selected and divided into seven groups with five pigs per group. Groups were housed separately in isolation rooms for one week prior to infection. At five weeks of age, pigs in group 1 were mock infected intratracheally with 4ml of MEM, while pigs in remaining groups were infected intratracheally with 4ml of MEM containing 1×10^5 PFU/ml or 1×10^6 PFU/ml of SIV/SK-WT, SIV/SK/02-R345V or SIV/SK/R345A (Table 1). Pigs in all groups were monitored daily for five days and then were sacrificed. All animal experiments were conducted at the Vaccine and Infectious Disease Organization, University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

3.2.7 Clinical observation and sampling.

Clinical signs including lethargy, apathy, inappetence, reluctance to move, coughing, sneezing, nasal discharge and labored abdominal breathing were monitored for five days. Rectal temperatures were recorded daily, nasal swabs were taken from each pig and placed in 1.5 ml MEM containing antibiotic/antimycotic solution (Invitrogen) and were frozen at -80 ºC until the study was completed.

3.2.8 Necropsy and macroscopic examination of lungs.

Animals in all groups were euthanized five days post infection by intravenous administration of Euthanyl (Sodium pentabarbitol 25mg/ml). At necropsy, lungs were removed *in toto* and evaluated for the percentage of the lung affected with purple-red, firm lesions typical for SIV infection. The percentage of the areas affected with pneumonia was estimated visually for each lung lobe. Total percentage for the entire lung was calculated based on weight proportions of each lung lobe to the total lung volume (Richt, Lager et al. 2003). Tissue samples from the right apical, cardiac and diaphragmatic lobes were taken for virus isolation and histopathology examination.

3.2.9 Virus titration from nasal swabs and lung tissue.

Lung tissue was processed by mincing with scissors and then homogenized. Processing of the tissue was done in MEM supplemented with antibiotic/antimycotic solution at the final concentration of 10% weight to volume. Each nasal swab and lung sample was subsequently thawed and vortexed for 15 sec, centrifuged at 1600 *g* for 25 min at 4 ºC. Supernatants were collected and ten-fold serial dilutions were prepared in MEM. Each dilution in replicates of 5 was plated onto confluent MDCK cells in 96-well plates. After 1 hr incubation at 37 ºC, the diluents were replaced by 200µl of MEM supplemented with 0.2% BSA and 1 µg/ml TPCK-treated trypsin or 0.5 µg/ml human neutrophil elastase. Plates were evaluated for cytopathic effect (CPE) between 24 and 96 hrs post infection (h.p.i.). Virus titers were calculated according to the Reed and Muench method (Reed L.J 1938).

3.2.10 Histopathology evaluation.

Tissue sections of lungs were routinely stained with hematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or extent of lesions within the sections examined as follows: 0: no visible changes; 1: mild focal or multifocal change; 2: moderate multifocal change; 3: moderate diffuse change; 4: severe diffuse change. A single pathologist scored all slides and was blinded for the experimental groups.

3.2.11 Statistical analysis.

Statistical analysis of body temperatures, macroscopic lesion scores, microscopic lesion scores and virus titers were performed using GraphPad Prism5 statistical software. Differences between means of each group in each assay were determined by using Mann Whitney analysis of variance methods. If the mean values of at least one group differed from others with a $P < 0.05$ they were considered statistically significant.

3.3 RESULTS

3.3.1 Generation of elastase dependent SIV/SK viruses.

To establish a reverse genetics system for SIV/SK, we cloned the eight viral RNA segments from SIV/SK virus into the reverse genetics vector pHW2000 (Hoffmann, Neumann et al. 2000). Upon transfection of these plasmids into MDCK and 293T co-cultured cells, infectious virus was recovered. The reverse genetics recovered WT SIV/SK (SIV/SK-WT) and the parental WT SIV/SK grew to similar titers in embryonated eggs and caused similar degree of lesions in pigs (Data not shown). To generate a trypsin-resistant virus, we exchanged the nucleotides corresponding to positions 1030 and 1031 in the SIV/SK HA gene from AG to GT and positions 1030 to1032 from AGA to GCG (Figure 3.1A), respectively. These mutations resulted in the replacement of Arg residue at the position 345 by Val and Ala, respectively. According to the literatures, leukocyte elastase has a narrow specificity. It cleaves preferentially Val-X bonds and to a lesser extent Ala-X bonds, which are preferred by pancreatic elastase. (Narayanan and Anwar 1969; Castillo, Nakajima et al. 1979). While mutant virus SIV/SK-R345V was rescued by transfection in the presence of human neutrophil elastase, we were unable to rescue mutant viruses by transfection with porcine pancreatic elastase even though Ala-Gly cleavage site was expected to be sensitive to this protease. However, SIV/SK-R345A could be rescued when human neutrophil elastase was provided, although with slower progression: SIV/SK-R345V virus was rescued at 36 hrs after transfection whereas SIV/SK-R345A virus was recovered at 72 hrs after transfection. The genotype of the mutant viruses were characterized and confirmed by DNA sequencing of the RT-PCR product derived from the HA gene of mutant viruses. Mutant viruses could not be recovered by transfection in the presence of trypsin.

3.3.2 Mutant viruses are strictly elastase dependent and exhibit the same growth properties as WT SIV in tissue culture.

Multi-cycle growth potential of influenza virus is dependent on proteolytic activation of HA (Klenk, Rott et al. 1975). To examine the replication potential of mutant viruses SIV/SK-R345V and SIV/SK-R345A, the plaque size and multiple cycle growth kinetics were compared to that of WT virus. To this end, confluent MDCK cells were infected with SIV/SK-WT, SIV/SK-R345V or SIV/SK-R345A at MOI of 0.001, supernatants were harvested at indicated time points until 72 h.p.i. and virus titers were determined by plaque assay on MDCK cells. As shown in Figure 3.1B, all of the viruses reached a plateau at 24 h.p.i. The growth kinetics and titers of the mutants in MDCK cells were similar to those of the WT virus. These results indicate that mutation in the HA protein cleavage site did not result in attenuation of virus growth in MDCK cells. To investigate the protease dependence of SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A, we performed plaque assays in the presence of either trypsin or neutrophil elastase in the plaque overlay or in the absence of an exogenous protease on MDCK cells. As shown in Figure 3.1C, while WT SIV/SK virus was able to form clear big plaques in the presence of trypsin (panel a), SIV/SK-R345V and SIV/SK-R345A viruses formed similar sized plaques in the presence of neutrophil elastase (panel e and f). In contrast, SIV/SK-R345V and SIV/SK-R345A viruses were resistant to trypsin (panel b and c). Neither the WT nor the mutant viruses were activated without the presence of exogenous proteases (panel g, h and i).

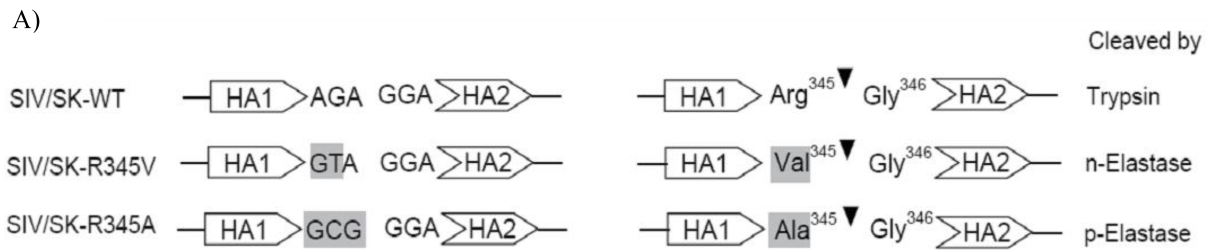
We also tested the growth potential of the two mutant viruses in the presence of porcine pancreatic elastase. SIV/SK-R345V did not grow at all, suggesting that this virus is entirely dependent on human neutrophil elastase activation. Interestingly, although SIV/SK-R345A could not be rescued by porcine pancreatic elastase, it grew in the presence of this protease. After passage of SIV/SK-R345A five times with porcine pancreatic elastase, we sequenced the RT-PCR product of HA derived from SIV/SK-R345A. We found that in front of the cleavage site, Ser-344 was replaced by Pro.

3.3.3 Mutant viruses are genetically stable.

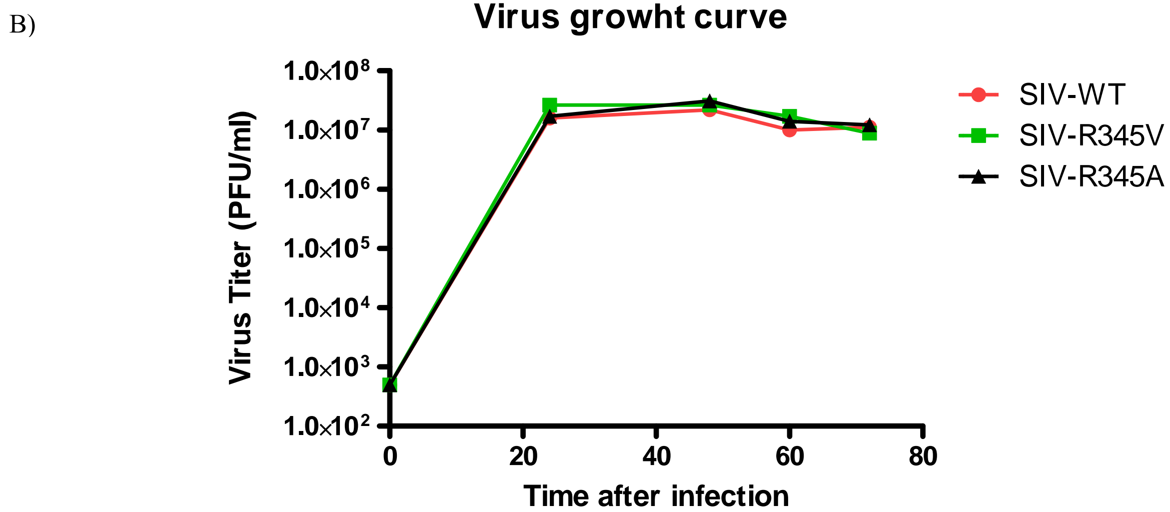
To address the genetic stability of the mutant viruses, they were passaged five times on MDCK cells at MOI of 0.001 in the presence of both trypsin and neutrophil elastase. Plaque assays were then carried out with ten-fold serial dilutions of the supernatants from the fifth

passage in the presence of either elastase or trypsin. Figure 3.1D showed the plaque assay results from 10^6 dilutions. Well defined plaques were seen in the presence of elastase, however no plaques were detected in the presence of trypsin. At lower dilutions of the supernatants, while cell monolayers were completely disrupted by a higher number of infectious viral particles in the presence of elastase, no infectious particles could be detected in the presence of trypsin (data not shown). After the fifth passage, sequencing results showed that both mutant viruses retained the introduced mutations at the HA cleavage site without any other unwanted mutations, suggesting high level of genetic stability of the mutant viruses in cell culture.

A)



B)



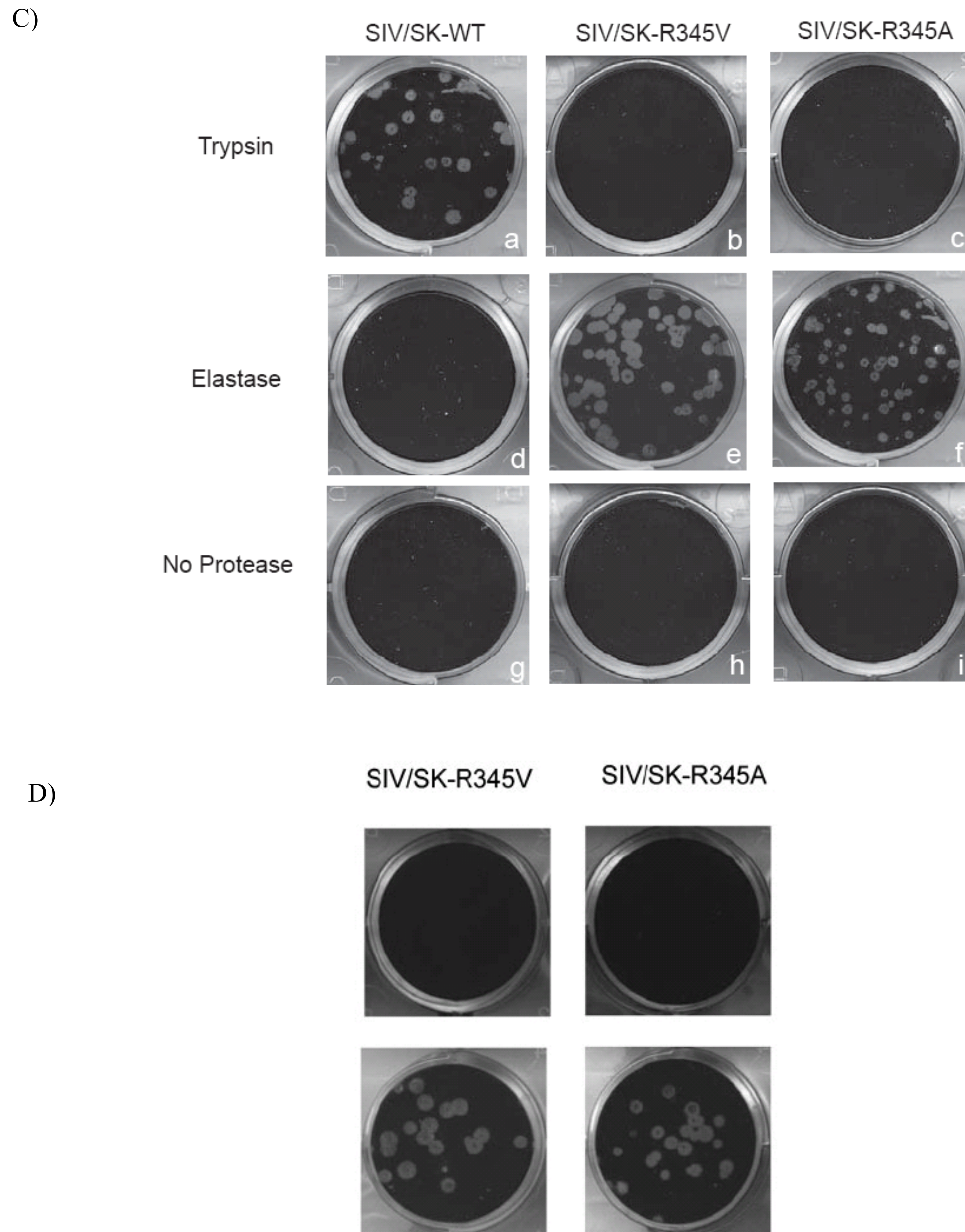
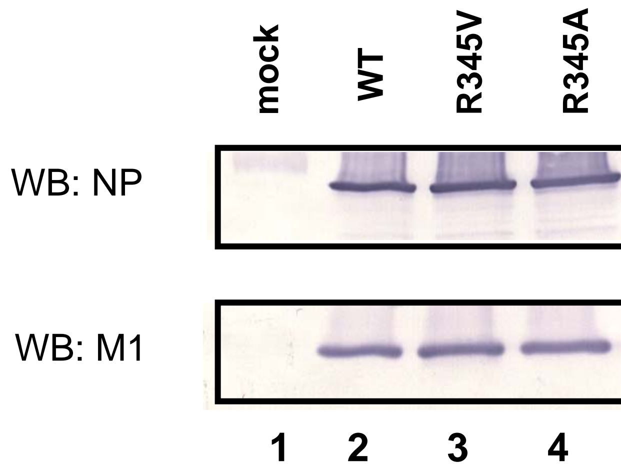


Figure 3.1. **Generation, growth property and genetic stability of mutant viruses.** (A) Schematic diagram showing the modifications of the HA cleavage site. (B) Multiple cycle growth curves of SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A on MDCK cells. Cells were infected in triplicate with each virus at an MOI of 0.001 in the presence of 1μg/ml TPCK treated trypsin (for WT) or 0.5μg/ml human neutrophil elastase (for mutant viruses). Supernatants were collected at indicated time points until 72 h.p.i. and titers were determined by plaque assay onMDCK cells. (C) Plaques formed by SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A viruses on MDCK cells in the presence of trypsin, neutrophil elastase or in the absence of exogenous protease. (D) Mutant viruses were passaged on MDCK cells at low MOI (0.001) for five times in the presence of both trypsin and neutrophil elastase. The supernatants from the fifth passage were serial diluted and plaque assays were performed in the presence of either trypsin or neutrophil elastase. Representive of plaque assay results with 10^6 dilutions were shown here.

3.3.4 Mutant viruses are able to infect cells but their replication is restricted due to the uncleaved HA0.

As a candidate for live attenuated vaccine, a virus should be able to enter cells and complete limited replication cycles. To examine whether this was the case with the mutant viruses SIV/SK-R345V and SIV/SK-R345A, MDCK cells were infected by SIV/SK-R345V or SIV/SK-R345A at MOI of 10. After 1 hr virus absorption, cells were washed extensively, medium without any extraneous proteases was added. At 8 h.p.i., supernatants were harvested and subjected to virus purification; whereas cells were lysed for Western blotting analysis using NP or M1 antibody. As seen in Figure 3.2A, NP and M1 expression could be detected in the cells infected with SIV/SK-R345V and SIV/SK-R345A (lane 3 and 4). The expression levels were similar to that in WT virus infected cells (lane 2). To examine the status of HA present in virus particles, purified virons grown in the presence or absence of corresponding protease were separated on SDS-PAGE followed by staining with commassie blue. As shown in Figure 3.2B, HA remains in the form of HA0 in SIV/SK-R345V and SIV/SK-R345A when virus particles were grown without adding elastase (lane 4 and 6). In contrast, the majority of HA0 was cleaved into HA1 and HA2 in SIV/SK-R345V and SIV/SK-R345A virus particles when grown in the presence of neutrophil elastase, although traces of HA0 were visible (lane 3 and 5). As a positive control, HA1 was evident in purified WT SIV/SK grown in the presence of trypsin (lane 2).

A)



B)

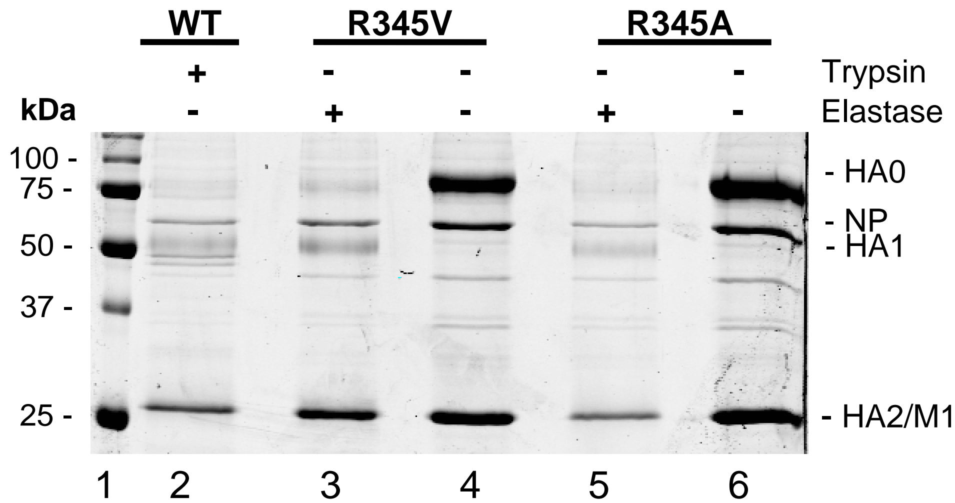


Figure 3.2. Characterization of mutant viruses in tissue culture. (A) MDCK cells were mock or infected with SIV/SK-R345V or SIV/SK-R345A at an MOI of 10. At 8 h.p.i. cell lysates were prepared and subjected to Western blotting using NP or M1 antibody. **(B)** Viruses grown either in the presence or in the absence of appropriated protease were harvested from tissue culture supernatants and purified. Purified virions were resolved on SDS-PAGE followed by Commassie blue staining.

3.3.5 Elastase dependent SIV/SK-R345V and SIV/SK-R345A viruses are attenuated in pigs.

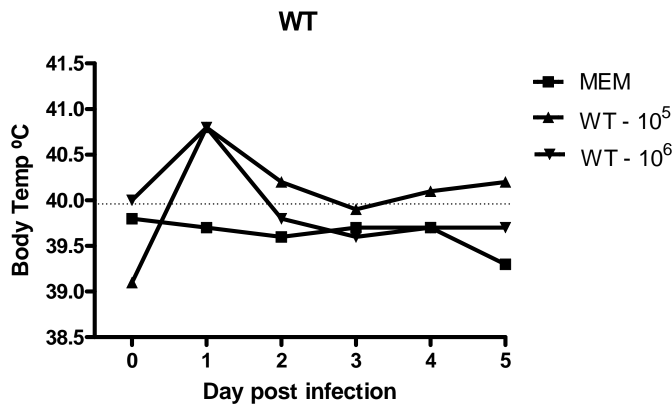
Thirty-five four-week old, SIV sero-negative pigs were randomly grouped into 7 groups. Groups of 5 pigs were infected intratracheally with 4ml of MEM containing $1x10^5$ PFU/ml or $1x10^6$ PFU/ml of SIV/SK-WT, SIV/SK-R345V or SIV/SK-R345A. The animals in control group were mock infected with medium only (Table 3.1). Clinical signs, rectal temperature were monitored and nasal swabs were taken daily after virus infection. On day 5 p.i., pigs were euthanized and necropsies were performed. During the five-day observation period, clinical signs characteristic for SIV infection were observed only on days 1, 2 and 3 p. i. in the groups infected with the SIV/SK-WT at both doses. Animals in all other groups did not show any signs of respiratory distress, weight loss or nasal discharge. As shown in Figure. 3.3, mean rectal temperatures in both groups infected with the SIV/SK-WT (4×10^5 PFU and 4×10^6 PFU) increased to 40.8 °C on day 1 p. i. The temperature change is significant compared to the control group ($P = 0.0465$) (panel A). On the following days, the temperature gradually decreased and then slightly rose up. By the end of the experiments, the mean temperatures of animals infected with high dose and low dose were 40.2 °C ($P = 0.0278$) and 39.7°C ($P = 0.0651$), respectively. Similar temperature kinetics were observed in the group infected with the high dose of SIV/SK-R345A virus (Figure 3.3C), where mean temperature reached 40.6 °C on day 1 p.i. and then remained in the range of 39.5 °C to 40.5 °C. However, pigs in the remaining groups (SIV/SK-R345V infection dose: 4×10^5 PFU and 4×10^6 PFU, SIV/SK-R345A infection dose: 4×10^5 PFU) did not show significant differences in mean rectal temperatures compared to the control group (Figure 3.3B and C).

Nasal swabs were taken daily from all pigs in assigned groups. Only pigs infected with the SIV/SK-WT shed virus during all five days. The number of animals that shed virus and virus titers are shown in Figure 3.4. The highest number of animals that shed virus was on days 3 and 4 p.i. in high dose SIV/SK-WT infected group (panel A). Virus could be recovered from only one animal on days 3, 4, and 5 p.i. in low dose SIV/SK-WT infection group (Panel B). We could not detect any virus in nasal secretion from the pigs infected with SIV/SK-R345V and SIV/SK-R345A.

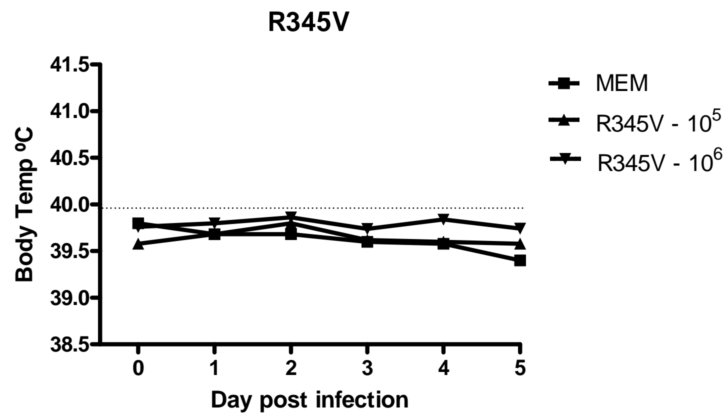
Table 3.1. **Assignment of pigs, dose and route of virus infection**

Group N=5	Inoculum	Concentration	Dose Volume	Route
1	MEM		4ml	Intratracheal
2	SIV/SK-WT	10^5 PFU/ml	4ml	Intratracheal
3	SIV/SK-WT	10^6 PFU/ml	4ml	Intratracheal
4	SIV/SK-R/V	10^5 PFU/ml	4ml	Intratracheal
5	SIV/SK-R/V	10^6 PFU/ml	4ml	Intratracheal
6	SIV/SK-R/A	10^5 PFU/ml	4ml	Intratracheal
7	SIV/SK-R/A	10^6 PFU/ml	4ml	Intratracheal

A)



B)



C)

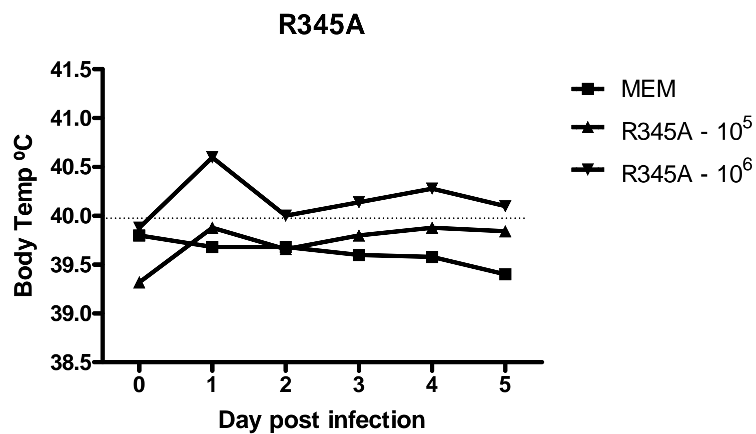
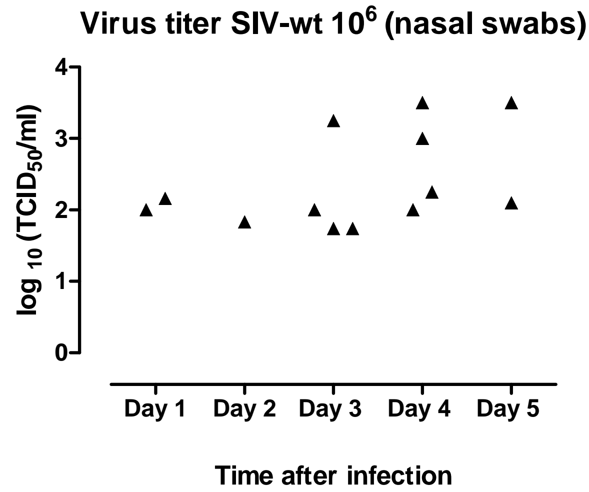


Figure 3.3. Median group rectal temperatures of Pigs. Median daily temperatures of pigs infected with high dose (4×10^6PFU) or low dose (4×10^5 PFU) of SIV/SK-WT **(A)**, SIV/SK-R345V **(B)**, or SIV/SK-R345A **(C)** were compared with those of pigs infected with MEM.

A)



B)

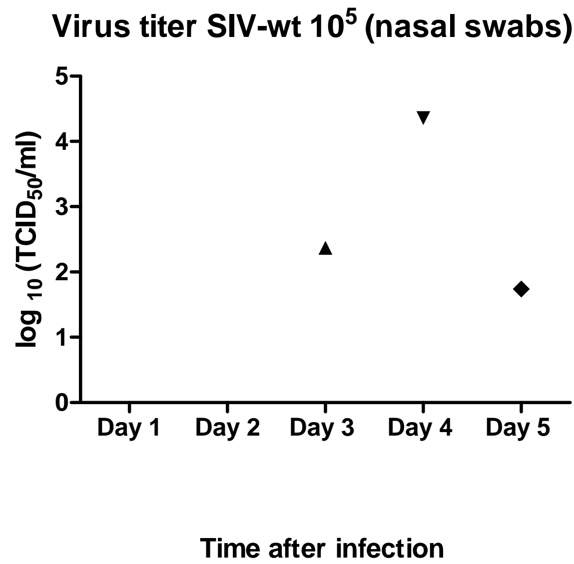


Figure 3.4. Virus titers in nasal secretion of pigs. Nasal swabs were collected daily from pigs infected with viruses or MEM. Virus titer was determined on MDCK cells. Virus shedding was only detected in pigs infected with high dose (A) and low dose (B) of SIV/SK-WT.

At necropsy, the percentage of each lung surface with macroscopic lesions was evaluated. The mock, SIV/SK-R345V and the SIV/SK-R345A (low or high dose) infected pigs did not show any typical macroscopic lung lesions. In contrast, in SIV/SK-WT infected pigs, gross lesions characterized as purple to plum-colored, consolidated areas were observed most dominant in the apical and cardiac lobes while diaphragmatic lobes were less affected. We also observed hyperemic and enlarged mediastinal lymph nodes in WT virus infected pigs. The percentage of macroscopic lung lesions in low dose and high dose SIV/SK-WT infected pigs were 16.2 % and 28.8 %, respectively. These were significantly higher compared to the control group ($P = 0.0043$, $p = 0.0043$ respectively) (Figure 3.5A). In agreement with these results, we could recover SIV/SK-WT from lung tissue of all animals infected with the WT virus. Mean values for the virus titer isolated from lungs infected with high dose and low dose were $10^{4.5}$ $TCID_{50}$/gr and $10^{3.5}$ $TCID_{50}$/gr, respectively. No virus could be detected in the lungs of animals infected with SIV/SK-R345V and SIV/SK-R345A viruses (Figure 3.5B).

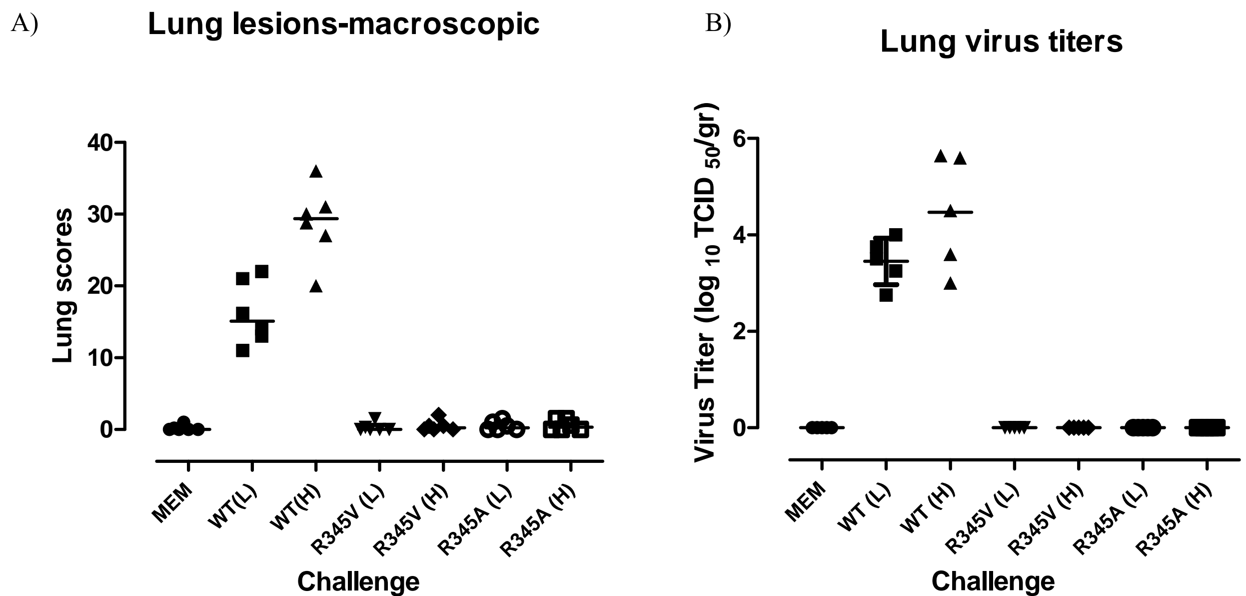


Figure 3.5. Percentage of lung lesions and virus titers. Pigs were infected with high dose or low dose of viruses. On day 5 post infection, pigs were euthanized and the percentage of lung lesions was evaluated. Median percentage of lung lesions were shown in (A). Lung tissues were then collected, homogenized and virus titers were determined on MDCK cells (B).

Histopathology results were consistent with previous studies of SIV infection in swine (B.E.Straw 1999; Richt, Lekcharoensuk et al. 2006). Microscopic lesions were observed most consistently in medium-sized airways. Thus, results obtained from the medium bronchioles were used for comparison of SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A viruses. The lungs of mock infected as well as mutant viruses infected pigs showed no, or minimal, microscopic lesions, whereas moderate to severe lesions were detected in animals infected with the SIV/SK-WT virus (Figure 3.6). Virus damage varied from severe necrotizing bronchiolitis and interstitial pneumonia to very mild bronchointerstitial pneumonia. Epithelial lining was most prominent in SIV/SK-WT infected pigs due to peribronchiolar lymphocyte and neutrophil infiltration in the airway. Interstitial thickening and lymphocyte infiltration in alveolar walls were minimal and inconsistently present in all groups (Table 3.2.). In consistence with the macroscopic lung lesions, SIV/SK-R345V and SIV/SK-R345A infected pigs showed significantly less lung damage than that in SIV/SK-WT infected pigs by microscopic histopathology.

Table 3.2. Histopathology lung scores

Inoculum (4ml)	Necrosis	Attenuation[§]	Hyperplasia	Infiltration of inflammatory cells in the lumen of bronchioles	Bronchiolar-associated lymphoid tissue proliferation (BALT)
MEM	0.00±0.00	0.00±0.00	0.00±0.00	0.4±0.55	0.8±0.45
R345V 10^5 PFU/ml	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.8±0.45
R345V 10^6 PFU/ml	0.00±0.00	0.00±0.00	0.00±0.00	0.2±0.45	1.00±0
R345A 10^5 PFU/ml	0.00±0.00	0.00±0.00	0.00±0.00	0.2±0.45	0.8±0.45
R345A 10^6 PFU/ml	0.2±0.45	0.00±0.00	0.00±0.00	0.00±0.00	0.4±0.55
WT 10^5 PFU/ml	1.6±0.55***	1.4±0.55 ***	1.2±0.45 ***	2.2±1.09 ***	1.0±0.00
WT 10^6 PFU/ml	1.4±0.55***	1.00±0.00 ***	1.6±0.55 ***	1.6±0.45 ***	0.8±0.45

Inoculum	Peribronchial lymphocyte and neutrophil infiltration	Perivascular lymphocyte infiltration	Atelectasis	Interstitial thickening and lymphocyte infiltration in alveolar wall	Overall Score
MEM	0.20±0.45	0.40±0.55	0.60±0.55	1.00±0.00	1.00±0.00
R345V 10^5 PFU/ml	0.20±0.45	0.60±0.55	0.20±0.45	0.80±0.45	0.80±0.45
R345V 10^6 PFU/ml	0.40±0.55	0.20±0.45	0.80±0.45	1.00±0.00	1.00±0.00
R345A 10^5 PFU/ml	0.60±0.89	0.20±0.45	0.60±0.89	1.20±0.45	1.20±0.45
R345A 10^6 PFU/ml	0.40±0.55	0.20±0.45	0.40±0.55	0.60±0.55	0.60±0.55
WT 10^5 PFU/ml	2.00±0.71 ***	1.00±0.00	2.00±0.71 ***	1.20±0.45	3.00±0.70 ***
WT 10^6 PFU/ml	1.80±0.84 ***	0.60±0.55	2.00±0.00 ***	1.20±0.45	3.00±0.00 ***

§: Attenuation of Bronchiolar Epithelium: flattening of the epithelial cells lining the bronchioles as part of the repair process following injury.

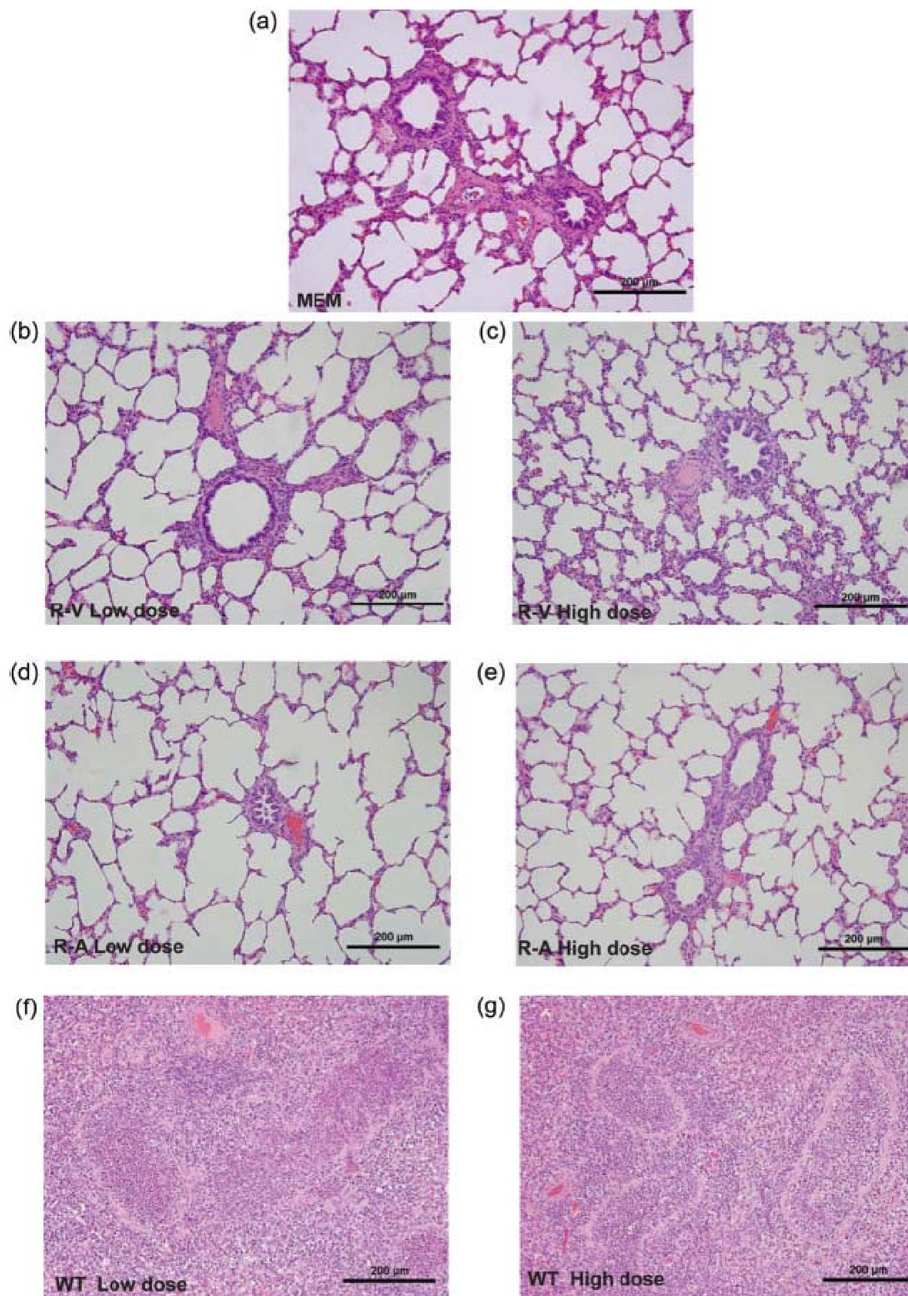


Figure 3.6 Histopatholog examination of lungs from infected pigs 5 days post infection. Medium-sized bronchioles from the lung of a control pig infected by MEM (A). Normal bronchioles and surrounding blood vessels from the lung of pigs inoculated with SIV/SK-R345V mutant virus at low dose (B) and high dose (C). Normal bronchioles from the lung of pigs inoculated with SIV/SK-R345A mutant virus at low dose (D) and high dose (E). The lung of pigs infected with SIV/SK-WT at low dose (F) and high dose (G) showed severe acute necrotizing bronchiolitis and interstitial pneumonia, severe bronchiolar necrosis with basophilic debris, and severe neutrophil infiltration in the lumen of bronchioles and bronchi. Neutrophils and macrophages in the alveolar spaces and a mild perivascular lymphoid infiltration. Magnification, x 20, bar 200 µm.

3.4 DISCUSSION

At present, all SIV vaccines are inactivated and their application does not provide the desired immune response and cross-protection (Brown, 1994; Macklin *et al.*, 1998). LAIV are not available for swine, although results of recent studies on NS1 gene deleted vaccines have been reported (Richt *et al.*, 2006; Solorzano *et al.*, 2005; Vincent *et al.*, 2007). One advantage of live vaccines is that they are delivered intranasally and only replicate to a limited extent, thus they may induce balanced cross-reactive cell-mediated immunity and humoral antibody responses, providing superior immunity to that induced by conventional inactivated vaccines (Gorse, Campbell et al. 1995). Data from studies conducted after the application of cold-adapted LAIV in humans and horses showed that live vaccines are capable of inducing stronger immune response and long-term protection (Cox, Brokstad et al. 2004; Paillot, Hannant et al. 2006). In contrast, one concern about these LAIV would be possible reassortment between field strains and the vaccine virus, generating new reassortant virus with unpredicted infectivity.

Stech *et al*. described an approach to generate a live attenuated virus. Accordingly, a mutant of strain A/WSN/33 with a modified cleavage site within its HA was generated which was dependent on proteolytic activation by elastase (Stech, Garn et al. 2005). This mutant was strictly dependent on elastase and grew as well as WT in tissue culture, but was entirely attenuated in mice at a virus dose of 10^6 PFU. At a dose of 10^5 PFU it induced complete protection against lethal infection. These promising results prompted us to investigate whether the strategy was applicable for SIV in its natural host.

Here we generated two elastase-dependent mutant SIVs. Initially, we constructed plasmid pHW-SIV/HA-R345V, which encodes HA with a modified cleavage site that is susceptible to human neutrophil elastase. Being concerned that upon infection with the virus, neutrophil infiltration could trigger and support virus replication *in vivo* by releasing the elastase, we designed plasmid pHW-SIV/HA-R345A, which encodes HA with a porcine pancreatic elastase cleavage site at the junction of HA1 and HA2. Both mutant viruses could be rescued only in the presence of human neutrophil elastase. Although SIV/SK-R345A could grow in the presence of pancreatic elastase, it underwent the adaptation when passaged five times with pancreatic elastase. Sequencing results showed that an optimal cleavage motif of Pro^{344}-Ala-Gly instead of Ser^{344}-Ala-Gly was generated under the selection pressure, suggesting that when modifying the

HA cleavage site, the adjacent amino acid sequence of HA cleavage site should also be considered in order to achieve the most favorable protease recognition and cleavage.

The two mutant viruses were further characterized *in vitro*. Both mutant viruses were solely dependent on neutrophil elastase activation and grew to analogous titers in the presence of the appropriate protease as to the WT virus (Figure 3.1A-C), suggesting that their growth property was preserved in tissue culture. Furthermore, tissue culture grown mutant viruses were able to infect cells and synthesize similar amount of viral proteins, as did the WT virus (Figure 3.2). Most importantly, the mutant viruses are genetically stable (Figure 3.1D). These features enable the two mutant viruses to be great candidates to serve as live vaccines.

The next step towards the aim of development of a live vaccine would be to examine whether the mutant SIVs were attenuated and did not cause significant illness in pigs. Biologically it might be possible that one or both mutants could cause an unusual infection and disease, if the host can provide appropriate enzymes with substrate specificities. Data from our clinical observation showed that only pigs infected with the SIV/SK-WT showed signs of respiratory distress and elevated temperatures typical for SIV infections. SIV/SK-R345A at high dose (4×10^6 PFU) caused slightly high body temperature (Figure 3.3). Our records for each individually infected animal in this group showed that two out of five animals responded with increased temperature for 1°C on day one post infection. The remaining three animals did not show significant body temperature changes (increased 0.2°C-0.5°C). The one time body temperature increase could be due to the stress factors or individual immune variation.

Results obtained at necropsy and histopathology revealed that SIV/SK-R345V and SIV/SK-R345A viruses were not able to induce macroscopic or microscopic lesions in lung tissue. In addition, failure to isolate SIV/SK-R345V and SIV/SK-R345A viruses but not the WT SIV/SK, from lungs and nasal swabs contribute to the conclusion that these viruses were attenuated in pigs. Lack of elastase in the lungs and inability to cleave HA most likely enables only limited replication cycles for SIV/SK-R345V and SIV/SK-R345A viruses thus preventing SIV disease. In our study, we tested two doses of mutant viruses in pigs. Our data showed that even at a high dose (4×10^6 PFU), both viruses did not cause any disease in pigs, relieving the concerns that neutrophil infiltration to the site of infection may provide elastase to support mutant virus propagation.

Taken together, our results confirmed and extended the approach proposed by Stech *et al*. (Stech, Garn et al. 2005) by applying the idea to SIV and testing in its natural host. The mutant viruses maintained the growth property in the presence of appropriate protease in tissue culture, but were highly attenuated in pigs. Currently we are testing the protective immune response of SIV/SK-R345V and SIV/SK-R345A in pigs.

4.0 ELASTASE-DEPENDANT LIVE ATTENUATED SWINE INFLUENZA A VIRUSES ARE IMMUNOGENIC AND CONFER PROTECTIONS TO SWINE INFLUENZA A INFECTION IN PIGS

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4.1 INTRODUCTION

Swine influenza virus (SIV) is the causative pathogen of swine influenza, a highly contagious, acute respiratory viral disease of swine. Mortality of SIV infected pigs is usually low while morbidity may approach 100%. Swine influenza is characterized by sudden onset, coughing, respiratory distress, weight loss, fever, nasal discharge and rapid recovery (B.E.Straw 1999). SIV is a member of the *Orthomyxoviridae* family, classified into the genus of influenza virus A, containing eight segments of a single-stranded RNA genome of negative polarity (Palese P 2007). Epithelial cells in the swine respiratory tract have receptors for both avian and mammalian influenza viruses (Ito, Couceiro et al. 1998), thus pigs could potentially serve as "mixing vessels" for the generation of new reassortant strains with pandemic capacity. There are a number of reports where direct transmission of influenza viruses from pigs to humans occurred (Hinshaw, Bean et al. 1978; Dacso, Couch et al. 1984; Wentworth, McGregor et al. 1997) and several cases had a fatal outcome on infected individuals (Smith, Burgert et al. 1976; Top and Russell 1977; Wentworth, Thompson et al. 1994; Kimura, Adlakha et al. 1998). Consequently, effective control of SIV would be beneficial to both humans and animals.

Classical H1N1 SIVs were main isolates from pigs in U.S and Canada until 1998 (Chambers, Hinshaw et al. 1991; Olsen 2002). A dramatic change in the epidemiologic pattern of SIV began in 1997-1998. Serological studies conducted by Olsen and colleagues in 1997-1998 detected significant increase in H3 seropositivity and H3N2 SIV have been isolated from pigs in both U. S and Canada (Zhou, Senne et al. 1999; Karasin, Schutten et al. 2000). Furthermore, reassortment between H3N2 viruses and classical H1N1 SIV resulted in the appearance of H1N2 reassortant viruses (Karasin, Olsen et al. 2000; Karasin, Landgraf et al. 2002). In addition to H4N6 viruses of duck origin isolated from pigs in Canada (Karasin, Olsen et al. 2000), there are reports on wholly avian viruses H3N3 and H1N1 subtypes isolated also from Canadian pigs (Karasin, West et al. 2004). In general, there are three major SIV subtypes H1N1, H1N2 and H3N2 with multiple genetic and antigenic variants within each subtype circulating in North

America swine populations (Olsen 2002; Karasin, West et al. 2004). Increased incidence of avian-like or human-like SIV reassortants raises concerns for public health and requires research towards development of cross-protective SIV vaccines.

Currently available swine influenza vaccines are based on inactivated whole virus containing both H1N1 and H3N2 subtypes. Application of these vaccines reduces the severity of disease but does not provide consistent protection from infection (Brown 1994; Macklin, McCabe et al. 1998). In contrast to killed vaccines administered intramuscularly, the intranasally administered, live attenuated influenza vaccines (LAIV) induce the immune response at the site of natural infection. Therefore, a LAIV has the potential to induce broad humoral and cellular immune responses which could provide protection against antigenically different influenza viruses. LAIV against influenza viruses based on attenuation of the virus by cold-adaptation are available for human (Belshe 2004) and equine species (Townsend, Penner et al. 2001). However to date no SIV LAIV are commercially available for use in swine in North America. Recent studies by Richt *et al.* showed that mutant SIV with truncated NS1 protein was highly attenuated in pigs (Solorzano, Webby et al. 2005). In addition, this SIV/NS1 LAIV was capable of stimulating a protective immune response against homologous and partial protection against heterologous subtypic wild type (WT) SIVs (Richt, Lekcharoensuk et al. 2006; Vincent, Ma et al. 2007). Stech *et al*. demonstrated that the conversion of conserved cleavage site in influenza HA from trypsin-sensitive to elastase-sensitive results in *in vivo* attenuation of influenza virus in mouse models (Stech, Garn et al. 2005; Gabriel, Garn et al. 2008). Furthermore, these elastase-dependent LAIV were able to induce protective systemic and mucosal immune response. Recently we showed that two elastase-dependent SIV viruses R345V and R345A derived from A/Sw/Saskatchewan/18789/02 (SIV/Sk02) are attenuated in their natural host pigs (Masic, Babiuk et al. 2009). In the current study we addressed the immunogenic and cross-protective abilities of these mutants.

4.2 MATERIALS AND METHODS

4.2.1 Cells and viruses.

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). H1N1 mutant viruses SIV/Sk-R345V (R345V) and SIV/Sk-R345A (R345A) used in our study were generated as previously described

(Masic, Babiuk et al. 2009). These two viruses were grown in MDCK cells in the presence of 0.5 μg/ml human neutrophil elastase (Serva Electrophoresis GmbH). Stock virus, after purification, reached titers of $1.7x10^8$ and $2x10^8$ PFU/ml respectively. The other SIV isolates used in the study were H1N1 A/Sw/Saskatchewan/18789/02 (SIV/Sk02); H1N1 A/Sw/Indiana/1726/88 (SIV/Ind88) and H3N2 A/Sw/Texas/4199-2/98 (SIV/Tx98). These viruses were propagated at 37ºC in the allantoic cavities of 11-day-old embryonated chicken eggs. Stock virus titers were $1.4x10^7$, $1.33x10^6$, $1.7x10^7$ PFU/ml respectively. Titers for all viruses used in the study were determined on MDCK cells by plaque assay as described previously (Shin, Liu et al. 2007).

4.2.2 Experimental design and clinical sampling.

For the purpose of this study we designed two animal trials. In the first trial (Table 4.1), thirty-five, five-week old SIV negative landrace crossbred pigs were obtained from Prairie Swine Center (Floral, Saskatchewan, Canada). Pigs were randomly selected and divided into five groups with seven pigs per group. At six weeks of age (day 0), pigs in group 1 were mock vaccinated with 4 ml of MEM, while pigs in group 2 and 4 received $4x10^6$ PFU of R345V, pigs in group 3 and 5 received $4x10^6$ PFU of R345A. The viruses or MEM were administered intratracheally (IT), assuring consistent infection. Three weeks later (day 21), pigs in group 4 and 5 received a second dose of vaccine containing the same amount of virus, while pigs in group 2 and 3 and three pigs from the control group were euthanized by intravenous administration of Euthanyl (Sodium pentabarbitol 25 mg/ml) and subjected to necropsy. Ten days after the second vaccination (day 31), pigs in group 4 and 5, and the remaining animals in group 1 were euthanized and subjected to necropsy. At necropsy, lungs were evaluated and scored for the presence of SIV characteristic lesions, tracheo-bronchial lymph nodes were extracted and broncho-alveolar fluid (BALF) was collected by lavaging the lungs with 20 ml of phosphate-buffered saline (PBS, 0.137 M NaCl, 2.7 mM KCl, 8 mM $NaHPO_4$, 1.47 mM KH_2 PO_4, pH 7.3). Prior to testing for the presence of virus, BALF samples were incubated at 37°C for 1 h with an equal amount of 10 mM dithiothreitol (DTT) to disrupt mucus. Serum samples and nasal swabs were collected before and after each vaccination and at necropsy.

In the second trial (Table 4.2) forty-nine four week old SIV negative pigs were assigned into seven groups with seven animals per group. Animals in groups 1 to 4 were mock vaccinated IT with 4 ml of MEM while pigs in groups 5 to 7 were vaccinated IT with $4x10^6$ PFU of R345V.

Three weeks after the first vaccination animals in groups 1 to 4 received MEM whereas vaccinated groups (5 to 7) received a second dose of $4x10^6$ PFU of R345V. Ten days after the second vaccination (day 31), pigs in all groups were challenged IT with $8x10^5$ PFU of homologous or heterologous subtypic SIVs (Table 4.2). After the challenge, pigs were monitored for the presence of clinical signs characteristic for SIV infection and then sacrificed on day 5 post challenge. Tissue samples from the right apical, cardiac and diaphragmatic lung lobes were taken for virus isolation and histopathology examination. Serum samples were collected prior to, after the second vaccination and after viral challenge. BALF samples were obtained at necropsy. All animal experiments were conducted at the Vaccine and Infectious Disease Organization, University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

4.2.3 Isolation of Lymphocytes from tracheo-bronchial lymph nodes.

Tracheo-bronchial lymph nodes were dissected *in toto* at necropsy and stored on ice in AIM-V medium containing L-glutamine, 50 mg/ml streptomycin sulfate and 10 mg/ml gentamicin sulfate (Invitrogen, Burlington ON, Canada) supplemented with 10% FBS. Lymph node cells (LNC) were isolated by finely mincing tissues with a scalpel, filtering the cell suspension through 40-μm nylon cell strainers (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washing with AIM-V medium supplemented with 2% of FBS. Prior to seeding, cells were resuspended in AIM-V 2% FBS media containing 50 μM of β-mercaptanol and were counted to quantitate the cell numbers.

4.2.4 Detection of SIV-specific IFN-γ secreting cells by ELISPOT.

Nitrocellulose microtiter plates - UNIFILTER 350 (Whatmann, Florham Park, NJ) were coated with mouse anti-porcine IFN-γ monoclonal antibodies (Endogen, Rockford, IL, USA) in coating buffer at a concentration of 5 μg/ml for 16 h at 4°C. Wells were washed and LNC were seeded directly at $1x10^6$ cells/well in a final volume of 200 μl/well AIM-V containing 2% FBS. LNC were stimulated for 10 h at 37°C with 25 μg/ml of purified and UV inactivated SIV/Sk02 virus, 5 μg/ml of Con A (Sigma-Aldrich), or media only. After stimulation, plates were washed five times with PBST (PBS with 0.05% Tween 20) and incubated with rabbit anti-porcine IFN-γ (Endogen, Rockford, IL, USA) at a concentration of 2 μg/ml for 16 h at 4°C. Plates were then

washed and incubated with biotinylated goat anti-rabbit IgG (H+L) (DiAMED, South San Francisco, CA, USA) at a dilution of 1:5000 for 2 h at room temperature. Wells were washed five times and incubated with streptavidin alkaline solution (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:5000 for 1.5 h at room temperature. After washing eight times with double distilled water (ddH$_2$O), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Sigma-Aldrich) insoluble alkaline substrate solution was added (100 µl/well) and incubated for 5 min. Plates were washed again with ddH$_2$O and left to dry overnight at room temperature. Spots were counted manually under an inverted light microscope and recorded. Number of spots observed in media only stimulated wells were counted and subtracted as a background. Data was reported as number of IFN-γ secreting cells per 10^6 somatic cells.

4.2.5 Lymphocyte proliferative responses (LPR) assay.

LNC were resuspended in AIM-V culture medium at a concentration of 2.5×10^5 cells/well and stimulated for 72 h at 37°C with 25 µg/ml of SIV/Sk02 antigen, 5 µg/ml of Con A or media. Six hrs prior to the 72-h incubation, LNC were pulsed with 0.4 µCi [5'-^{3}H]Thymidine (Amersham Pharmacia, Piscataway, NJ, USA) as previously described (Mena, Nichani et al. 2003). Cells were then harvested using standard liquid scintillation protocols and uptake of ^{3}H-thymidine was assessed in a beta counter (Topcount, Packard Instrument Company, Meriden, CT). The LPR was calculated as the mean counts per minute (c.p.m) of triplicate cultures and expressed as a stimulation index (c.p.m in the presence of stimulus/c.p.m in the absence of stimulus).

4.2.6 ELISA for antigen-specific IgG, IgA antibodies and hemagglutination inhibition (HI) assay.

For antigen-specific ELISAs, 2.5 µg/ml of purified and UV inactivated SIV/Sk02, SIV/Ind88 and SIV/Tx98 antigen were coated onto 96-well Immulon-2 (Dynex Technology INC, Chantilly, USA) plates and incubated overnight at 4°C. Plates were blocked for 1 h at room temperature with 100 µl of 1% skim milk in TBST (0.1 M Tris, 0.17 M NaCl, 0.05% Tween 20) and washed four times with PBST. Serum, nasal and BALF samples were added (100 µl/well) in triplicate at appropriate dilutions and incubated for 1.5 h at room temperature. Samples of previously defined positive control sera and appropriate negative controls were run on each plate.

Subsequently, the plates were incubated with mouse anti-porcine IgA monoclonal antibody (AbD Serotec) or alkaline phosphatase labeled goat anti-porcine IgG (KPL, Gaithersburg, MD, USA). The IgA ELISAs were developed by the addition of biotinylated goat anti-mouse IgG (H+L) (DiAMED, South San Fransisco, CA, USA) antibodies and streptavidin alkaline phosphatase solution (Jackson ImmunoResearch, West Grove, PA, USA). After washing with PBS-T, IgG and IgA ELISAs were developed by the addition of PNPP substrate [10mg/ml p-nitro-phenyl phosphate di(tris) salt crystalline (Sigma-Aldrich), 1% dieathanolamine (Sigma-Aldrich), 0.5 mg/ml $MgCl_2$, pH 9.8]. The optical density of the reaction product was measured at 405 nm (a 490 nm reference filter used to detect background which was subtracted from the measurement reading) on a microplate reader (Molecular Devices, SpectraMax Plus 384). The titer of sample was defined as the highest dilution at which the OD of that sample was higher than the defined cut off (the mean OD of a known negative sample plus 2 times standard deviation).

To measure the HI titers, serum samples were treated overnight with receptor-destroying enzyme (Cholera filtrate-C8772, Sigma Aldrich) at 37°C to eliminate non-specific HI factors. Viruses used in HI assays were SIV/Sk02, SIV/Ind88 and SIV/Tx98. HI assay was performed as described elsewhere (OIE 2004) .

4.2.7 Histopathology evaluation.

Necropsy, macroscopic examination of lungs and tissue processing for virus isolation was performed as described previously (Masic, Babiuk et al. 2009). Tissue sections of lungs were routinely stained with hematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or extent of lesions within the sections examined as follows: 0: no visible changes; 1: mild focal or multifocal change; 2: moderate multifocal change; 3: moderate diffuse change; 4: severe diffuse change. Two independent pathologists scored all slides and they were blinded for the experimental groups.

4.2.8 ELISA for IFN-α, IL-1 and IL-6 cytokines.

For detecting IFN-α, IL-1 and IL-6 cytokines, polystyrene microtiter plates (Immulon 2; Dynex Technology INC, Chantilly, USA) were coated with the following capture antibodies:

mouse anti-recombinant porcine IFN-α clone K9 (R&D # 27100-1), goat anti-recombinant porcine interleukin 6 (R&D AF 686), or mouse anti-recombinant porcine IL-1β (R&D MAB 6811), respectively at a concentration of 1 μg/ml in coating buffer. Recombinant porcine IFN-α (Endogen rPo IFN-α; 2000 pg/ml), recombinant porcine IL-6 (R&D 686-PI-025 rPoIL-6; 5000 pg/ml) and recombinant porcine IL-1β (R&D 681-PI-010 rPo IL-1β; 10,000 pg/ml) were used as standards. Standards and homogenized lung samples were diluted in TBST-0.1% skim milk and added to the coated plates. After overnight incubation at 4°C, detection antibodies mouse anti-recombinant porcine IFN-α clone F17 biotin (R&D #27105-1; 1/1000), goat anti-recombinant porcine IL-6 biotin (R&D BAF686; 0.2 μg/ml), or goat anti-recombinant porcine IL-1β biotin (R&D BAF681; 0.25 μg/ml) were added, respectively. Finally, the plates were developed and measured as described above. Sample concentrations were calculated using Softmax Pro 5.2 version software (Molecular Devices).

4.2.9 Statistical analysis.

Statistical analysis were performed using GraphPad Prism5 (San Diego, CA, USA) and Statistix7 (Tallhassee, FL, USA) software. Differences between means in two groups (vaccinated vs. unvaccinated) in each assay were determined using Mann-Whitney nonparametric t-test. To compare two vaccines, data from LPR, ELISPOT assays and serum antibodies were transformed and One-way ANOVA for RANK data was applied. If the median values of at least one group differed from others with a $P < 0.05$, they were considered statistically significant.

4.3 RESULTS

4.3.1 Live attenuated viruses induced cell mediated immune response.

To examine whether the mutant viruses were immunogenic, SIV negative pigs were divided into 5 groups and were intratracheally vaccinated with MEM, R345V, or R345A, respectively (Table 4.1). Two groups of pigs received one immunization and euthanized on day 21; whereas two groups of pigs received a second immunization on day 21 and were euthanized on day 31. Animals in the control group received MEM and were euthanized on day 21 and 31 respectively. At necropsy, tracheo-broncihal lymph nodes and BALF were collected.

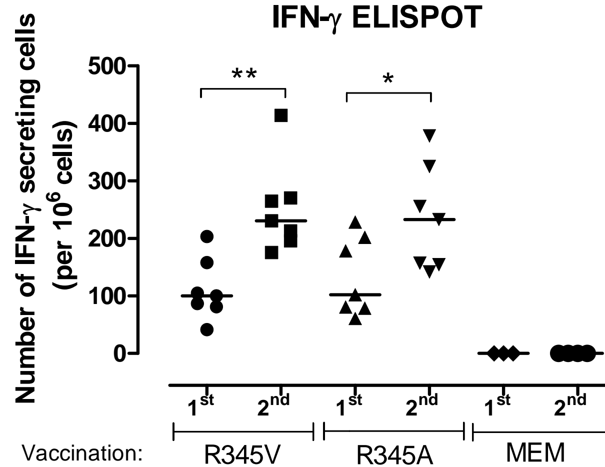
To assess the ability of R345V or R345A viruses to induce cell mediated immune response upon vaccination, LNC were isolated from vaccinated and control pigs and antigen-specific

responses were measured by IFN-γ ELISPOT and LPR assay. As shown in Figure 4.1, both R345V and R345A were able to induce significantly high numbers of antigen specific IFN-γ secreting cells after only one vaccination. A second vaccination with the same dose of previously administered vaccines resulted in further increase of local IFN-γ secreting cells and this was significantly higher than in groups received a single vaccination ($p=0.002$ for R345V and $p=0.05$ for R345A, respectively) (Figure 4.1A). To further measure cell-mediated responses, we conducted a LPR assay where we assayed the antigen-specific proliferation in LNC. Consistence with the IFN-γ ELISPOT results, lymph node cells proliferated in response to specific antigen after the first vaccination with the median stimulation index of 9.57 (R345V) and 38.04 (R345A) respectively. Moreover, a second vaccination resulted in a significant increase in the stimulation index ($P=0.02$ and $P=0.05$ respectively) (Figure 4.1B). Statistical analysis showed that there was a significant difference in cell mediated immune responses between the first and the second vaccination in both groups vaccinated with R345V and R345A. However, there was no statistically significant difference in the cell-mediated immune responses between the two vaccines using these two assays.

Table 4.1. Assignment of pigs in each group – Immunogenicity study

Group N=7	Day 0	Day 21	Day 31
1.	MEM 4ml	Necropsy (N=3)	Necropsy N=4
2.	R345V 4 x 10^6 PFU	Necropsy	-
3.	R345A 4 x 10^6 PFU	Necropsy	-
4.	R345V 4 x 10^6 PFU	R345V 4 x 10^6 PFU	Necropsy
5.	R345A 4 x 10^6 PFU	R345A 4 x 10^6 PFU	Necropsy

A)



B)

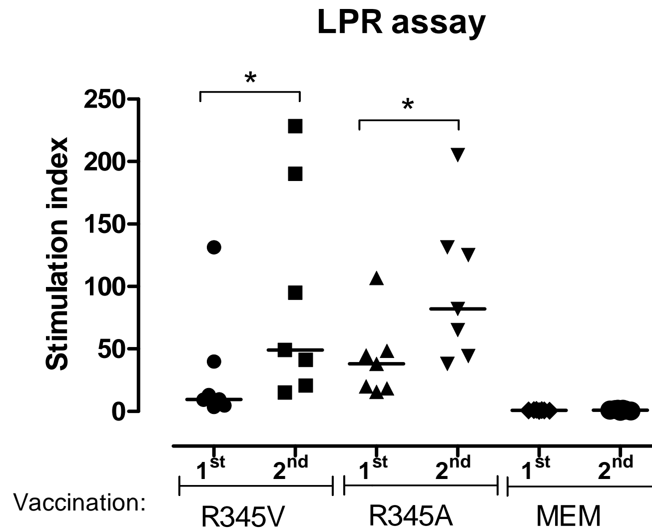


Figure 4.1. Cell mediated immune response induced by mutant viruses. (A) Numbers of SIV-specific IFN-γ secreting cells in pigs after the first and the second vaccination with R345V and R345A, as measured by ELISPOT. The results are reported as the average number of spots observed in wells with SIV antigen stimulated LNC (seeded in triplicates) minus the average number of spots in negative control wells (LNC simulated with media alone). **(B)** SIV-specific lymphocyte proliferation response of LNC in pigs after the first and the second vaccination with R345V and R345A. The LPR were calculated as the mean counts per minute (c.p.m) of triplicate cultures and expressed as a stimulation index (c.p.m in the presence of stimulus/c.p.m in the absence of stimulus). Each data point represents an individual animal, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; **P<0.001.

4.3.2 Live attenuated viruses induced humoral immune responses.

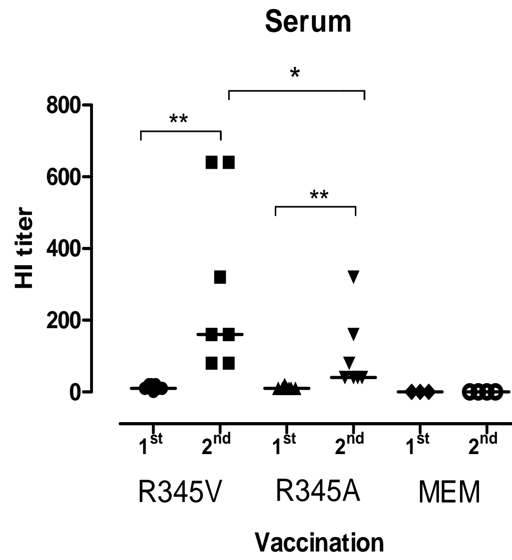
Sera were collected prior to the first vaccination, on 21 days after the first vaccination and 10 days after the second vaccination. SIV specific antibodies were first examined by HI assay. All pigs were negative for H1N1 SIV antibodies (HI <1:10) at the start of the experiment, and the seven unvaccinated pigs remained seronegative at all times examined. After the first vaccination with R345V or R345A, all pigs had low levels of antibodies against the parental H1N1 strain (HI = 20). The second vaccination resulted in the significant increase of HI titer (HI = 160 for R345V and HI = 80 for R345A). Pigs vaccinated with R345V had statistically significant higher HI titers compared to the R345A vaccinated group (P = 0.04) (Figure 4.2A).

Antigen-specific serum IgG and IgA were then measured on day 21 and 31 by ELISA using inactivated H1N1 SIV/Sk02 as the capture antigen. As seen in Figure 4.2B, both R345V and R345A viruses induced a moderate level of IgG after the first vaccination (titer = 321 for R345V and 164 for R345A), moreover, the second vaccination led to a considerable increase in the level of IgG (titer = 1611 for R345V and 955 for R345A). Similarly, the first vaccination could induce a detectable level of IgA (titer = 181.8 for R345V and 84.5 for R345A), with the second vaccination leading to an increase in the production of IgA (titer = 746.3 for R345V and 628.7 for R345A) (Figure 4.2C). At each time point, serum IgG titers were approximately two to three times higher than IgA titer (Figure 4.2B and 4.2C).

To assess the presence of IgG and IgA antibodies specific to H1N1 SIV/Sk02 at mucosal surfaces in the upper and lower respiratory tract, nasal swabs and BALF samples from pigs in all groups were tested by ELISA. As shown in Figure 4.2D and 4.2E, the antigen-specific IgA and IgG titers in nasal swabs remained low (<10) after the first vaccination. After the second vaccination, IgA titer increased significantly to a median titer of 80 (R345V) and 60 (R345A) while IgG titers rose to just above 10.

Similarly, IgA was the dominant antibody subtype in the lower respiratory tract. In BALF, the IgA titres were significantly increased after the second immunization compared to that after the first vaccination (median IgA titre = 29.0 vs 2450, p = 0.0006 for R345V; and 52.2 vs 2279, p = 0.001 for R345A) (Figure 4.2F). The IgG level in BALF was also increased after the second immunization (median IgG titre = 2.3 vs 108, p = 0.02 for R345V; and 3.0 vs 112, p = 0.02 for R345A) (Figure 4.2G). However, the magnitude of the increase was less than that of IgA.

A)



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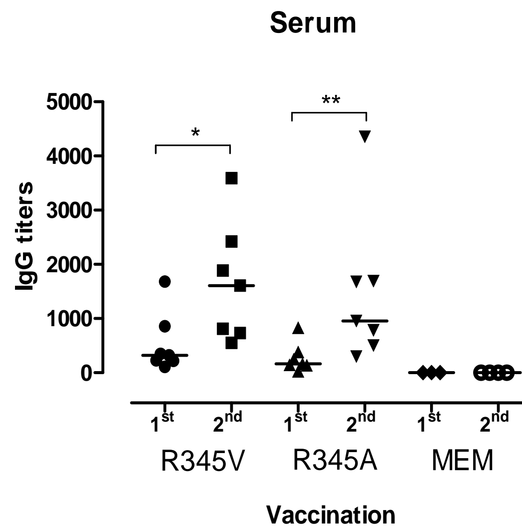
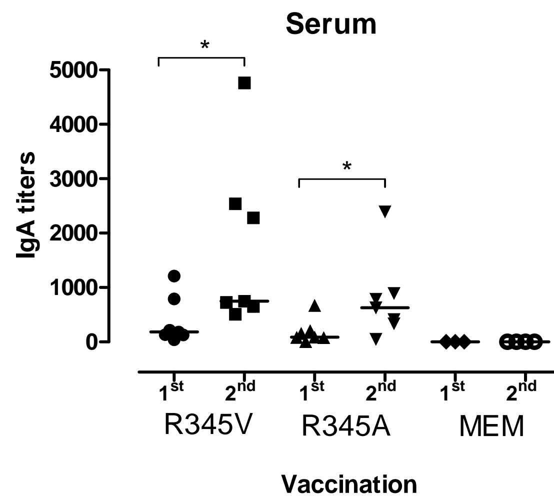


C)



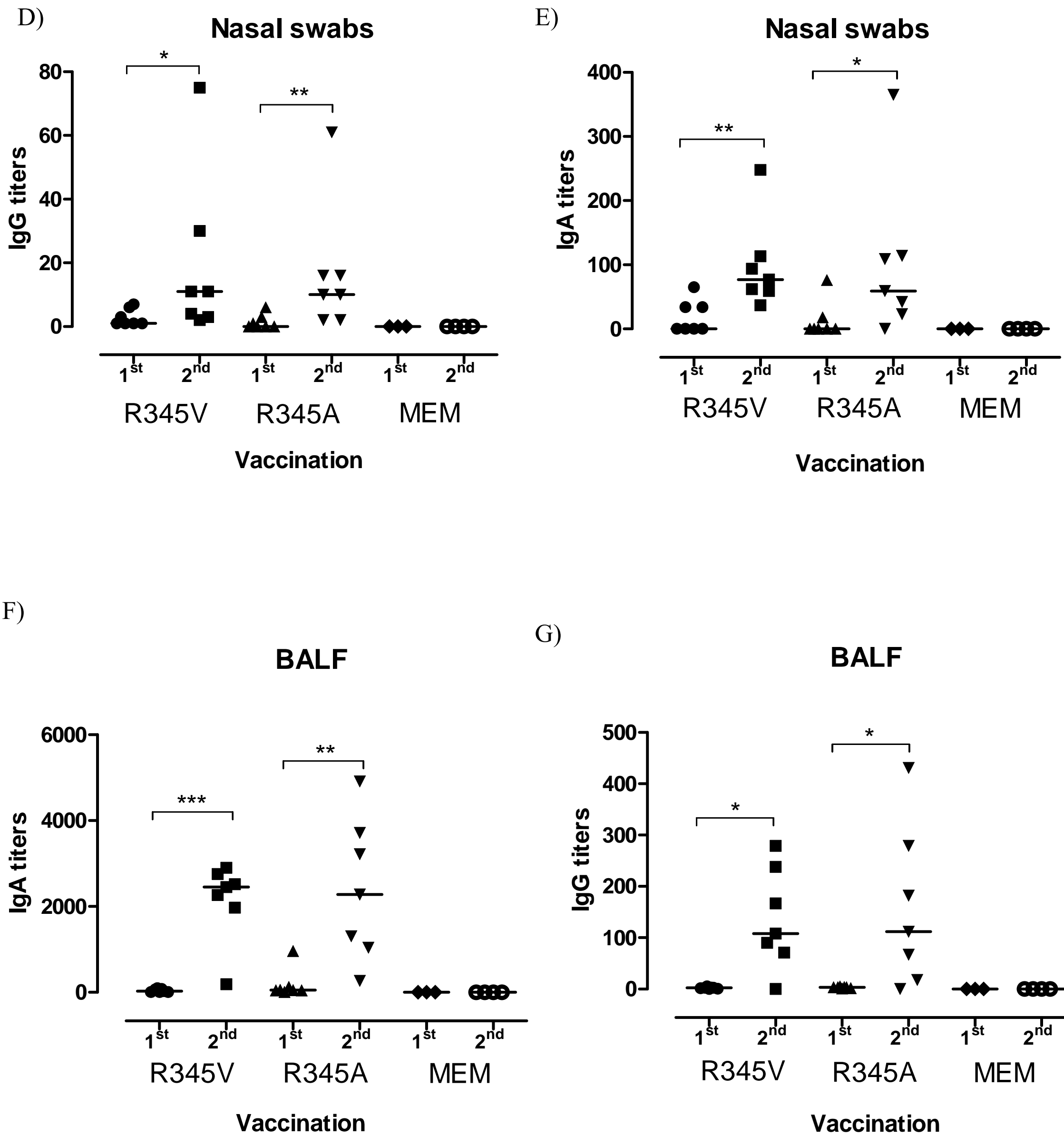


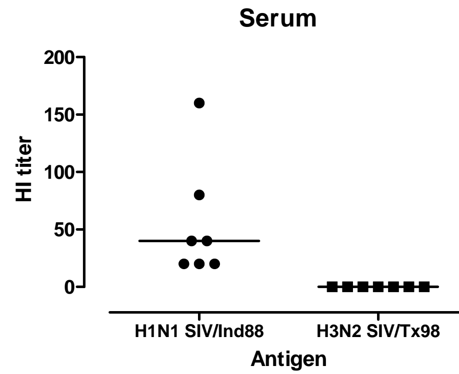
Figure 4.2. SIV specific antibodies induced by the mutant viruses. SIV/Sk02 specific HI (A), IgG (B) and IgA (C) levels induced by R345V and R345A were detected in serum after the first and the second vaccination. Mucosal IgG (D and G) and IgA (E and F) antibody titers from nasal swabs (D and E) and from BALF (F and G) were also determined. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

4.3.3 Live attenuated virus induced cross-reactive antibodies.

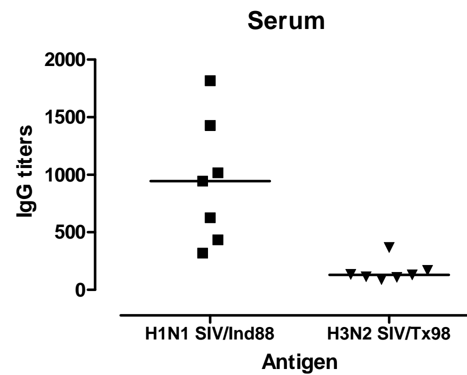
All pigs were negative prior to the start of the experiment for H1N1 and H3N2 antibodies by HI assay (HI titer <10). Pigs vaccinated with R345V seroconverted by HI assay to SIV/Ind88 H1N1 antigens during the time of study (Figure 4.3A). The median HI titer to the SIV/Ind88 was 1:40. However there were no detectable HI antibodies against SIV/Tx98 H3N2 after two vaccinations with R345V.

The presence of cross-reactive antibodies in serum and BALF samples was measured by using purified and UV-inactivated SIV/Ind88 and SIV/Tx98 as capture antigens in ELISA assay. Similarly to HI titers, high level of serum IgG recognizing H1N1 SIV/Ind88 was detected. Although H3N2 cross-reactive serum IgG titer was detectable, the level was ten times lower than IgG recognizing H1N1 antigen (Figure 4.3B). In the lower respiratory mucosa (BALF), R345V vaccinated pigs had a significant higher level of IgA antibody and moderate level of IgG antibody cross-reacting with homologous antigenic variant H1N1 SIV/Idn88 and heterologous subtypic H3N2 SIV/Tx98 (Figure 4.3C).

A)



B)



C)

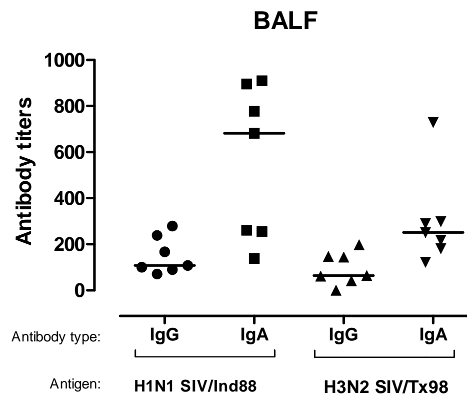


Figure 4.3. Cross reactive antibody induced by R345V virus. Serum HI (A), IgG (B) and mucosal IgA and IgG (C) that cross reacting to SIV/Ind88 and SIV/Tx98 were determined after the second immunization. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

4.3.4 Vaccination reduced macroscopic lung lesions after SIV challenge.

Our foregoing results indicated that (i) both viruses were immunogenic, and (ii) two vaccinations were required to induce significant high levels of immune responses. Considering that R345V exhibited slightly more consistent and induced stronger immune response than did R345A, in the next protection trial we used the following vaccine strategy. Pigs were immunized with R345V twice with a 3 week-interval (Table 4.2). Ten days after the second immunization, pigs were challenged intratracheally with homologous or heterologous subtypic SIVs and observed for 5 days. Pigs were then euthanized and necropsies were performed. During the five-day observation period, fever and mild respiratory signs such as abdominal breathing, sneezing and nasal discharge were observed only in the unvaccinated animals challenged with the H1N1 SIV subtypes (Table 4.2, groups 2 and 3) while unchallenged pigs, pigs were vaccinated and challenged with H1N1 SIVs did not show any clinical signs (groups 1, 5, 6). No apparent clinical signs with respect to respiratory distress and nasal discharge were observed in H3N2 challenged pigs (groups 4 and 7). Typical SIV gross lesions characterized as sharply-demarcated, purple to plum-colored, consolidated areas were observed in all pigs of unvaccinated but challenged groups (groups 2, 3 and 4). Lesions were most dominant in the apical and cardiac lung lobes while diaphragmatic and intermediate lobes were less affected. Lungs of pigs vaccinated with R345V and challenged with H1N1 viruses (group 5 and 6) had no lung gross lesions and appeared similar to normal lungs. The average lung lesion scores of group 5 and 6 were significantly less than that of group 2 and 3 with $p=0.0017$ (challenged with Sk02) and $p=0.002$ (challenged with Ind88), respectively (Figure 4.4). Although lung lesions were seen in pigs vaccinated and challenged with heterologous subtypic H3N2 virus (group 7), the severity was significantly less than its corresponding unvaccinated but challenged group (group 4), the p value of the lung lesion score between these two groups were less than 0.05 (Figure 4.4).

Table 4.2. Assignment of pigs in each group – Immuno-protection study

Group N=7	1st vaccination (day=0)	2nd vaccination (day=21)	Challenge (day=31)
1.	MEM	MEM	MEM
2.	MEM	MEM	SIV/Sk02 H1N1
3.	MEM	MEM	SIV/Ind88 H1N1
4.	MEM	MEM	SIV/Tx98 H3N2
5.	R345V	R345V	SIV/Sk02 H1N1
6.	R345V	R345V	SIV/Ind88 H1N1
7.	R345V	R345V	SIV/Tx98 H3N2

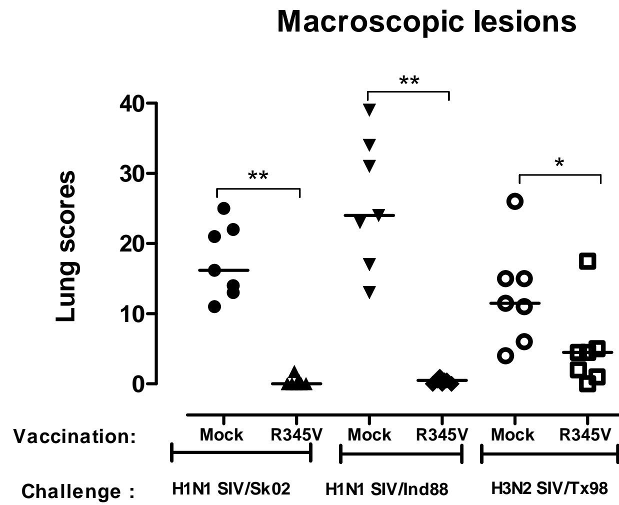
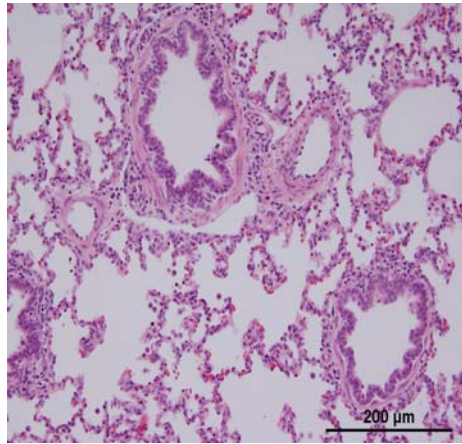


Figure 4.4. Macroscopic lung lesions. The percentage of the areas affected with pneumonia was estimated visually for each lung lobe. Total percentage for the entire lung was calculated based on weight proportions of each lung lobe to the total lung volume. Results are the median score of lung lesions of seven animals in each group. *P<0.05; **P<0.01; ***P<0.001.

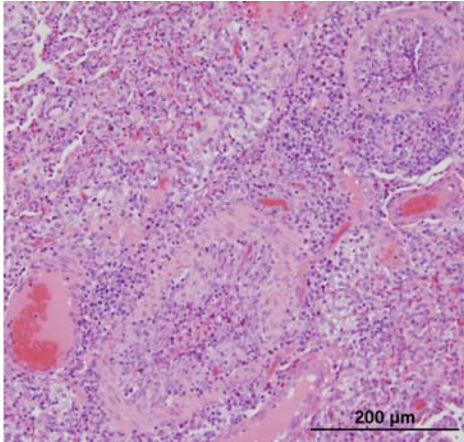
4.3.5 Vaccination reduced microscopic lung lesion after SIV challenge.

SIV induced histopathological lung lesions are characterized by detachment of large areas of bronchial/bronchiolar epithelium and accumulation of necrotic epithelial cell debris and neutrophils in the airways (van Reeth and Nauwynck 2000). We examined the microscopic lung pathology. As shown in Figure 4.5, consistent with the macroscopic lung lesion observed above, unvaccinated pigs challenged with H1N1 SIV/Sk02 (group 2) or H1N1 SIV/Ind88 (group 3) had the most severe histopathological changes characterized by severe necrotizing bronchiolitis with moderate multifocal necrosis, attenuation of surviving bronchiolar epithelium and hyperplasia of bronchial/bronchiolar mucosa. Severe peribronchiolar and perivascular lymphoid infiltration was accompanied with a severe neutrophil infiltration in the lumen of most bronchioles. Furthermore, severe locally-extensive interstitial pneumonia with atelectasis, focal alveolar necrosis and neutrophil infiltration as well as infiltration of lymphocytes and macrophages in the alveolar walls and air spaces were also observed (panel B and D). In contrast, as seen in MEM injected pigs, very mild histopathological changes such as mild perivascular lymphoid infiltration and occasional mild bronchiolar-associated lymphoid tissue (BALT) proliferation was observed in vaccinated and H1N1 challenged (groups 5 and 6) pigs (panel A, C and E). Focal necrosis of small bronchiole and mild, locally-extensive interstitial pneumonia with atelectasis and mixed alveolar inflammatory cell infiltration was found in one animal vaccinated and H1N1 SIV/Ind88 challenged (group 6). Severe peribronchiolar and perivascular lymphoid infiltration and moderate alveolar atelectasis due to infiltration of mixed inflammatory cells in alveolar walls and spaces proliferation were observed in all seven unvaccinated and H3N2 SIV/Tx98 challenged pigs (group 4, panel F). In addition, histopathological changes such as moderate bronchiolitis with focal necrosis, mild attenuation and moderate hyperplasia of bronchiolar epithelium were also found in animals in this group. In contrast, four out of seven vaccinated pigs challenged with H3N2 SIV/Tx98 (group 7) developed mild to moderate bronchiolitis with rare epithelial necrosis, moderate bronchiolar hyperplasia and severe peribronchiolar and perivascular lymphoid infiltration and mild BALT (panel G). Lesions and epithelial damage in the remaining three pigs were similar to that of control mock-challenged pigs (groups 1, panel A).

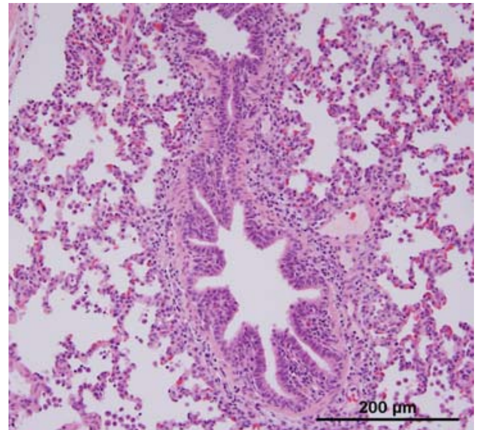
A)



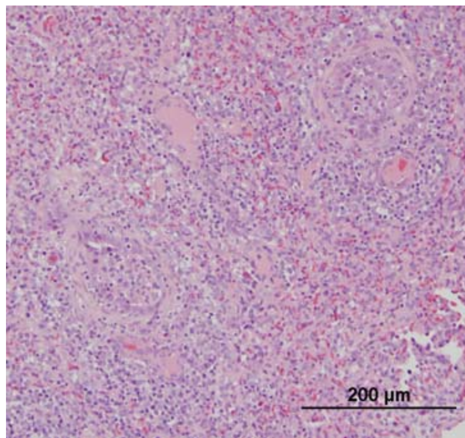
B)



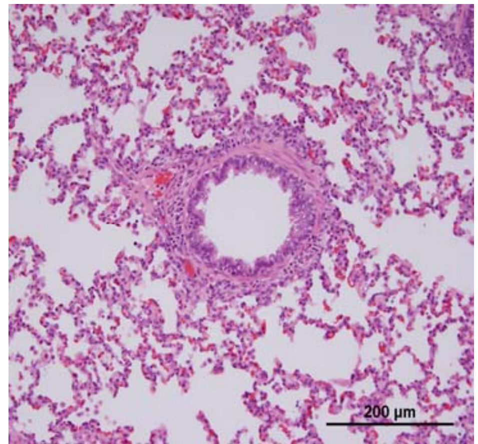
C)



D)



E)



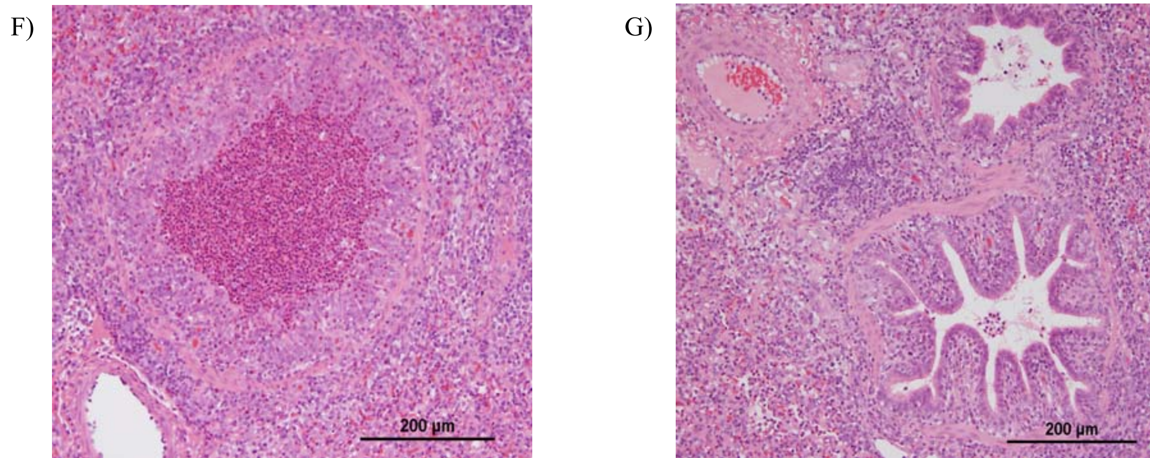


Figure 4.5. Microscopic lung lesions. (A) Medium-sized bronchioles from the lung of a control pig inoculated with MEM only. (B) Severe necrotizing bronchiolitis with moderate multifocal necrosis, attenuation of surviving bronchiolar epithelium in lungs of un-vaccinated and SIV/Sk02 challenged pigs. (C) Normal bronchioles and surrounding blood vessels from the lung of pigs vaccinated with R345V and challenged with H1N1 SIV/Sk02. (D) Severe acute necrotizing bronchiolitis and interstitial pneumonia, severe bronchiolar necrosis in unvaccinated and H1N1 SIV/Ind88 challenged pigs. (E) Normal bronchioles from the lung of pigs vaccinated with R345V and challenged with H1N1 SIV/Ind88. (F) Moderate bronchiolitis with focal necrosis and severe neutrophil infiltration in the lumen of bronchioles and bronchi in unvaccinated and H3N2 SIV/Tx98 challenged pigs. (G) Mild to moderate bronchiolitis with rare epithelial necrosis in R345V vaccinated and H3N2 SIV/Tx98 challenged pigs. Magnification: x 20; scale bar: 200 µm.

4.3.6 Vaccination reduced virus replication in lungs.

Tissue sections from right apical, cardiac and diaphragmatic lung lobes were used for virus isolation. Tissue processing and virus titration was done as previously described (Masic, Babiuk et al. 2009). The data are shown in Figure 4.6. Consistent with results obtained from necropsies and histopathology, we were able to recover viruses from all unvaccinated SIV challenged pigs as well as from five animals that were vaccinated and challenged with heterologous subtypic H3N2 (group 7). The median virus titers from unvaccinated H1N1 (group 2 and 3) or H3N2 (group 4) challenged groups were $10^{4.2}$ $TCID_{50}$/gr, $10^{3.8}$ $TCID_{50}$/gr and $10^{5.1}$ $TCID_{50}$/gr, respectively. The median virus titers from pigs vaccinated and challenged with heterologous subtypic H3N2 (group 7) were $10^{3.1}$ $TCID_{50}$/gr and were significantly lower compared to virus titers from pigs in group 4 (P =0.004). No virus could be detected in the lungs sections of mock-challenged or vaccinated and H1N1 challenged pigs (groups 1, 5 and 6).

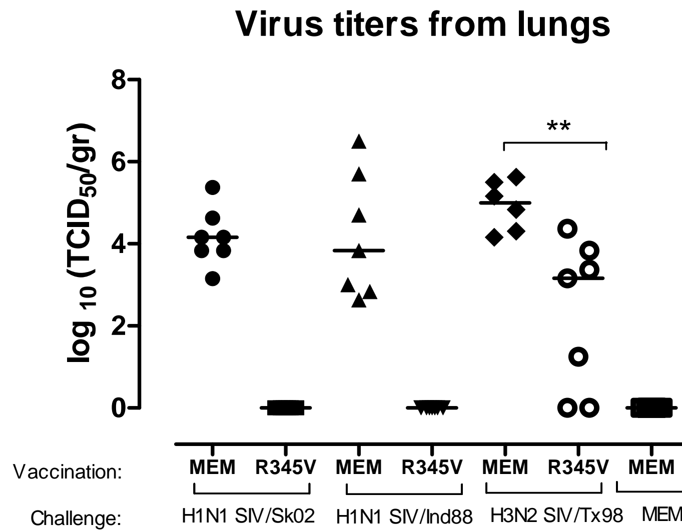


Figure 4.6. Lung virus titers. Lung tissues from right apical, cardiac and diaphragmatic lobe were collected, homogenized and virus titers were determined on MDCK cells. Titers are calculated according to the Reed and Muench method. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

4.3.7 Vaccination reduced the production of proinflammatory cytokines in lower respiratory tract.

Cytokines TNF-α, IFN-α, IL-1β and IL-6 are produced in lungs during SIV infection and are involved in lung pathology (Van Reeth, Labarque et al. 1999; Van Reeth, Van Gucht et al. 2002). Supernatants from lungs (SNL) after tissue homogenization were subjected to ELISA to determine the cytokine levels in lungs. The same samples collected at necropsy 5 days post challenge that were used for virus isolation were used for cytokine profiling. TNF-α, IFN-α, IL-1β and IL-6 were undetectable in SNL of control pigs. TNF-α was undetectable in all groups, probably due to the very narrow window of production (data not shown). In contrast, IFN-α, IL-1β and IL-6 were detectable on the fifth day after challenge in all vaccinated and challenged animals, as well as in unvaccinated and challenged pigs. Cytokine levels were significantly higher in all unvaccinated and challenged groups compared to the corresponding vaccinated and challenged animals (Figure 4.7). Production of all three detected cytokines was correlated with neutrophil infiltration and coincided with the onset of typical SIV clinical signs and lung pathology.

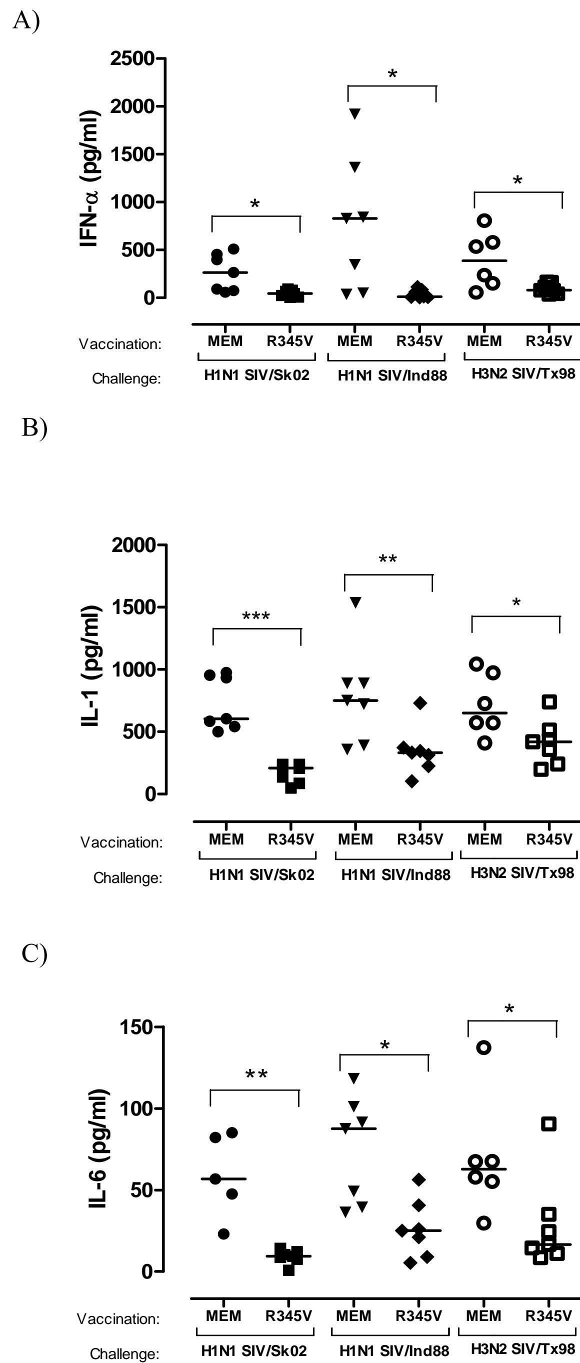


Figure 4.7. Levels of pro-inflammatory cytokines in lung. IFN-α (A), IL-1β (B) and IL-6 (C) in lung of unvaccinated, R345V vaccinated and challenged pigs. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

4.3.8 Antibody titers after virus challenge.

The level of antibodies in serum and in BALF was determined after pigs were vaccinated and challenged with different viruses. As shown in Figure 4.8, high levels of HI (titer = 320) and IgG (titer = 2390) that are reactive to the homologous virus H1N1 SIV/Sk02 were detected in serum (Figure 4.8A and 4.8B). Cross-reactive IgG antibodies in serum to homologous antigenic variant virus H1N1 SIV/Ind88 were significantly increased after virus challenge [from median titer of 945, (Figure 4.3) to 2251 (Figure 4.8B); $p=0.002$] and reached similar levels to that seen in the H1N1 SIV/Sk02 challenged group. HI antibody that recognized homologous antigenic variant virus H1N1 SIV/Ind88 was also significantly increased [from 40 (Figure 4.5) to 80 (Fig.8A); $p = 0.05$]. While serum cross-reactive IgG antibody to heterologous virus H3N2 SIV/Tx98 was low before virus challenge (titer = 130, Figure 4.3), after virus challenge the median titer increased to 335.3 [Figure 4.8B ($p = 0.03$)]. As seen in the previous trial, we could not detect any serum HI antibody against H3N2 SIV/Tx98. In lower respiratory mucosa, reactive IgA antibodies to homologous virus SIV/Sk02 were highly induced after vaccination (Titer = 2750, Figure 4.2F) and virus challenge (titer = 5874, $p = 0.0006$), and cross-reactive IgA antibodies to the SIV/Ind88 and SIV/Tx98 also showed significant increase ($p = 0.007$ and $p = 0.03$ respectively) after the challenge with WT viruses.

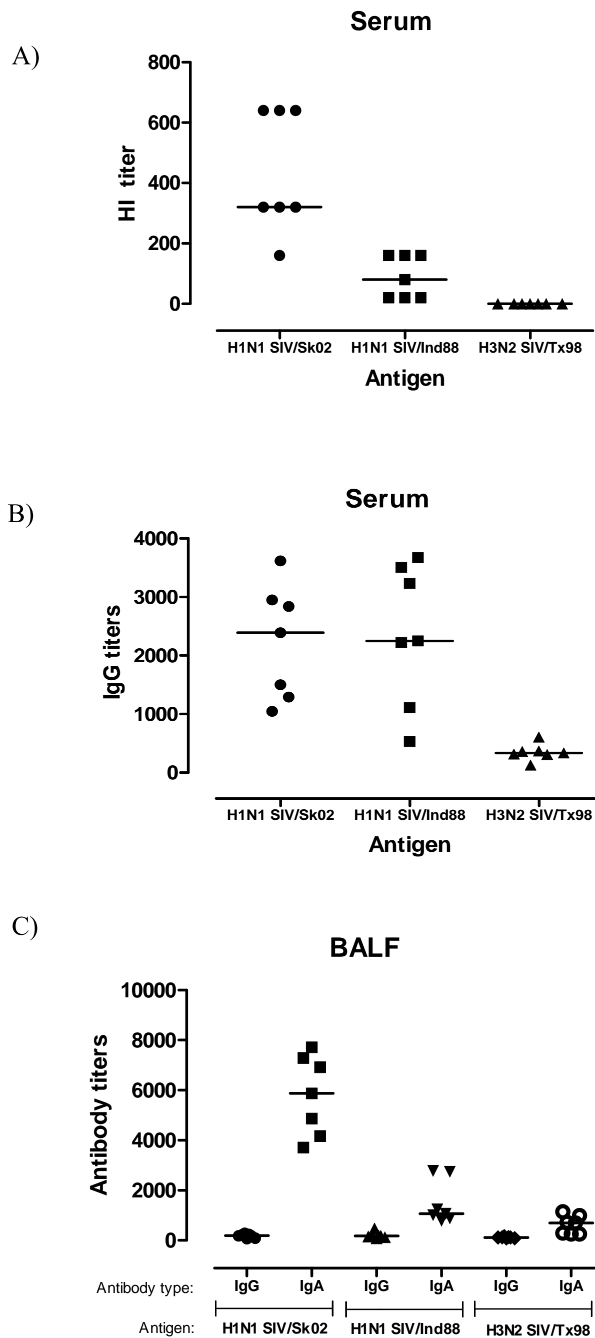


Figure 4.8. Antibody titres after vaccination and challenge. Serum HI (A), IgG (B) and mucosa IgA titers from BALF (C) specific for SIV/Sk02, SIV/Ind88 and SIV/Tx98 were examined after pigs were vaccinated twice with R345V and challenged with WT SIVs. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars.

4.4 DISCUSSION

Attenuation of elastase-dependant LAIV are based on genetically engineered an atypical hemagglutinin cleavage site that is resistant to activation during natural infection. Replacement of the original trypsin-sensitive (Arg-Gly) cleavage site with the elastase-sensitive (Val-Gly or Ala-Gly) motif resulted in *in vivo* attenuation in pig (Masic, Babiuk et al. 2009) and mouse models (Stech, Garn et al. 2005; Gabriel, Garn et al. 2008). Theoretically, lack of an appropriate protease at the site of infection results in the influenza virus uncleaved HA, thus disabling fusion of progeny viruses with the endosome which in turn blocks virus replication. Previously we generated two elastase-dependent mutant SIVs (R345V and R345A). The two mutant viruses are genetic stable and are highly attenuated in pigs. Neither the mutant viruses nor the revertant viruses were isolated from lungs of the infected pigs (Masic, Babiuk et al. 2009), thus they may have potential to serve as live attenuated vaccines. In this study, we first assessed their immunogenic properties by examining the cell-mediated and humoral immune responses after one and two vaccinations. Afterwards, we tested the ability of R345V to induce protective immunity against challenge with homologous and heterologous subtypic SIVs in pigs.

It has long been believed that recovery of influenza virus infection is mediated by the cellular immune responses (McMichael, Michie et al. 1986; Graham and Braciale 1997; Heinen, de Boer-Luijtze et al. 2001), whereas prevention of viral infection correlates with serum and mucosal anti-influenza virus antibody titers (Burlington, Clements et al. 1983). In addition, there is a large body of evidence showing that cell-mediated responses are an important contributor to heterologous subtypic immunity in mice and pigs (Epstein, Lo et al. 1997; Flynn, Belz et al. 1998; Nguyen, Moldoveanu et al. 1999; Heinen, de Boer-Luijtze et al. 2001). IFN-γ, produced by $CD4^{+}$ T helper cell type 1 (Th1) lymphocytes, $CD8^{+}$ cytotoxic lymphocytes and NK cells (Bach, Aguet et al. 1997), is the major immuno-modulator that coordinates the immune responses and establishes an antiviral state of longer duration (Schroder, Hertzog et al. 2004). In our study one vaccination with R345V or R345A was sufficient to induce significant numbers of local IFN-γ secreting cells. The two-fold increase in IFN-γ response after the secondary vaccination suggested that both virus candidates are capable of inducing T-cell activation (Figure 4.1A). In addition, results from the LPR assay showed that one vaccination with R345V or R345A induced low proliferation of lymphocytes while a second vaccination resulted in significant LNC proliferation (Figure 4.1B). Previous reports from experimental infection with

WT SIVs showed that second exposure to virus antigen did not result in an increase of lymphocyte proliferation or IFN-γ secretion (Larsen, Karasin et al. 2000; Heinen, de Boer-Luijtze et al. 2001), these might be attributed to the multiple replication of WT SIVs. In our study, one vaccination is not sufficient to induce maximum immune response, possibly due to one or limited replication cycles of R345V *in vivo*.

Antibody responses to R345V and R345A in the serum and at the respiratory mucosa were measured after the first and the second immunization. While one vaccination induced low levels of antigen specific IgG, IgA and HI antibodies in serum and BALF, secondary vaccination induced considerable high titers of antigen-specific IgG, IgA and HI (Figure 4.2). This could also be attributed to the restricted replication and short antigen exposure. These data suggest that two vaccinations might be required for immune response and could mimic natural immunity after SIV infection and be protective against challenge with the WT homologous and heterologous SIVs.

Both R345V and R345A viruses could induce cellular and humoral immunity and showed similar antigenic properties. After comparison of statistical analyses from all performed assays, SIV mutant R345V showed enhanced serum HI and antigen-specific IgG titers, while serum antigen-specific IgA antibody titers, numbers of IFN-γ secreting cells and LPR stimulation index were at a similar level as in R345A vaccinated pigs. Therefore, we decided to choose R345V as a vaccine candidate in our protection trial.

Two vaccinations via the IT route with R345V were sufficient to confer complete protection from challenge with the homologous subtypic H1N1 SIV/Sk02 and H1N1 variant SIV/Ind88 (groups 5 and 6). Vaccinated and challenged pigs did not show any of the SIV characteristic clinical signs or elevated rectal temperatures compared to the unvaccinated challenged controls (data not shown). Virus was not detected in lungs of any pigs in these groups, macroscopic (Figure 4.4) and microscopic (Figure 4.5) lesions were undetectable or minimal. To demonstrate the ability of the elastase-dependant R345V SIV to induce immunity against an antigenically distinct SIV subtype, vaccinated pigs were challenged with a heterologous subtypic H3N2 SIV/Tx98. On 5 days post infection, 5 out of the 7 pigs in R345V vaccinated and H3N2 SIV/Tx98 challenged group had detectable virus in their lungs. However, median virus titers were significantly lower than in the unvaccinated H3N2 SIV/Tx98 challenged group (Figure 4.6). In addition, macroscopic and microscopic lesions were significantly reduced.

There is growing evidence that the early cytokines are the cause of the SI clinical signs and lung epithelial damage (Van Reeth, Nauwynck et al. 1998; Van Reeth, Van Gucht et al. 2002; Van Reeth, Van Gucht et al. 2002). Early cytokines are produced by non-immune cells at the site of infection and they are responsible for local inflammatory reactions, as well as some systemic effects. IFN-α, TNF- α and IL-1α and β are the first released in the early cytokine cascade (Van Reeth, Nauwynck et al. 1998). These cytokines are rapidly followed by IL-6 and a number of chemokines (Van Reeth 2000). IFN-α, TNF-α, IL-1 and IL-6 are cytokines with multifunctional activities and they have been associated with fever, sleepiness and anorexia. Furthermore peak cytokine levels directly correlate with virus replication and epithelial lung damage (Van Reeth 2000; van Reeth and Nauwynck 2000). To further strengthen our data on immuno-protection study, we assessed the production of IFN-α, TNF-α, IL-1*a* and IL-6 cytokines in pigs. On day 5 post infection, we could not detect any released TNF-α (data not shown) probably due to the very narrow window of TNF-α production (first 6-8 h p.i). In all unvaccinated and H1N1 SIV challenged pigs, there was a significant increase in pro-inflammatory cytokines (IFN-α, IL-1 and IL-6) compared to the R345V vaccinated and challenged animals. However, in pigs challenged with heterologous subtypic H3N2 SIV/Tx98, the statistical difference between unvaccinated and vaccinated groups was exactly 0.05 for IFN-α and IL-1β which would be consistent with the partial protection ability of R345V. Taken together our data obtained from macroscopic, microscopic lesions, virus titers and cytokine release clearly showed that two administrations of R345V virus vaccine confer full protection against homologous and antigenic variant H1N1 SIVs and partial protection from antigenically distinct H3N2 SIV infection.

Induction of heterologous subtypic immunity after the experimental or natural infection with influenza A virus in several species has been described (Schulman and Kilbourne 1965; Webster and Askonas 1980; Reeth, Brown et al. 2004). There is mounting evidence showing that the presence of cross-reactive antibodies, especially IgA induced in the respiratory mucosa after natural infection or vaccination with live attenuated vaccines are strongly correlated to protection from challenge with homologous and heterologous subtypic influenza viruses (Liew, Russell et al. 1984; Vincent, Ma et al. 2007). The importance of cross-reactive IgA is also supported by experiments where passive transfer of IgA to non-immune mice conferred protection (Tamura, Funato et al. 1991) whereas mucosal administration of anti-IgA to immune mice blocked protection from re-infection with the same virus (Renegar and Small 1991). In our study, the

R345V mutant virus was capable of inducing a significantly higher level of IgA, that reacts with homologous SIV/Sk02 (titer = 2450, Figure 4.2F), SIV/Ind88 (titer = 682, Fig. 3C) and moderate levels of IgA that reacts to heterlogous subtypic SIV/Tx98 (titer = 251, Figure 4.3C) in BALF. In addition to the contribution of IgA, cross-reactive IgG antibody was found in the lungs along with IgA and has been considered a correlate of heterotypic protection (Tumpey, Renshaw et al. 2001). Vaccination with R345V also induced a rapid appearance of serum IgG antibody not only reacts to homologous SIV/Sk02 (titer = 1611, Fig. 2B), but also reacts to H1N1 antigenic variant SIV/Ind88 (titer = 945, Figure 4.3B) and heterologous subtypic H3N2 SIV/Tx98 (titer = 130, Figure 4.3B). Cross reactive IgG was also detected in BALF although at low level (titer = 108 to SIV/Ind88; 64 to SIV/Tx98, Figure 4.3C). These data suggest that immunization of live R345V induced influenza-specific IgA in the mucosa and IgG in serum may contribute to complete protection of homologous and partial protection to heterologous SIV infection.

The presence of cross-reactive IgG in serum and IgA in BALF after the vaccination and virus challenge suggests that the live attenuated virus could prime immune cells to generate antibodies against common virus epitopes. The significant increase of cross-reactive IgG antibodies in serum and IgA in BALF observed after the challenge (Figure 4.8) could be due to primed B cells against common viral antigens following vaccination. This observation is in agreement with the previous findings by Heinen *et al.* who found that infection of pigs with H1N1 SIV and re-infection with H3N2, significantly increase serum IgG recognizing M2 epitopes or mucosal IgA recognizing NP epitopes (Heinen, de Boer-Luijtze et al. 2001). In our study, two vaccinations with modified live virus induced a pattern and kinetics of immune response that contains protective characteristics similar to past natural infection. The exact mechanism and pathway involved in immune-protection against SIV by live vaccine remain elusive and further studies are needed to determine to what extent cross-talk between cell-mediated and humoral immune response play a role in SIV protection.

A mutant H3N2 SIV with truncated NS1 protein has shown to be highly attenuated and immunogenic in swine. It is potential to be a modified live-virus vaccine (Solorzano, Webby et al. 2005; Richt, Lekcharoensuk et al. 2006; Vincent, Ma et al. 2007). This potential vaccine is due to the decreased ability of NS1 to antagonize the interferon production and its downstream effectors. Our studies provide an additional attenuating approach leading to development of live vaccines to combat SIV infection. Both NS1-truncated and elastase dependent LAIVs are highly

attenuated in swine and are immunogenic. Vaccination with both LAIVs was fully protective against infections from homologous SIV and homologous SIV with antigenic variant; and was partially protective against heterologous subtypic SIV infection. NS1-trancated LAIV can be propagated in embryonated chicken eggs, whereas the elastase dependent LAIV can be propagated solely in cell culture; in either case, the viruses can grow to high titres. Taken together, both LAIVs meet the criteria required to be successful live vaccines.

The common objection against the use of live attenuated virus as vaccine is the possibility of reversion to pathogenicity. The absence of appropriate protease for R345V *in vivo* allows only few replication cycles leading to restricted replication. An important advantage of the short and limited replication is the decreased probability of any reversion and reassortment between vaccine virus and WT virus. Although current vaccination requires two administrations and was delivered intratracheally (to ensure all the viruses are delivered to the respiratory tract of the pigs), the advantage of the elastase-dependent live attenuated virus has been characterized by its ability to induce humoral and cell mediated immune responses. Most importantly, vaccination with this virus led to reduction of homologous and heterlogous subtypic SIV virus load and pathogenesis. In addition, the heterologous subtypic immunity induced by elastase-dependent live attenuated virus could have a significant impact on the epidemiology of novel SIVs emerging in the swine population by reducing viral shedding and potentially limiting the spread of novel SIVs. Currently we are planning to test more practical and optimal route of vaccination, such as intranasal immunization.

5. IMMUNOGENICITY AND PROTECTIVE EFFICACY OF AN ELASTASE-DEPENDENT LIVE ATTENUATED SWINE INFLUENZA VIRUS VACCINE ADMINISTERED INTRANASALLY IN PIGS

(As submitted to Journal of Virology, May 2010)

5.1 INTRODUCTION

Swine influenza virus (SIV) infections are common and an important cause of bronchointerstitial pneumonia and respiratory disease in pigs (B.E.Straw 1999). SIV belongs to the genus influenza A of the family *Orthomyxoviridae* (Peter Palese 2007). The genome consists of eight single-stranded RNA segments of negative polarity. Due to their segmented genome, influenza A viruses undergo infrequent antigenic shift or reassortment, leading to generation of novel viruses. Epithelial cells in the swine respiratory tract have receptors for both avian and mammalian influenza viruses (Ito, Couceiro et al. 1998) therefore pigs could serve as “mixing vessel” for the generation of new reassortant strains with pandemic capacity. This has been demonstrated for the newly emergent pandemic human H1N1 influenza A virus that evolved by reassortment of swine influenza viruses (Garten, Davis et al. 2009). This new H1N1 virus contains six genes of the North American swine triple reassortant lineage with the M and NA acquired from the Eurasian swine lineage H1N1. In addition, these swine origin human H1N1 viruses are characterized by increased human transmission and high pathogenicity in young adults constituting a significant public health concern (Garten, Davis et al. 2009; Smith, Vijaykrishna et al. 2009). The emergence of the swine origin pandemic H1N1 influenza A virus in humans now demonstrated that the flow of genes and viruses between swine and humans is bidirectional (Garten, Davis et al. 2009). Consequently, effective control and prevention of SIV infections is required for both human and animal health.

Classical H1N1 (cH1N1) SIVs were major isolates from pigs in U.S and Canada until 1997-1998 (Chambers, Hinshaw et al. 1991; Olsen 2002) when a novel H3N2 virus arose in the North American swine herd and quickly became endemic. The new H3N2 viruses acquired HA, NA and PB1 genes from human influenza virus, PA and PB2 from avian influenza viruses and the remaining genes, NP, M and NS, from swine influenza viruses. Therefore, these viruses are designated as triple reassortant viruses (TRV) (Zhou, Senne et al. 1999). Reassortment between TRV H3N2 and cH1N1 viruses resulted in the generation of novel distinct genetic and antigenic clusters (Vincent, Lager et al. 2006). Presently, there are a number of reassortant influenza viruses that have been identified in pigs, such as H3N2 drift variants (Webby, Swenson et al. 2000; Webby, Rossow et al. 2004), H1N2 (Karasin, Landgraf et al. 2002), H1N1(Webby, Rossow et al. 2004), and H3N1 (Ma, Gramer et al. 2006) viruses. Furthermore, there are reports of wholly avian H3N3 and H1N1 isolates from Canadian pigs (Karasin, West et al. 2004) as well as the introduction of genetically and antigenically distinct H1N1 and H1N2 viruses containing surface genes from the current human seasonal influenza A viruses (Karasin, Carman et al. 2006). As a result of interspecies reassortments and antigenic drift, H1 SIV phylogenetic clusters α, β, γ and δ are endemic in North American pigs (Vincent, Swenson et al. 2009). In general, SIV diversity has been expanding to include the cH1N1, H3N2, H1N1 and H1N2 triple reassortnant SIVs with multiple genetic and antigenic variants that have become endemic and continue to co-circulate in most major swine producing regions in North America (Olsen 2002; Karasin, West et al. 2004). Increased incidence of human-like and avian-like SIV reassortants raises the importance of surveillance for SIV and requires rapid research to develop cross-protective SIV vaccines.

Currently available swine influenza vaccines are inactivated whole virus or split virus preparations for intramuscular administration. They contain representatives of the H1N1 and H3N2 subtypes in combination with an oil adjuvant and have not been changed for more than 15 years. Application of these vaccines reduces the severity of disease but does not provide consistent protection from infection (Brown 1994; Macklin, McCabe et al. 1998). Protection achieved by these vaccines is associated with the induction of antibodies to the two major viral envelope glycoproteins, HA and NA (Rimmelzwaan, Baars et al. 1999). However, HA and NA point mutation known as antigenic drift occurs frequently resulting in the antigenic changes in that can diminish protection ability and, cause vaccine failure and indeed, contribute to disease and pathology in subsequently infected pigs. These vaccines induce little or no heterologus cross-reactive immunity between influenza subtypes or genetically different viruses within subtype (Tamura, Tanimoto et al. 2005). In contrast to inactivated vaccines mucosally delivered live attenuated influenza vaccines (LAIV) induced immune responses at the site of natural infection and demonstrate cross-reactive ability (Vincent, Ma et al. 2007). LAIV also have the potential to induce broad cell-mediated and humoral immune responses which could provide better protection against antigenically distinct influenza viruses. Currently, LAIV are available for human (Belshe 2004) and equine species (Townsend, Penner et al. 2001) but despite several reports (Vincent, Ma et al. 2007; Masic, Booth et al. 2009) there is no commercially available LAIV for pigs in North America. Recently we showed that elastase-dependent LAIV R345V derived from A/Sw/Saskatchewan/18789/02 (H1N1) (SIV/Sk02) is attenuated, immunogenic and protective in pigs after intratracheal administration (Masic, Babiuk et al. 2009; Masic, Booth et al. 2009). However, intratracheal vaccination is not routinely used in the swine industry.

Intranasal vaccination is a more practical way of immunization and has application to mass herd vaccination since it enables use of smaller volumes and delivery of antigens

mimicking natural infection to provide stronger immune responses (Cox, Brokstad et al. 2004; O'Hagan and Rappuoli 2004). In the current study we first assessed the immunogenic and protective abilities for two different doses of R345V LAIV after two intranasal vaccinations against homologous SIV/Sk02. Secondly, after establishing the proper intranasal R345V vaccine dose we evaluated the cross-protective capacity for R345V by challenging pigs with two antigenically distinct SIVs.

5.2 MATERIALS AND METHODS

5.2.1 Cells, viruses and vaccine.

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The H1N1 mutant virus SIV/Sk-R345V (R345V) used in this study was generated as previously described (Masic, Babiuk et al. 2009). This virus contained a modified HA cleavage site thus is only sensitive to human neutrophil elastase. The other SIVs used in the study were homologous H1N1 A/Sw/Saskatchewan/18789/02 (SIV/Sk02); antigenic subtype H1N1 A/Sw/Indiana/1726/88 (SIV/Ind88) and heterologous H3N2 A/Sw/Texas/4199-2/98 (SIV/Tx98). Propagation and titration of these viruses were described previously (Masic, Booth et al. 2009) .

5.2.2 Experimental design and sampling.

All study procedures and animal care activities were conducted at the Vaccine and Infectious Disease Organization, University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

For the purpose of this study we designed two animal trials. The first trial was designed to compare immune responses and protection ability of two different doses of R345V administrated intranasally. The second trial served to evaluate cross-protection after intranasal vaccination with R345V against antigenically different H1N1 and heterologus H3N2 SIVs. Four-week old, SIV negative landrace crossbred pigs were obtained from the Prairie Swine Center (Floral, Saskatchewan, Canada).

In the first trial, fifteen pigs were randomly selected and assigned to three groups with five pigs per group (Table 5.1). At five weeks of age, pigs in groups 1 and 2 were vaccinated intranasally with 1ml (0.5ml/nostril) of MEM containing either 1×10^7 PFU or 5×10^6 PFU of R345V while the remaining five pigs in group 3 were mock vaccinated intranasally with 1ml of MEM only. Intranasal vaccination was performed using a regular lever tip 3ml syringe with $1^1/_3$ J-12 teat infusion cannula attached. After three weeks (day 21), pigs in all groups were given a secondary intranasal vaccination with the same dose respectively. Ten days after the second vaccination (day 31), pigs in all groups were challenged intratracheally with $8x10^5$ PFU of homologous wild type (WT) SIV/Sk02.

In the second trial, twenty seven pigs were assigned to five groups with six animals in the first four groups, and three animals in the fifth group (Table 5.2). Vaccinated and unvaccinated pigs challenged with the same WT SIV were housed in the same room but in separate pens, while three unvaccinated/mock challenged pigs were housed in a separate isolation room. Animals in all five groups were given two intranasal inoculations at three weeks interval with either 1ml of MEM only (groups 1, 3 and 5) or with 1ml MEM containing $1x10^7$ PFU of R345V (groups 2 and 4). On day 31, pigs in groups 1 and 2 were challenged intratracheally with $8x10^5$ PFU of homologous antigenic variant H1N1 SIV/Ind88 while pigs in groups 3 and 4 were challenged with heterologus subtypic H3N2 SIV/Tx98 (Table 5.2). Pigs in group five received mock inocula only.

After the challenge pigs in both trials were monitored for five consecutive days for the presence of clinical signs of SIV infection and then humanely euthanized with a lethal dose of pentobarbital sodium (Euthanyl 25mg/ml) and subjected to necropsy. At necropsy, lungs were evaluated and scored for the presence of SIV characteristic lesions, tracheo-bronchial lymph nodes were extracted and broncho-alveolar fluid (BALF) was collected by lavaging the lungs with 20 ml of phosphate-buffered saline (PBS, 0.137 M NaCl, 2.7 mM KCl, 8 mM $NaHPO_4$,

1.47 mM KH_2PO_4, pH 7.3). Blood and nasal swab samples were collected before and after each vaccination and at necropsy. Tissue samples from the right apical, cardiac and diaphragmatic lung lobes were collected for virus isolation and histopathology examination.

Table 5.1 Assignment of pigs in each group – Immunogenic and antigenic characteristics for two IN doses (1 x 10^7 pfu/ml and 5 x 10^6 pfu/ml) of R345V LAIV

Group N=5	1st vaccination IN	2nd vaccination IN	Challenge IT
1.	R345V 1 x 10^7PFU/ml	R345V 1 x 10^7PFU/ml	SIV/Sk02 H1N1
2.	R345V 5 x 10^6 PFU/ml	R345V 5 x 10^6 PFU/ml	SIV/Sk02 H1N1
3.	MEM	MEM	SIV/Sk02 H1N1

Table 5.2 Assignment of pigs in each group –Cross-protection study

Group N=6	1st vaccination	2nd vaccination	Challenge
1.	MEM	MEM	SIV/Ind88 H1N1
2.	R345V	R345V	SIV/Ind88 H1N1
3.	MEM	MEM	SIV/Tx98 H3N2
4.	R345V	R345V	SIV/Tx98 H3N2
5. **N=3**	MEM	MEM	MEM

5.2.3 Histopathology evaluation and virus isolation.

Necropsy, macroscopic examination of lungs and tissue processing for virus isolation was performed as described previously (Masic, Babiuk et al. 2009; Masic, Booth et al. 2009). Tissue sections of lungs were routinely stained with hematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or extent of lesions within the sections examined as follows: 0: no visible changes; 1: mild focal or multifocal change; 2: moderate multifocal change; 3: moderate diffuse change; 4: severe diffuse change. Two independent pathologists scored all slides and they were blinded for the experimental groups.

5.2.4 Antigen specific IFN-γ ELISPOT.

Tracheo-bronchial lymph nodes were dissected *in toto* at necropsy and cells were isolated as previously described (Masic, Booth et al. 2009). For the ELISPOT assay, nitrocellulose microtiter plates - UNIFILTER 350 (Whatmann, Florham Park, NJ) were coated with 5 µg/ml of mouse anti-porcine IFN-γ monoclonal antibodies (Endogen, Rockford, IL, USA) and incubated overnight at 4°C. Lymph node cells (LNC) were seeded at a concentration of $1x10^6$ cells/well in a final volume of 200 µl/well AIM-V media containing 2% FBS. LNC were stimulated for 10 h at 37°C with 25 µg/ml of purified and UV inactivated H1N1 SIV/Sk02, H1N1 SIV/Ind88 or H3N2 SIV/Tx98 viruses, 5 µg/ml of Con A (Sigma-Aldrich), or media only. After 10h incubation, plates were washed five times with PBST (PBS containing 0.05% Tween 20) and incubated with 2 µg/ml of rabbit anti-porcine IFN-γ (Endogen, Rockford, IL, USA) overnight at 4°C. Biotinylated goat anti-rabbit IgG (H+L) antibodies (DiAMED, South San Francisco, CA, USA) were added at a dilution of 1:5000 and incubated for 2 h at room temperature. Plates were washed five times and incubated with streptavidin alkaline phosphotase solution (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:500 for 1.5 h at room temperature. In the final step, plates were washed eight times with double distilled water (ddH_2O), and 100 µl/well of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Sigma-Aldrich) insoluble alkaline substrate solution was added. After 5 min incubation, plates were washed again and left to dry overnight at room temperature. Spots were counted manually under an inverted light microscope and recorded. Number of spots observed in media only stimulated

cells were counted and subtracted as a background. Data was reported as number of IFN-γ secreting cells per 10^6 somatic cells.

5.2.5 ELISA for antigen-specific IgG, IgA antibodies, pro-inflammatory cytokines and hemagglutination inhibition (HI) assay.

Serum samples were collected by jugular venipuncture at following time points: before the first vaccination, before the second vaccination, before the challenge and at the necropsy on 5 day post infection (d.p.i.). To evaluate the serum HI titers, sera were heat inactivated at 56°C for 30 min, then treated overnight at 37°C with receptor-destroying enzyme (Cholera filtrate-C8772, Sigma Aldrich) to eliminate non-specific HI factors. Viruses used in HI assays were SIV/Sk02, SIV/Ind88 and SIV/Tx98. HI assay was performed as described elsewhere (OIE 2004).

Titers of antigen specific IgG and IgA antibodies in serum, BALF and nasal swabs samples were determined by whole virus based ELISA assay as described previously (Masic, Booth et al. 2009). The optical density of the reaction product was measured at 405 nm (a 490 nm reference filter used to detect background which was subtracted from the measurement reading) on a microplate reader (Molecular Devices, SpectraMax Plus 384). The titer of sample was defined as the highest dilution at which the OD of that sample was higher than the defined cut off (the mean OD of a known negative sample plus two times standard deviation).

An ELISA used to detect pro-inflammaroty cytokines in the respiratory tract was performed as previously described (Masic, Booth et al. 2009). Sample concentrations were calculated using Softmax Pro 5.2 version software (Molecular Devices, SpectraMax Plus 384).

5.2.6 Statistical analysis.

Statistical analysis were performed using GraphPad Prism5 (San Diego, CA, USA) and Statistix7 (Tallhassee, FL, USA) software. Differences between medians in two groups (vaccinated vs. unvaccinated) in each assay were determined using Mann-Whitney nonparametric t-test. To compare dose effect, data from ELISPOT assays and antibody titers were transformed and One-way ANOVA for RANK data was applied. If the mean values of at least one group differed from others with a $P < 0.05$, they were considered statistically significant.

5.3 RESULTS

5.3.1 Clinical efficiency of intranasal vaccination of R345V against homologous H1N1 SIV/Sk02 challenge.

In the first trial, pigs were intranasally immunized at two times with the same dose of R345V. To test the dose effect of R345V on eliciting immune responses and protective efficacy against parental wild-type SIV infection , one group of pigs was immunized with 10^7 PFU/ml of R345V (high dose, group 1) and another group of pigs was immunized with $5x10^6$ PFU/ml of R345V (low dose, group 2) (Table 5.1). Ten days after the second vaccination, pigs were challenged with homologous H1N1 SIV/SK02 and observed for 5 days. During the five-day observation period, fever and mild respiratory signs including abdominal breathing, sneezing and nasal discharge were observed up to 4 d.p.i. in the unvaccinated/challenged animals (group 3). No obvious respiratory clinical signs (nasal and ocular discharge and respiratory distress) were seen in R345V vaccinated/challenged pigs (groups 1 and 2). However, pigs in group 1 and 2 had slightly elevated body temperatures at 24 h.p.i. suggesting limited viral replication (Figure 5.1A).

SIV gross lesions characterized as sharply-demarcated, purple to plum-colored, consolidated areas were observed in all 5 pigs in the unvaccinated/challenged group (Figure 5.1B). Presence of the lesions was most dominant in the apical and cardiac lung lobes while diaphragmatic and intermediate lobes were less affected. Lungs of R345V vaccinated/homologous virus challenged pigs (Table 5.1 groups 1 and 2) showed mild or no gross lung pathology. Lung scores were significantly lower in the vaccinated groups compare to unvaccinated control group ($p<0.0001$) (Figure 5.1B). However, there was no significant difference in macroscopic lung pathology and lung scores between the two vaccinated groups.

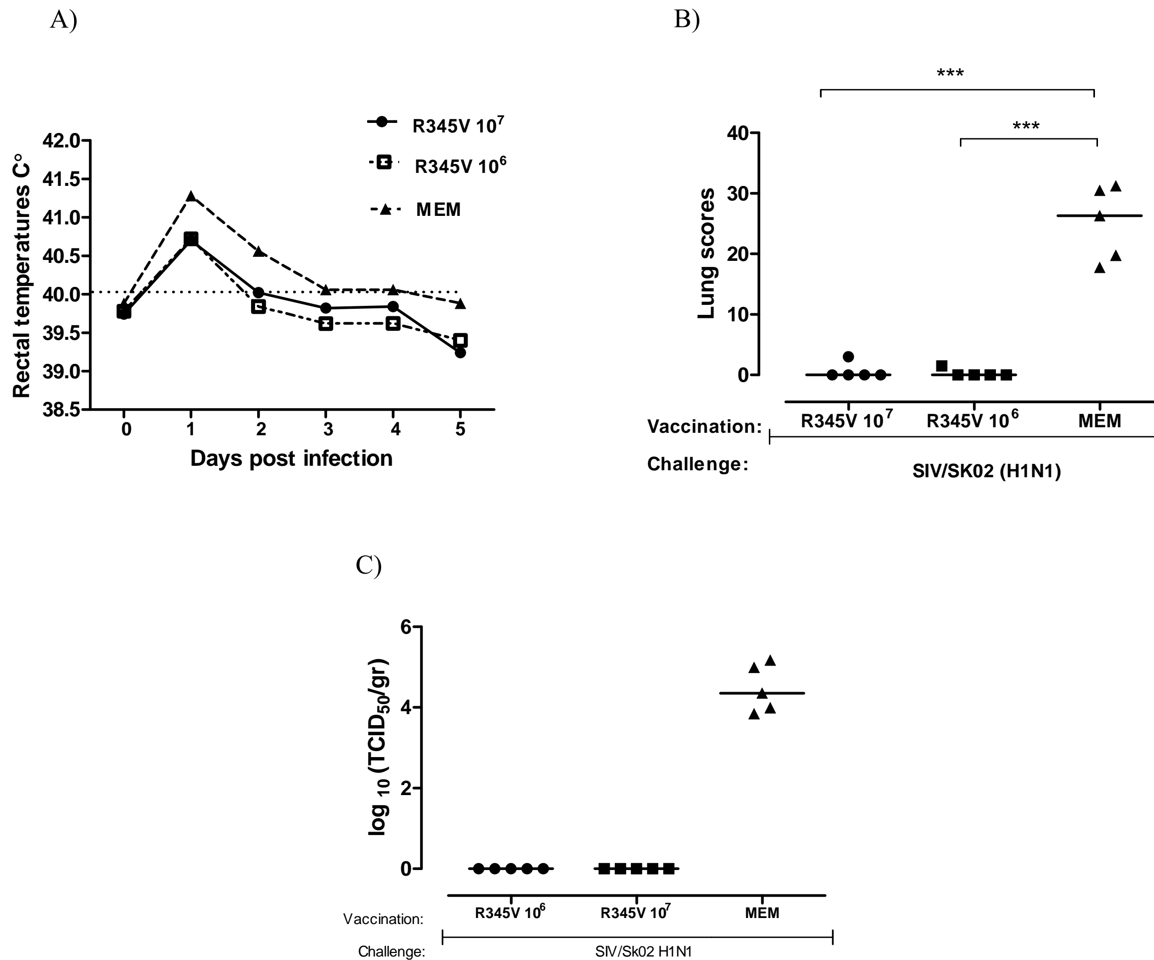


Figure 5.1 Rectal temperatures, macroscopic lung lesions and virus titers of IN vaccinated/unvaccinated and homologous H1N1 SIV/Sk02 challenged pigs. (A) Rectal temperatures from IN vaccinated (R345V 10^7 and R345V 10^6) and unvaccinated (MEM) groups were recorded daily for five consecutive days after the challenge with wild type H1N1 SIV/Sk02. Results are shown as a median group temperatures. The cut-off for a febrile response was calculated as median rectal temperatures of all pigs at day 0 (dashed horizontal line). **(B)** Macroscopic lung lesions. The percentage of the areas affected with pneumonia was estimated visually for each lung lobe. Total percentage for the entire lung was calculated based on weight proportions of each lung lobe to the total lung volume. Results are the median score of lung lesions of five animals in each group. *P<0.05; **P<0.01; ***P<0.001. **(C)** Lung virus titers**.** Lung tissues from right apical, cardiac and diaphragmatic lobe were collected, homogenized and virus titers were determined on MDCK cells. Titers are calculated according to the Reed and Muench method. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001

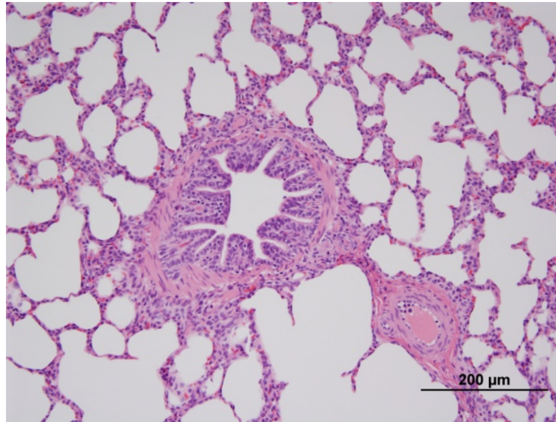
5.3.2 Virus replication in lungs.

Lung sections from right apical, cardiac and diaphragmatic lobes were collected at necropsy and used for virus isolation. Tissue processing and virus titration was completed as previously described (Masic, Babiuk et al. 2009). Consistent with the previous results obtained from intratracheal vaccination (Masic, Babiuk et al. 2009; Masic, Booth et al. 2009), virus was isolated from lungs of all five unvaccinated/SIV/Sk02 virus challenged pigs. The median group virus titer in lungs was $10^{4.35}$ $TCID_{50}$/g. No virus could be detected in the lung of pigs in group 1 and 2 (Figure 5.1C).

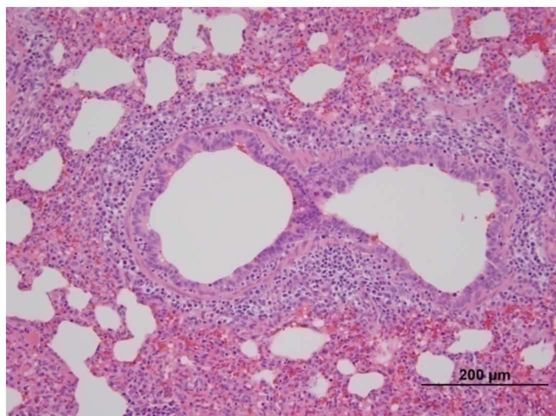
5.3.3 Microscopic lung lesion.

To evaluate the protection abilities of two different doses of R345V in greater detail, we examined the microscopic lung pathology. Consistent with the macroscopic lung pathology observed above, unvaccinated/SIV/Sk02 challenged pigs had prominent histopathological changes characterized by severe bronchointerstitial pneumonia with moderate subacute necrotizing bronchiolitis typical of SIV infection. Furthermore, peribronchiolar and perivascular lymphoid infiltration was accompanied with a severe neutrophil infiltration in the lumen of most bronchioles (Figure 5.2C). In contrast, in both R345V vaccinated/SIV-SK/02 challenged groups there was a significant reduction in the presence and severity of microscopic lesions compared to the unvaccinated/challenged group (Figure 5.2A and 5.2B). Examination of lung sections of the pigs vaccinated with R345V higher dose revealed minimal interstitial pneumonia and mild pleural thickening (Figure 5.2A). Yet, only one animal in this group had moderate peribronchiolar lymphoid infiltration and interstitial thickening due to inflammatory cell infiltration in alveolar walls (changes similar to group 2). Microscopic changes observed in pigs vaccinated with the lower dose were characteristic for moderate bronchointerstitial pneumonia with prominent peribronchiolar lymphoid infiltration. However, bronchiolar necrosis was not observed (Figure 5.2B). Microscopic lung scores in both vaccinated groups were significantly different compared to the control group ($p<0.0001$; Figure 5.2D).

A)



B)



C)

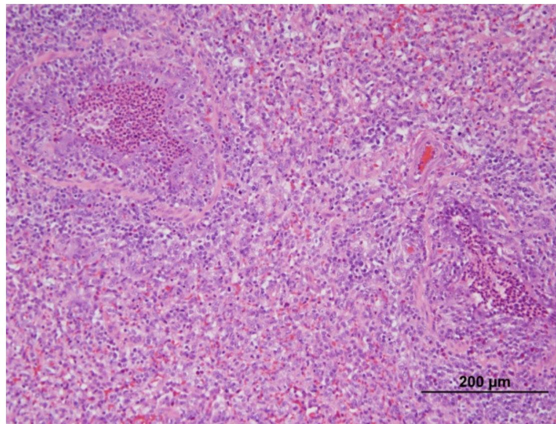


Figure 5.2 Microscopic lung lesions. (A) Normal bronchioles and surrounding blood vessels from the lung of pigs vaccinated with R345V 10^7 and challenged with H1N1 SIV/Sk02; (B) Mild to moderate bronchiolitis in lungs of pigs IN vaccinated with R345V 10^6 and challenged with H1N1 SIV/Sk02; (C) Severe necrotizing bronchiolitis with moderate multifocal necrosis, attenuation of surviving bronchiolar epithelium in lungs of unvaccinated and SIV/Sk02 challenged pigs; Magnification, x 20, bar 200 μm.

Table 5.3 Histopathology lung scores

Group	Vaccination	Scores
1.	R345V 10^7	1.2±0.45 a
2.	R345V 10^6	1.8±0.45 a
3.	MEM	3.8±0.45

Table 5.3 Microscopic lung lesions scores. Median group scores that represent the severity of histopathological changes in lungs of pigs R345V vaccinated IN R345V 10^7 and R345V 10^6) and SIV/Sk02 challenged groups and unvaccinated and challenged group (MEM). Severity of lesions was scored as follow: 0 - no visible changes; 1 - mild focal or multifocal change; 2 - moderate multifocal change; 3 - moderate diffuse change; 4 - severe diffuse change.

5.3.4 Production of pro-inflammatory cytokines in respiratory tract.

Production of IFN-α, IL-1β and IL-6 cytokines in lungs is correlated with neutrophil infiltration and the onset of typical SIV clinical signs and lung pathology (Van Reeth, Labarque et al. 1999; Van Reeth, Van Gucht et al. 2002). BALF samples collected at necropsy on 5 d.p.i. were assessed by ELISA to determine the cytokine levels in the lower respiratory tract. Consistent with previous observations (Masic, Booth et al. 2009), IFN-α, IL-1β and IL-6 were detectable on day 5 after challenge in all challenged pigs. However, Levels of pro-inflammatory cytokines (IFN-α, IL-1β and IL-6) in the lower respiratory tract were significantly higher in all pigs from the unvaccinated/challenged group compared to the two vaccinated/SIV/Sk02 challenged groups (Figure 5.3). No statistically significant differences were observed between the two vaccinated groups.

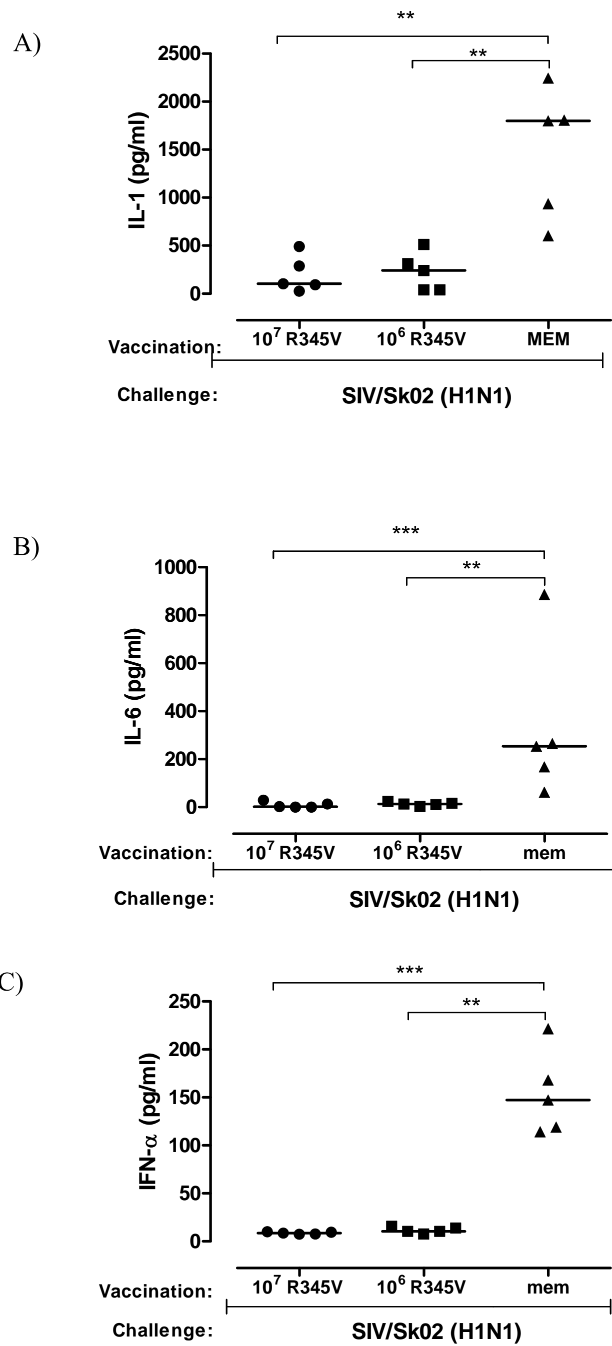


Figure 5.3 Levels of pro-inflammatory cytokines in lung. IL-1β (A), IL-6 (B) and IFN-α (C) in lungs of vaccinated (R345V 10^7 and R345V 10^6) and unvaccinated (MEM), and H1N1 SIV/Sk02 challenged pigs. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

5.3.5 Intranasal vaccination of R345V induced cell mediated immune response.

To evaluate cell-mediated immune responses after intranasal vaccination with R345V, tracheobronchial lymph nodes were extracted at necropsy from vaccinated and unvaccinated pigs. LNC were isolated and were exposed to homologous subtypic SIV/SK02, homologous antigenic variant H1N1 SIV/Ind88 and heterologous subtypic H3N2 SIV/Tx98 antigens. The antigen-specific response was measured by an IFN-γ ELISPOT assay. As shown in Figure 5.4, both doses of R345V vaccine were able to induce significantly higher numbers of antigen specific IFN-γ secreting cells compared to the control group after stimulation with SIV/SK02. Furthermore, LNC from R345V vaccinated animals secreted IFN-γ after stimulation with antigenic subtype H1N1 SIV/Ind88 and heterologous H3N2 SIV/Tx98 antigens. Thus, both vaccine doses were able to induce cross-reactive cell-mediated immune responses. Statistical analysis showed that there was a significant difference in cell mediated immune responses between vaccinated and control groups. However, there was no statistically significant difference in the number of IFN-γ secreting cells between the two vaccinated groups (Figure 5.4).

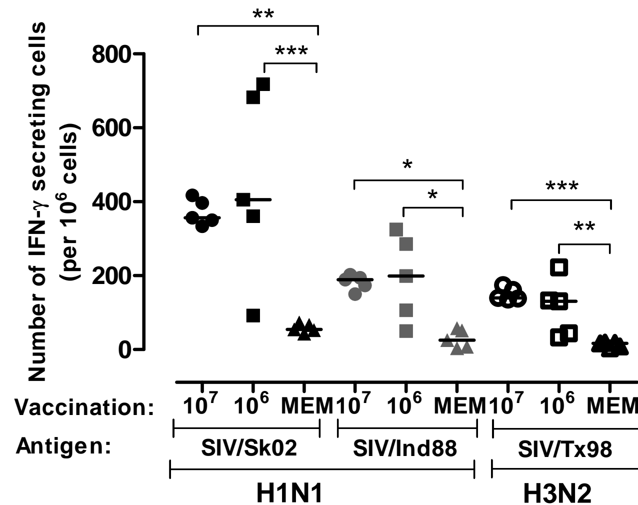


Figure 5.4 Cell mediated immune response induced by mutant virus. Numbers of SIV-specific IFN-γ secreting cells in pigs after two IN vaccinations with R345V 10^7 and R345V 10^6 and challenge with H1N1 SIV/Sk02, as measured by ELISPOT. The results are reported as the average number of spots observed in wells with SIV antigen stimulated LNC (seeded in triplicates) minus the average number of spots in negative control wells (LNC simulated with media alone). Each data point represents an individual animal, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

5.3.6 Intranasal vaccination of R345V induced humoral immune responses.

We first examined the presence of SIV specific antibodies by HI assay from the sera that were collected pre-vaccination, pre-boost, pre-challenge and at necropsy. All pigs were negative for H1N1 and H3N2 SIV antibodies (HI <1:10) at the start of the experiment, and the five unvaccinated pigs remained seronegative to all tested antigens (SIV/Sk02, SIV/Ind88 and SIV/Tx98) during the course of study. After the first intranasal vaccination (day 21), only 2 out of 5 pigs in each vaccinated group seroconverted to the SIV/Sk02 antigen with low levels (HI titer = 10). Following the second intranasal vaccination (day 31), all pigs in both vaccinated groups seroconverted to the parental SIV/Sk02 antigen with moderate titres (median HI = 80 in high dose group and 20 in low dose group). On 5 d.p.i., both vaccinated groups had statistically significant median titres greater than that in the unvaccinated/challenged group (HI titers of both groups were 160) (Figure 5.5A).

Next, we examined whether intranasal vaccination with R345V induced antigen specific IgG and IgA in sera and the respiratory mucosa by ELISA using inactivated whole H1N1 SIV/Sk02 virus as the capture antigen. As shown in Figure 5.5B, both R345V vaccine doses induced similar levels of serum IgG after the first vaccination (median titer = 245 in high dose group, and 264 in low dose group). Moreover, the second vaccination led to a considerable increase in the level of antigen specific IgG (median titer = 966 in high dose group and 797 in low dose group). The IgG titers in both vaccinated groups continued to rise after the challenge and on 5 d.p.i. reached the following values: median titer = 1967 in high dose group and 2313 in low dose group. In unvaccinated/challenged group, 4 out of 5 pigs seroconverted to SIV-SK02 with median group titer of 91.

Nasal swabs and BALF samples were assessed by ELISA for the presence of IgA antibodies specific to H1N1 SIV/Sk02 at mucosal surfaces in the upper and lower respiratory

tract. Figure 5.5C shows that after the first vaccination the antigen-specific IgA titers in nasal mucosa were low (median titer = 29.5 in high dose group and 11 in low dose group). Second intranasal vaccination resulted in an increased IgA titer to 83.4 (high dose group) and 79.6 (low dose group) while on 5 d.p.i. IgA titers rose significantly to value of 347.3 (high dose) and 417.5 (low dose). BALF was collected at necropsy on 5 d.p.i. As shown in Figure 5.5D, the IgA titres were significantly higher in both vaccinated groups compared to the unvaccinated control group (median titer = 2209 for high dose; 2598 for low dose and 22 for unvaccinated group). Altogether, there was a statistically significant difference in antigen specific serum IgG and mucosal IgA between vaccinated (groups 1 and 2) and unvaccinated groups (group 3). There was no significant difference between the animals in two groups vaccinated with different doses of R345V (group 1 and 2)

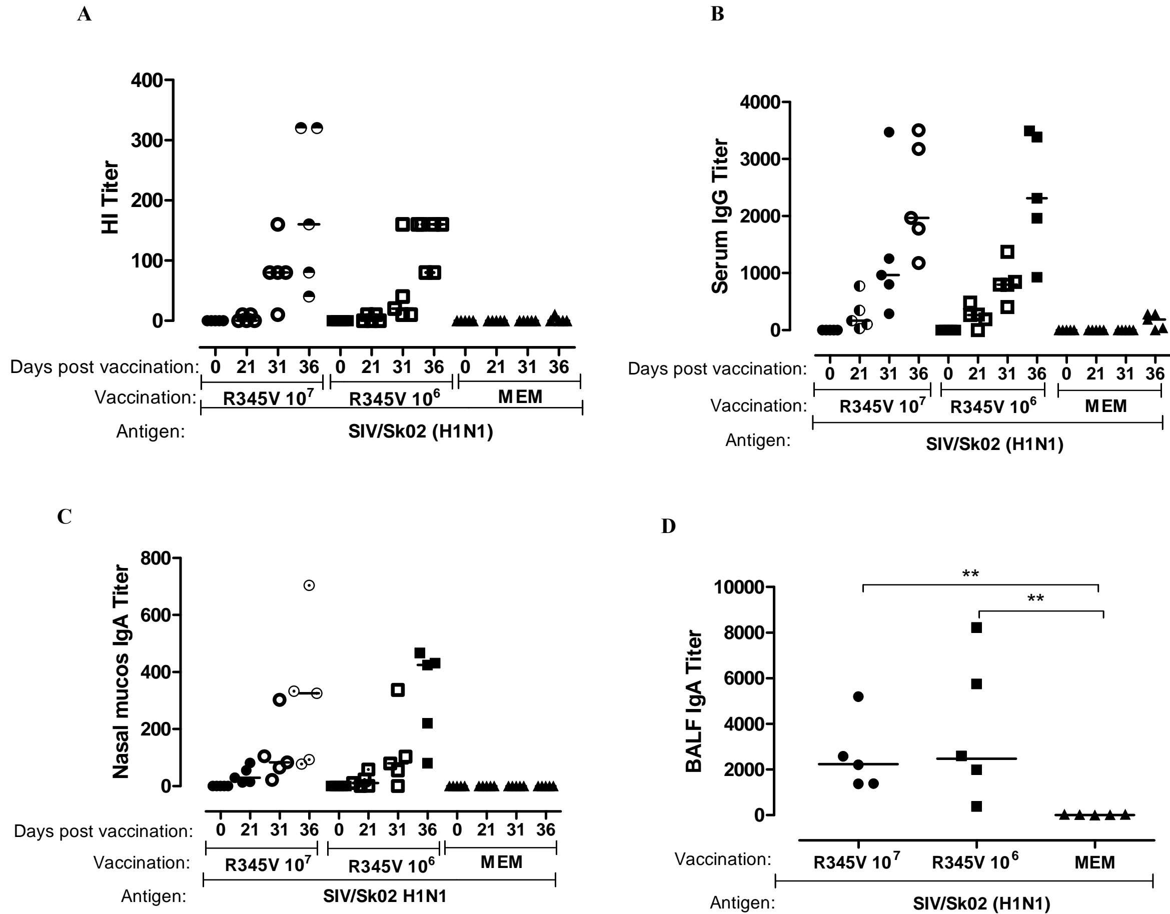


Figure 5.5. SIV specific antibodies induced by the IN vaccination of R345V mutant virus. H1N1 SIV/Sk02 specific HI (A) and IgG (B) levels induced by R345V 10^7 and R345V 10^6 detected in serum after the first, the second vaccination and after the challenge. Upper and lower respiratory mucosal IgA antibody titers from nasal swabs and BALF (C and D) were also determined. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

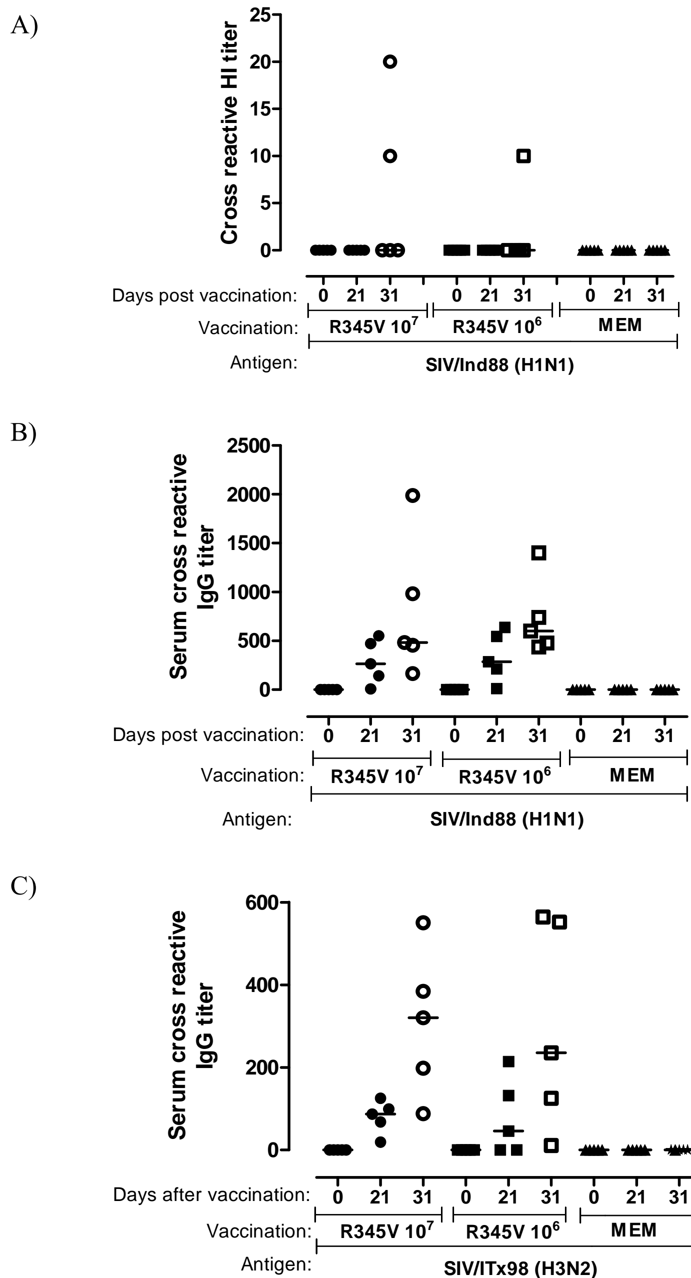
5.3.7 Intranasal vaccination of R345V induced cross-reactive antibodies in sera and respiratory mucosa.

The presence of cross-reactive antibodies in serum, nasal and respiratory mucosa was measured by using purified UV-inactivated H1N1 SIV/Ind88 and H3N2 SIV/Tx98 viruses as capture antigens in HI and ELISA assays. We could not detect any HI cross-reactive antibodies to the SIV/Ind88 H1N1 antigen after one vaccination in either vaccinated groups. After the second vaccination, SIV/Ind88 seroconversion was detected in 2 out of 5 pigs in the high dose vaccination group and 1 out of 5 pigs in low dose vaccination group (Figure 5.6A). However, there were no detectable HI antibodies against SIV/Tx98 H3N2 during the time of experiment (Data not shown).

Similar to HI titers, in both vaccinated groups a gradual increase in serum IgG recognizing H1N1 SIV/Ind88 was detected (Figure 5.6B). After the first vaccination the detectable levels of serum cross-reactive IgG were low (median titer = 166 in high dose group and 185 in low dose group), however the second vaccination led to an increase of serum IgG in both vaccinated groups (median titer = 483 in high dose group and 600 in low dose dose) (Figure 5.6B). A similar pattern was observed for the serum IgG recognizing H3N2 SIV/Tx98. After one intranasal vaccination, median group titers for H3N2 cross-reactive IgG antibodies in the two vaccinated groups were very low (titer = 87 in high dose group and 46 in low dose group), but increased after the second vaccination to titers of 320 and 235 respectively (Figure 5.6C).

Seroconversion kinetics and levels of cross-reacting antibodies to both H1N1 antigenic variant and H3N2 antigens in nasal mucosa are shown in Figure 5.6D and E. One intranasal vaccination with R345V induced very low IgA titre to SIV/Ind88 and SIV/Tx98. After the second vaccination (day 31) 4 out of 5 animals in each vaccinated group seroconverted to H1N1 and H3N2 antigens, reaching the following median titers: H1N1 SIV/Ind88 median titer was 32 in high dose group; 55.5 in low dose group and H3N2 SIV/Tx98 titer was 28.8 for high dose

group and 35.2 for low dose group (Figure 5.6D and E). Compared to the low level of IgA in nasal mucosa, significantly higher levels of H1N1 and H3N2 cross-reactive IgA antibodies were detected in the lower respiratory mucosa (BALF). At necropsy (36 days post vaccination, 5 d.p.i.) IgA antibodies recognizing H1N1 SIV/Ind88 antigen in BALF reached titers of 391 in the high dose group and 469 in the low dose group (Figure 5.6F). Similarly, median group IgA antibody titers against H3N2 SIV/Tx98 in lower respiratory mucosa were 303 in the high dose group and 393 in the low dose group (Figure 5.6G).



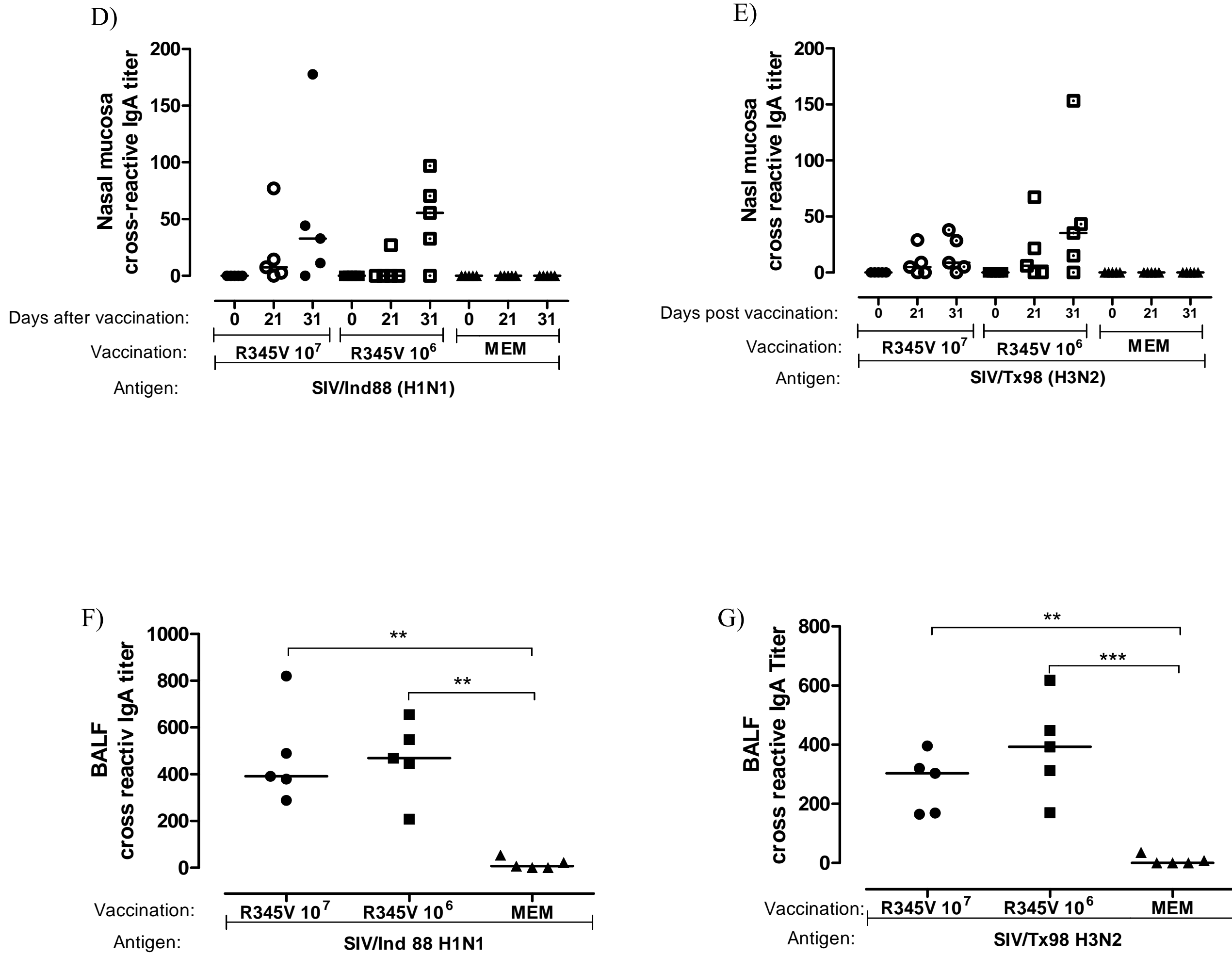


Figure 5.6 Cross reactive antibody induced by R345V virus. Serum HI (A), IgG (B) and mucosal IgA (D and F) that cross reacting to H1N1 SIV/Ind88 and serum, IgG (C) and mucosal IgA (E and G) that cross reacting to H3N2 SIV/Tx98 were determined after the second immunization. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001.

5.3.8 Protection efficacy of intranasal administration of R345V against H1N1 antigenic variant and heterologous subtypic H3N2 SIV strains.

Our above results showed that both R345V vaccine doses were immunogenic and protective against parental H1N1 SIV/Sk02 challenge in pigs. Data comparison showed no remarkable differences with respect to clinical signs, gross lesions, virus load, serum and mucosal antibodies or cytokine levels in pigs vaccinated with 1×10^7 PFU/ml or 5×10^6 PFU/ml of R345V. Since more consistent reduction in microscopic lung pathology was observed after challenge with SIV/Sk02 in the group vaccinated with 1×10^7 PFU/ml of R345V compared to the group vaccinated with 5×10^6 PFU/ml, we decided to use the $1x10^7$ PFU/ml dose as a vaccine inoculum in our next challenge study.

Twenty-seven SIV negative pigs were assigned to 5 groups and housed separately during the time of study. Pigs in designated groups were vaccinated intranasally two times with a three-weeks interval. Ten days after the second vaccination, pigs were challenged with either H1N1 SIV/Ind88 or H3N2 SIV/Tx98 viruses (Table 5.2). During the 5 days after virus challenge, fever and mild respiratory signs such as nasal discharge, and sneezing were observed only in the unvaccinated/SIV/Ind88 challenged group of pigs (group 1). The R345V vaccinated/SIV/Ind88 challenged group (group 2) as well as unvaccinated/mock challenged group (group 5) did not show any of the characteristic respiratory symptoms of SIV infection. Furthermore, no SIV characteristic clinical signs were observed during the 5 days of observation in H3N2 challenged pigs (groups 3 and 4).

On 5 d.p.i. pigs were humanely euthanized and necropsies were performed. Typical SIV gross lesions characterized as marked, purple-red colored, consolidated areas were observed on lung lobes of all unvaccinated/challenged pigs (Figure 5.7A). Again, the most severe lesions were found in the apical and cardiac lobes. While vaccinated/SIV/Ind88 challenged pigs (group 2) had only mild gross lungs lesions, vaccinated/ SIV/Tx98 challenged pigs showed mild to moderate lung gross lesions, but the median lung lesion scores of this group (group 4) was significantly lower than its corresponding unvaccinated/challenged group (group 3). Statistical analysis revealed that there was a significant reduction in lung scores between vaccinated/challenged groups (groups 2 and 4) compared to their corresponding

unvaccinated/challenged groups (groups 1 and 3) (Figure 5.7A). Unvaccinated/mock challenged pigs (group 5) were free of macroscopic gross lesions (data not shown).

Histopathological changes in lungs of pigs in all groups were in agreement with the macroscopic lesions. Unvaccinated/SIV/Ind88 challenged pigs had the most severe histopathological changes characterized by moderate to severe bronchointerstitial pneumonia with moderate subacute necrotizing bronchiolitis (Figure 5.7B). The exception in this group was one animal that had milder lesions with mild interstitial pneumonia and no evidence of bronchiolar necrosis. In contrast, in vaccinated/SIV/Ind88 challenged group, 4 out of 6 pigs showed mild bronchointerstitial pneumonia with moderate to severe peribronchiolar inflammation. The two remaining pigs showed moderate bronchointerstitial pneumonia with mild bronchiolar necrosis.

Similarly, in the unvaccinated/SIV/Tx98 challenged group, pigs' lung sections had changes typical for moderate to severe subacute bronchointerstitial pneumonia with severe peribronchiolar inflammation, atelectasis, bronchiolar necrosis and neutrophil infiltration in alveolar spaces. In the vaccinated SIV/Tx98 challenged group, 3 out of 6 pigs showed moderate bronchointerstitial pneumonia with prominent locally extensive peribronchiolar and alveolar lymphoid inflammation and mild focal bronchiolar necrosis. Damage of bronchial epithelial in the remaining three pigs corresponded to the mild bronchointerstitial pneumofronia without bronchiolar necrosis observed.

5.3.9 Virus load in lungs was reduced by vaccination with SIV LAIV.

Consistent with the results obtained from necropsies and histopathology, viruses were recovered from all unvaccinated/SIVs challenged pigs . In contrast, we only recovered the virus from 1 out of 6 vaccinated /SIV/Ind88 challenged pigs (titer is 10^3 $TCID_{50}$/gr) and from 3 out of 6 vaccinated/H3N2 challenged pigs (Figure 5.7C). The median virus titers from unvaccinated/SIV/Ind88 challenged group and unvaccinated/H3N2 challenged group were $10^{5.3}$ $TCID_{50}$/gr and $10^{5.1}$ $TCID_{50}$/gr, respectively. The median virus titer from vaccinated/H3N2 challenged pigs was $10^{1.4}$ $TCID_{50}$/gr, was significantly lower than that of unvaccinated/H3N2 challenged group ($10^{5.1}$ $TCID_{50}$/gr). Virus could not be detected in the lungs of mock challenged pigs.

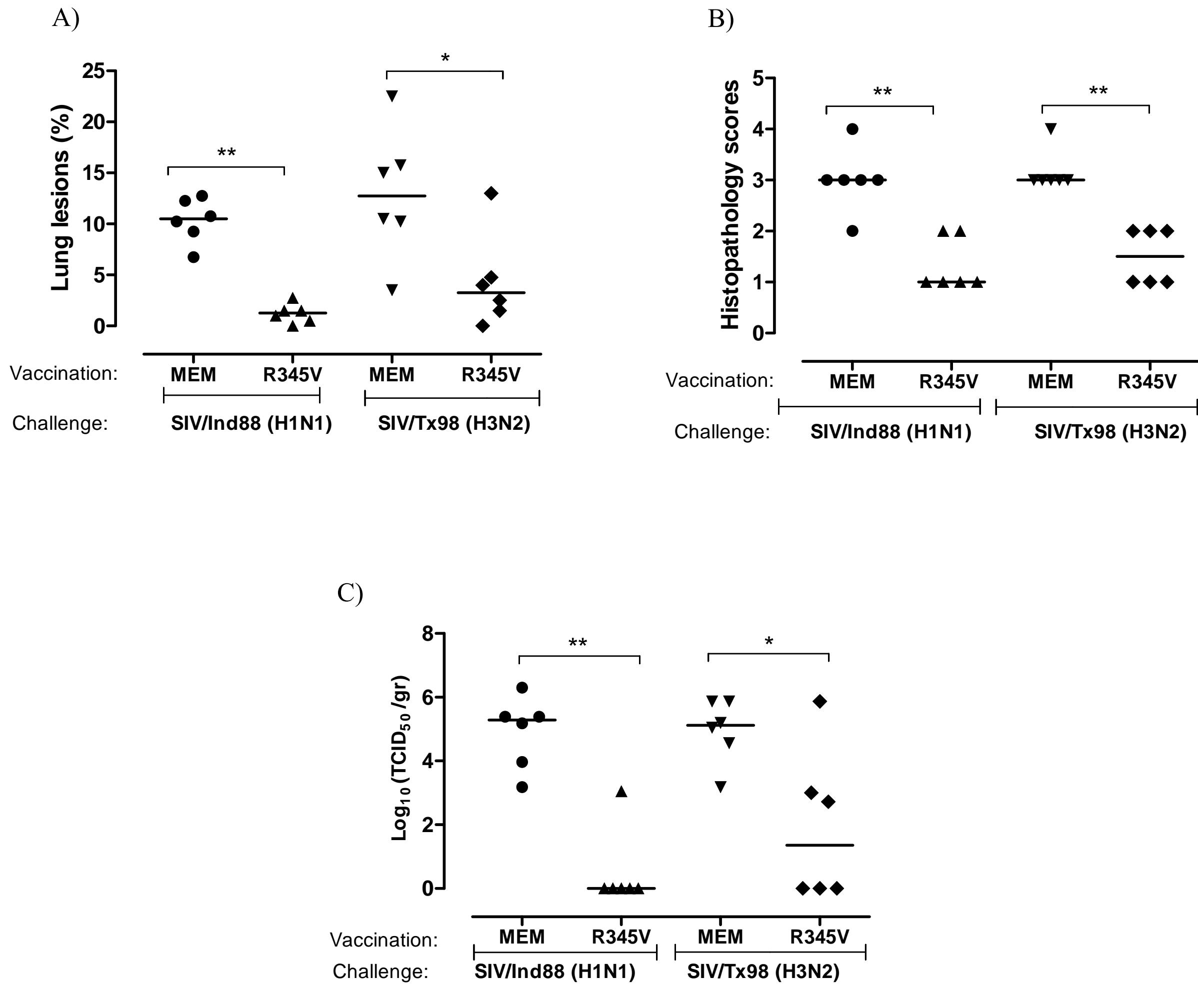


Figure 5.7 Macroscopic, microscopic lung lesions and virus titers in vaccinated (R345V) and unvaccinated (MEM) animals challenged with H1N1 antigenic variant and heterologous subtypic H3N2 SIVs. (A) The percentage of the areas affected with pneumonia was estimated visually for each lung lobe. Total percentage for the entire lung was calculated based on weight proportions of each lung lobe to the total lung volume. Results are the median score of lung lesions of six animals in each group. *P<0.05; **P<0.01; ***P<0.001. (B) Tissue sections of lungs were routinely stained with hematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or extent of lesions within the sections examined as follows: 0: no visible changes; 1: mild focal or multifocal change; 2: moderate multifocal change; 3: moderate diffuse change; 4: severe diffuse change. (C) Lung virus titers. Lung tissues from right apical, cardiac and diaphragmatic lobe were collected, homogenized and virus titers were determined on MDCK cells. Titers are calculated according to the Reed and Muench method. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *P<0.05; **P<0.01; ***P<0.001

5.3.10 Serum and mucosal antibody titers after challenge

The levels of antibodies in serum, upper and lower respiratory mucosa of vaccinated pigs were determined after challenge with homologous antigenic variant H1N1 SIV/Ind88 or heterologous virus H3N2 SIV/Tx98. Cross-reactive IgG antibodies in serum to SIV/Ind88 reached the median group titer of 1232 after challenge with H1N1 SIV/Ind88, and the final median titer of cross-reactive antibodies to H3N2 SIV/Tx98 virus reached value of 447 after the challenge with corresponding SIV (Figure 5.8A). Cross-reactive IgA from nasal mucosa in both vaccinated groups rose to a median titer of 137 after challenge with SIV/Ind88, and to 75 after challenge with H3N2 SIV/Tx98 (Figure 5.8B). All vaccinated and H1N1 or H3N2 challenged pigs had considerable levels of cross-reactive IgA in the lower respiratory tract. The median group IgA titer for pigs vaccinated/challenged with H1N1 SIV/Ind88 was 852, whereas pigs that were vaccinated/challenged with H3N2 SIV/Tx98 had a median IgA group titer of 436 (Figure 5.8C).

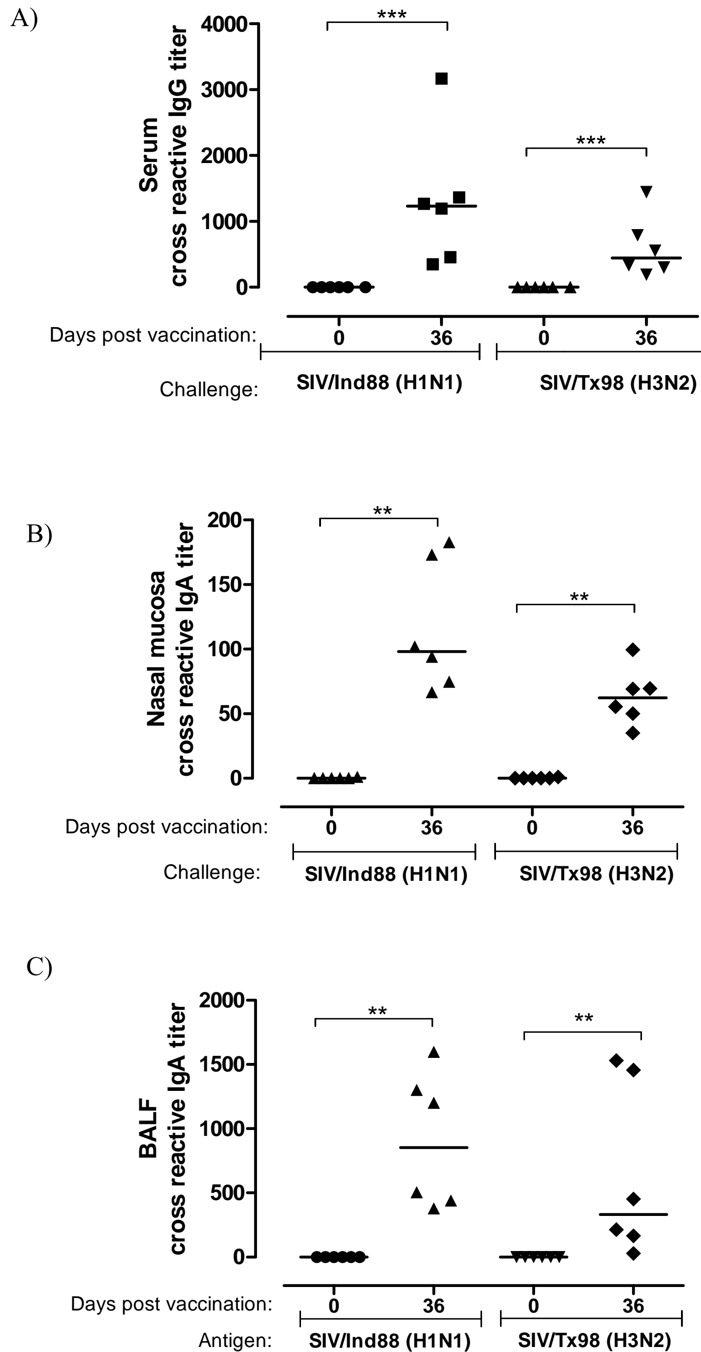


Figure 5.8. Antibody titres after vaccination and challenge. Serum IgG (A) and mucosal IgA titers from nasal passages (B) and BALF (C) specific for H1N1 SIV/Ind88 and H3N2 SIV/Tx98 were examined after pigs were vaccinated twice with R345V and challenged with WT SIVs. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars.

5.4 DISCUSSION

Influenza virus HA is initially synthesized as a precursor HA0 (Skehel and Wiley 2000). In order for virus to be infectious, HA0 must be cleaved by host proteases into HA1 and HA2 subunits (Steinhauer 1999). Previously we have generated a mutant SIV-R345V whose HA cleavage site was mutated from the original trypsin-sensitive cleavage site (Arg-Gly) to an elastase-sensitive (Val-Gly) cleavage site. Attenuated replication is achieved due to deficiency of a proper protease at the site of infection results in no cleavage of influenza virus HA0, thus preventing fusion of progeny viruses with the endosome which in turn restrain virus replication (Masic, Babiuk et al. 2009). When administered two times intratracheally, this virus elicited cell mediated and humoral mediated immune responses, which provided complete protection for homologous SIVs challenge and partial protection from heterologous SIV challenge (Masic, Booth et al. 2009). Since intratracheal administration is technically demanding for mass herd vaccination under field conditions or at large swine farms, we decided to test the immunogenicity and protection efficacy of R345V after an intranasal route of administration, a more practical way of vaccination.

In both animal trials, our R345V LAIV administered twice via the intranasal route, showed significant protection capability against three antigenically different SIVs. The protection was assessed by the presence and severity of SIV characteristic clinical signs, macroscopic and microscopic lung lesions, virus titers in lungs and levels of pro-inflammatrory cytokines. In general, all vaccinated/challenged pigs showed significant reduction in assessed protection parameters compared to unvaccinated/challenged groups. R345V was able to provide complete protection against parental homologous SIV/Sk02 (H1N1) and significantly high degree of heterologous protection to SIV/Ind88 (H1N1) and SIV/Tx98 (H3N2).

In the first trial, we evaluated the immunogenicity and protective ability against parental WT virus challenge with two different doses of R345V vaccine administered intranasally. Serum and mucosal anti-influenza virus antibodies are the major components of humoral immunity that protect the host from influenza infection (Burlington, Clements et al. 1983). Cell-mediated immunity promotes virus clearance and is an important factor in recovery from influenza virus infections (Thomas, Keating et al. 2006). While one intranasal vaccination induced low levels of antigen-specific serum HI, IgG and IgA in nasal secretions and lungs, the second vaccination resulted in a significant increase of serum antigen-specific HI and IgG; and antigen-specific IgA

at respiratory mucosa (Figure 5.5). Challenge with homologous parental SIV/Sk02 led to further increases in antigen-specific HI, IgG and IgA antibody titers suggesting that priming of immune cells by two times vaccination of R345V did not reach its maximum. This could be explained by the restricted replication cycles of our R345V and short antigen exposure.

The induction of cell-mediated immune responses were assessed by measuring the number of IFN-γ secreting cells from tracheo-bronchial lymph nodes determined by ELISPOT (Fig. 4). IFN-γ produced by $CD4^+$ T helper cell type 1 (Th1) lymphocytes, $CD8^+$ cytotoxic lymphocytes and NK cells (Bach, Aguet et al. 1997), is the major immuno-modulator that shapes the immune responses and establishes an antiviral state (Schroder, Hertzog et al. 2004). IFN-γ secretion by NK cells and possible by professional antigen-presenting cells is likely to be important in early host defense against infection, whereas T-lymphocytes become the major source of IFN-γ in adaptive immune response (Boehm, Klamp et al. 1997). In our previous study one intratracheal vaccination with R345V was able to induce significant numbers of local IFN-γ secreting cells but a two-fold increase in IFN-γ responses was achieved after the second vaccination (Masic, Booth et al. 2009). Similarly, in the current study we could detect significant numbers of antigen-specific local IFN-γ secreting cells after two intranasal vaccinations followed by SIV challenge. Both vaccine doses (high dose and low dose) induced similar numbers of IFN-γ secreting cells in vaccinated pigs and they were not statistically different from each other. The detection of the IFN-γ secreting cells (median number of cells = 54.3) from the pigs in the control group (unvaccinated/SIV/Sk02 challenged) could be due to the initial priming of immune cells at the site of infection after challenge and subsequent re-exposure to the same antigen during the ELISPOT assay which may mimic a second immunization.

The capacity of intranasally administered R345V to induce cross-reactive cell-mediated responses and antibodies against antigenic variant H1N1 SIV/Ind88 and heterologous H3N2 SIV/Texas98 were evaluated. The lymph node cells from R345V vaccinated pigs were stimulated with either inactivated H1N1 SIV/Ind88 or H3N2 SIV/Tx98. Considerable numbers of cross-reactive IFN-γ secreting cells were detected in all vaccinated groups compared to unvaccinated controls (Figure 5.4). Two intranasal vaccinations with R345V were able to induce moderate levels of serum IgG and mucosal IgA antibodies that were cross reactive to H1N1 Ind88 and H3N2 Tx98 (Figure 5.6). Furthermore, we detected low HI antibody titers (≤20) cross-reactive only to H1N1 Ind88 virus while HI antibodies to H3N2 Tx98 virus were below

detectable limit of the assay. Detection of cross-reacting IFN-γ secreting cells, and cross-reactive antibodies in serum and mucosal surfaces, especially IgA induced by the R345V at the respiratory mucosa suggested that vaccination with R345V via the intranasal route has the potential to provide heterosubtypic cross-protection against SIVs.

A second study was conducted to assess whether intranasal vaccination would protect against an antigenically different H1N1 and heterologous H3N2 SIV infection. Data obtained from the first trial showed that both $1x10^7$ and $5x10^6$ pfu/ml R345V vaccine doses induced cellular and humoral immunity; and both doses demonstrated similar protection capability to parental SIV infection. Statistical analyses and comparison of data from all conducted assays showed no significant differences in clinical signs, gross lesions, lung virus load, antigen-specific and cross-reactive antibodies in pigs vaccinated with the 1×10^7 PFU/ml and 5×10^6 PFU/ml of R345V dose. Since there was less individual variation between animals vaccinated with higher dose, suggesting this dose induced more consistent immune response than did the low dose, and epithelial lung injury after the challenge with WT SIV/Sk02 was less severe in the group that received the higher vaccine dose than those that received the lower dose, we decided to use dose of $1x10^7$ PFU/ml of R345V in the cross-protection study.

Similar to previous studies (Vincent, Ma et al. 2007; Masic, Booth et al. 2009), we could detect the presence of a significant cross-reactive IgG in serum and IgA at respiratory mucosa after two intranasal vaccination and challenge with corresponding WT SIVs (Figure 5.8). These results suggested that the intranasally administered R345V could prime B cells to produce antibodies against influenza viral antigens. In addition, the pre-existence of significant levels of IgA and IgG antibodies cross-reacting to H1N1 antigenic variant and H3N2 SIV could be responsible for the reduction in virus shedding and replication in the lungs (Figure 5.7). The importance of cross-protective immunity after experimental or natural infection with antigenically unrelated influenza A viruses has been described in several animal models (Schulman and Kilbourne 1965; Webster and Askonas 1980; Reeth, Brown et al. 2004). Several reports suggest that pre-existing antibodies are one of the mediators of immunity mechanisms. Particularly, cross-reactive IgA antibodies induced at the respiratory mucosa after natural infection or vaccination with live vaccines are involved in the protection from homologous and heterologous subtypic influenza A viruses challenge (Liew, Russell et al. 1984; Vincent, Ma et al. 2007)

The reversion to its pathogenic phenotype of live attenuated viruses is the major concern for their use as live vaccines. Theoretically, the absence of an appropriate protease required for replication of R345V *in vivo* results in limited virus replication thus resulting in the virus attenuation. The main advantage of the short and restricted replication is the reduced chance of any reversion and reassortment between wild type virus and vaccine virus. Even though current intranasal vaccination requires two administrations the benefit of the elastase-dependent live attenuated virus is its ability to induce considerable humoral and cell mediated immune responses. Moreover, intranasal vaccination with R345V resulted in the significant reduction of homologous and heterlogous subtypic SIV virus load and pathogenesis. Importantly, the cross-protective immunity induced by elastase-dependent R345V virus is an important property that might have a considerable impact on the epidemiology of new variant SIVs by reducing viral shedding and limiting spread. Another significant aspect of this study is that SIV infection of pigs with classical H1N1 (SIV/Ind88) or triple reassortant H3N2 (SIV/Tx98) was prevented or reduced by R345V which is derived from a novel H1N1 SIV with an avian genetic background (Karasin, West et al. 2004).

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

The goal of this study was to investigate the potential of developing efficient cross-protective LAIV against different SIV subtypes by reverse genetics. When the study began, no LAIV approved for pigs were available although various candidate vaccines have been tested (Richt, Lekcharoensuk et al. 2006). Current SIV vaccines are inactivated and adjuvanted whole virus preparations that are subtype specific and have limited protection efficacy (Brown 1994). The main advantage of LAIV compared to their inactivated counterparts is induction of both CMI and humoral immunity at the site of infection that resemble natural infection (Cox, Brokstad et al. 2004). In addition, the activation of CMI and strong mucosal antibody response are crucial in developing long-lived cross-protective immunity against influenza A infections.

Pigs can serve as an intermediate hosts for adaptation of AIV to humans or “mixing vessels” for the genetic reassortment between SIV, AIV and hIV that could lead to emergence of novel influenza virus subtypes. Therefore, the focus on inducing broad cross-protection in pigs has important public health implications and it is believed that reduction in spread of different SIV subtypes would indirectly decrease the occurrence of pandemic viruses and consequently human influenza disease.

Cleavage of influenza A virus HA0 polypeptide into HA1 and HA2 subunits by trypsin-like proteases is the crucial step for virus infectivity, pathogenicity and spread of infection. Stech and colleagues (Stech, Garn et al. 2005) demonstrated that the conversion of HA trypsin-sensitive to elastase-sensitive cleavage site does not alter replication or growth abilities of mutant viruses in tissue culture in the presence of elastase. However, this modification resulted in effective attenuation of the influenza A virus in *in vivo* mice models. Theoretically, *in vivo* attenuation is due to the lack of an appropriate protease at the site of infection. This will result in the influenza virus uncleaved HA, thus disabling fusion of progeny viruses with the endosome and blocks virus replication. Similarly, we decided to apply the same attenuation strategy towards development of LAIV for SI in pigs.

First, by using reverse genetics technique we generated two elastase-dependent mutant SIVs. Both SIV/R345V and SIV/R345A had mutations within HA cleavage site making it susceptible to human neutrophil elastase and porcine pancreatic elastase respectively. However, both mutant viruses could be rescued only in the presence of human neutrophil elastase.

Although SIV/R345A was able to grow in the presence of pancreatic elastase, it contained the additional mutation in the HA segment at position AA344 (Pro^{344}-Ala-Gly instead of Ser^{344}-Ala-Gly) which was most likely the result of adaptation to selection pressure of pancreatic elastase during the multiple passages in tissue culture.

In order to be considered as live vaccine candidate, genetically modified live virus should be able to maintain wild type phenotype (genetically stable, proteolytic activation, growth properties, pathogenicity, infectivity) and be able to induce immune response similar to that observed after natural infection. We characterized two mutant viruses comparing their, multicycle growth properties, protease dependence, pathogenicity and genetic stability to the wild type parental SIV in *in vitro* conditions. Both R345V and R345A viruses were solely dependent on neutrophil elastase activation and grew to equivalent titers in the presence of the appropriate protease as the WT virus. Furthermore, tissue culture grown R345V and R345A SIVs were genetically stable and able to infect cells, synthesizing similar quantities of viral proteins as the parental virus. These findings showed that the alteration of the cleavage site did not change wild type phenotype of both mutants in tissue culture when the appropriate protease was added. Most importantly, after testing the pathogenic abilities of these mutants in pigs (at high and low virus dose), we could not detect significant presence of SIV characteristic clinical signs, macroscopic, microscopic lesions and virus particles in the lungs of challenged animals. These observations suggested that both R345V and R345A were highly attenuated in pigs and that they could serve as potential LAIV for SIV in pigs.

Natural immunity to SIV depends on the activation of both cell-mediated and humoral responses triggered by the virus replication. Multiple factors such as virus pathogenicity, virus load, length of disease acute phase and innate immunity contribute to the strength and duration of natural immunity. Since both of our vaccine candidates were capable of completing a limited number of replication cycles *in vivo*, we assessed their immunogenic properties in pigs after one and two IT administration to check if they were capable of inducing any immune response. Immunogenicity of R345V and R345A was determined based on the capability of mutant viruses to induce both cell-mediated and humoral immune responses at the site of infection/inoculation. As a readout for induction of CMI we used IFN-γ ELISPOT and LPR. IFN-γ is the major immuno-modulator that coordinates the immune responses and establishes a long lasting antiviral state (Schroder, Hertzog et al. 2004). As shown in section four (4.3.1), both viruses

were able to induce considerable numbers of IFN-γ secreting cells and lymphocyte proliferation in response to SIV specific antigen. However, a second vaccination resulted in significantly better CMI than only one IT administration of R345V or R345A. Most likely one vaccination is not sufficient to induce maximum immune responses, possibly due to only one or limited replication cycles of mutant SIVs *in vivo*. Similarly to CMI, the induction of a humoral immune response in the serum and upper and lower respiratory mucosa was low or undetectable after only one vaccination in the majority of tested pigs. However, the levels of serum HI, IgG and IgA as well as mucosal IgA and IgG (from BALF and nasal passages) were significantly increased after a second vaccination in both R345V and R345A vaccinated groups. Although both R345V and R345A induced immunity to SIV to similar levels, comparison of collected data on immunogenicity from two vaccine candidates showed that R345V induced more consistent and slightly better immune responses. Furthermore, R345V was able to induce higher levels of serum and mucosal antibodies cross-reactive to antigenically distinct H1N1 and H3N2 SIV subtypes. Therefore, R345V was selected as a candidate for LAIV against SIV and its protection ability was further assessed.

As demonstrated in section 4.3.4-4.3.7, two vaccinations with R345V via IT route were sufficient to provide complete protection of pigs against homologous subtypic H1N1 SIV/Sk02 and H1N1 variant SIV/Ind88. However, R345V vaccinated pigs were only partially protected against heterologous subtypic H3N2 SIV/Tx98. The protection capability of R345V was assessed by determining the severity of clinical signs, percentage of gross and microscopic lung lesions, virus titers in lungs and levels of pro-inflammatroy cytokines after the challenge with wild type SIVs. All vaccinated animals showed significant reduction in all tested disease parameters compared to unvaccinated and challenged animals. In addition, serum and mucosal antibody responses in vaccinated pigs were high and correlated with previous reports that evaluated immune responses and protection ability to infection with SIV in pigs (Larsen, Karasin et al. 2000; Heinen, van Nieuwstadt et al. 2001).

The results using R345V as LAIV were encouraging for testing its immunogenicity and protection ability via the more feasible IN route. IN vaccination could be a choice for mass herd vaccination since it enables use of smaller volumes and antigen delivery similar to natural infection (Cox, Brokstad et al. 2004; O'Hagan and Rappuoli 2004).

Before we could test the efficacy of R345V LAIV via the IN route, we needed to

determine the IN vaccine dose. Previous results showed that two IT vaccinations were required for optimal immunity to SIV in pigs, thus IN vaccinations were performed twice. Unlike IT administration, IN vaccination does not deliver the entire vaccine dose to the major site of SIV replication (lungs). This is because the upper respiratory tract crosses with digestive tract via pharynx, the organ where the proximal part of the trachea and oesophagus begins, thus it is very likely that some of the vaccine will be delivered in the oesophagus and to the digestive tract decreasing vaccine efficacy. Therefore, we tested two different high vaccine doses of $1x10^7$ and $5x10^6$ PFU/ml to minimize possible vaccine failure due to the improper/mistargeted delivery. In addition, we assessed the immunogenicity and protection ability for both vaccine doses to homologous SIV after IN administration.

Consistent with previous observations (chapter 4.), IN administered R345V (at lower and higher dose) was capable of inducing CMI measured by the IFN-γ ELISPOT. Moreover, both vaccine doses of R345V given IN were sufficient to induce cross-reactive CMI to antigenically distinct H1N1 and H3N2 SIV antigens. Humoral immune responses measured in serum (HI, IgG) and mucosal surfaces (IgA), were undetectable to low after only one IN administration of R345V, while the re-vaccination via the same route resulted in a significant increase of SIV specific antibodies. In addition, both doses of R345V administered IN induced mucosal IgA and serum IgG cross-reactive to antigenically distinct H1N1 and H3N2 SIV antigens. Although we could not see any differences in immune responses between the two doses of R345V, results from histopathology that assessed the severity of microscopic lung injury after two IN vaccinations and challenge with wt homologous virus showed slight differences. Microscopic lesons in lower dose vaccinated and challenged group were characterized with mild to moderate interstitial pneumonia with severe peribronchial infiltration of PMN and neutrophils, consistent with exposure and immune response to SIV. In contrast, the higher dose vaccinated and challenged group showed mild histopathological changes, mostly mild peribronchial infiltration similar to one observed in the mock control group. Despite slightly increased lung histopathology, lower dose like the higher dose was sufficient to minimize SIV typical clinical signs and provide complete protection against parental SIV.

In agreement with the previous study (section 4), the IN administered R345V confered total protection against H1N1 antigenic subtype and partial protection against heterologous H3N2 SIVs. In addition, the antibody responses in serum and respiratory mucosa were at a

considerable high level while production of pro-inflammatory cytokines was significantly reduced in all vaccinated and challenged animals compared to unvaccinated-chalenged controls. However, the number of virus positive animals after challenge was higher than in the IT trial (section 4.) but still there was a significant reduction in virus titers compared to unvaccinated and challenged controls.

All together, our data showed that two administrations of R345V via IT or IN route are required to induce immune response and homologous and heterologous protection similar to one observed after natural SIV infection. Other LAIV vaccines can induce strong immune response after only one administration, which is attributed to the prolonged virus replication and antigen exposure(Townsend, Penner et al. 2001; Belshe 2004; Paillot, Hannant et al. 2006) . However, longer replication of vaccine strain extends the possibility for the influenza virus reassortment between vaccine and field strains. In our model, virus attenuation occurs most likely within the first 24 h.p.i and significantly reduces the timeframe for the possible reassortment with wild type field SIVs.

Limited virus replication and short antigen exposure were not sufficient to induce strong immunity after only one administration, but a second vaccination resulted in the desirable immunity and protection to three antigenically different SIVs. Our genetically modified and reverse genetics generated LAIV for SI can induce both cellular and humoral arms of adaptive immunity and provide broad cross-protection to anitgenically different SIVs, thus making it superior to current inactivated SI vaccines. In addition, the attenuation mechanism that enables only few replication cycles narrows the window for possible reversion or reassortment with wild type SIVs making this LAIV safer than other tested LAIV candidates. Another advantage of our R345V LAIV is that this mutant virus with avian-like genetic signature (parental SIV/Sk02 is wholly avian strain adopted to pigs) is capable of inducing strong cross-protective immunity to cH1N1 (SIV/Ind88 H1N1) and TR H3N2 (SIV/Tx98 H3N2). Moreover, in collaboration with Canadian Food Inspection Agencies- National Centre for Foreign Animal Diseases CFIA-NCFAD in Winnipeg, R345V has shown to be protective against current human pandemic quadruple reassortnat H1N1 (Mexico 2009 H1N1) virus in pigs (data not shown). All above confirmed that powerful cross-protective LAIV against SI such as R345V is highly desirable for not only controlling novel SIVs in pigs but for control of potential human pandemic strains that emerge from pigs.

6.2 GENERAL CONCLUSIONS

After summarizing and discussing all results shown in the chapters 3, 4 and 5 general conclusions would be:

- Genetically modified and reverse genetics generated R345V and R345A SIVs are strictly dependent on the presence of human neutrophil elastase in tissue culture.

- R345V and R345A mutant SIVs posses similar growth properties in terms of plaque size and growth kinetics when grown in tissue culture in the presence of human neutrophil elastase.

- Both R345V and R345A modified SIVs were highly attenuated in pigs after IT inoculation at doses of 10^5 PFU/ml and 10^6 PFU/ml.

- Two IT vaccinations with R345V or R345A are required to induce significant CMI and humoral immune responses and considerable levels of cross-reactive antibodies in serum and respiratory mucosa.

- Vaccination with R345V significantly reduces the presence of macroscopic and microscopic lung lesions, lung virus titers and pro-inflammatory cytokine responses in lungs of SIV infected pigs.

- Two IT given doses of R345V are sufficient to provide complete protection of pigs against homologous and antigenically distinct H1N1 SIV and partial protection from heterologous H3N2 SIV.

- Both doses 10^6 PFU/ml and 10^7 PFU/ml of R345V mutant virus are capable to induce humoral and CMI immune response after two IN administrations.

- Twice IN administered R345V at dose of 10^7 PFU/ml confer complete protection against homologous and antigenically distinct H1N1 SIV and partial protection from heterologous H3N2 SIV in pigs.

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