The Expression and Localization of the V and W Accessory Proteins in Porcine and Human Cells Infected with Nipah Virus

Comparing the Antiviral State and IFN signalling throughout a Nipah Virus Infection

Melissa Nalini Goolia

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

The Expression and Localization of the V and W Accessory Proteins in Porcine and Human Cells Infected with Nipah Virus

Comparing the Antiviral State and IFN signalling throughout a Nipah Virus Infection

BY

Melissa Nalini Goolia

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

Table of Contents

Acknowledgements

I would like to thank my supervisor, Dr. Hana Weingartl for the opportunity to carry out my research in her lab.

I thank my committee members, Dr. Heinz Feldmann and Dr. Julia Rempel for their advice and interest in this project. Their input and suggestions were extremely helpful in addressing many of my concerns throughout my degree.

This work would have not been completed without the continuous assistance and patience of Dr. Yohannes Berhane. I thank him for answering my daily questions, the advice, guidance and support throughout my degree.

Many people at the Canadian Food Inspection Agency have helped me in some way or the other throughout my stay here. I thank everyone who has done so, especially those in containment for all of the entertainment and everyone in the Special Pathogen Unit for the help. I would especially like to thank Jason Gren and Greg Smith for technical assistance, training and a tremendous amount of support and advice that helped me to survive the last couple of years.

In addition I would like to extend my gratitude to the Vesicular Disease Unit in CFIA, in particular, Dr. Alfonso Clavijo, Kate Hole and Tim Salo, for technical assistance, advice and support.

I thank Dr. Kevin Coombs and Dr. Joanne Embree for their much needed guidance and assistance.

I thank the Natural Sciences and Engineering Research Council of Canada (NSERC) and CFIA for funding to support this research project.

Finally, I would like to thank my parents and my brother for the overwhelming encouragement, patience and having faith in me over the years.

List of Tables

List of Figures

List of Abbreviations

2'5 OAS	2'5' Oligoadenylate Synthetase
βME	Betamercaptoethanol
AA	amino acid
AMEM	Alpha Modified Eagle's Medium
BEI	Binary Ethyleneimine
BP	Base Pairs
BSA	Bovine Serum Albumin
BSL4	Biosafety Level 4
C	Nipah Virus C protein
C terminal	Carboxy terminal
CMC	Carboxymethylcellulose
CMV	Cytomegalovirus
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
CSF	Cerebrospinal Fluid
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxynucleic Acid
DPI	Days post infection
dsRNA	Double standed RNA
EDTA	Ethylene Dinitrilotetracetic Acid
FBS	Fetal Bovine Serum
GMEM	Glasgow's Modified Eagle's Medium
HeV	Hendra Virus
HRP	Horse Radish Peroxidase
IFA	Immunofluorescence Assay
IFN	Interferon
INFAR	Interferon alpha receptor
IPTG	Isopropyl β-D-1 Thiogalactopyranoside
IRF3	Interferon Regulatory Factor 3
ISGF	Interferon Stimulated Gene Factor
ISRE	Interferon Stimulated Response Element
JAK	Janus Kinase
JEV	Japanese Encephalitis Virus
KB	Kilobases
LB	Luria-Bertani
MAb	Monoclonal antibody
MOI	Multiplicity of Infection
MW	Molecular Weight
N	Nucleoprotein
N terminal	Amino terminal
NiV	Nipah Virus
NLS	Nuclear Localization Signal

P	Phosphoprotein
p38-MK2	p38 mitogen activated kinase 2 pathway
P58	Anti-Nipah P antibody
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
PKR	Protein Kinase R
PK15	Porcine Kidney Cells
PRR	Pathogen Recognition Receptor
PT K75	Porcine Turbinate Cells
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SJPL	St. Jude's Porcine Lung Cells
ST	Swine Testis
STAT1	Signal Transducer and Activator Transcription
TBS-T	Tris Buffered Saline Tween-20
TCID50	Tissue Culture Infectious Dose 50%
TIR	Toll Interleukin Receptor
TLR3	Toll like Receptor 3
TRIF	Toll like Receptor Adaptor Molecule inducing IFN alpha
Tyk2	Tyrosine Kinase 2
U	Units
V	Nipah V protein
V76	Vero 76
VSV	Vesicular Stomatitis Virus
W	Nipah W protein

ABSTRACT

Nipah Virus (NiV) is a zoonotic pathogen in the *Paramyxoviridae* family which infects several species with different virulence. The virus p gene codes for three proteins (P, V, and W) stated to have STAT1 binding activity, indicating potential of NiV to evade the IFN-STAT1 signalling pathway.

The presented work shows that in vitro, NiV is sensitive to the antiviral action of IFN α, Comparison of human and porcine cells indicated that porcine cells established exogenous IFN-induced antiviral state at 18 hrs post treatment with 160 U/ml of IFN α while human cells needed 24 hrs and 329 U/ml.

In contrast to data published based on recombinant techniques, the intracellular localization and expression of NiV V/W by western blot and immunofluorescence detected the protein(s) and their co-localization with STAT1 only within the cytoplasm on infected porcine and human cells.

1. Introduction

1.1 Nipah Virus

1.1.1 Taxonomy

Nipah Virus (NiV) is a recently emerged and highly deadly pathogen classified within the *Paramyxoviridae* family, order called *Mononegavirales* (Mayo, 2002). The family is divided into *Pneumovirinae* and *Paramyxovirinae* subfamilies (Mayo, 2002) and contains many viruses that are significant human and animal pathogens. Measles, mumps, and human parainfluenza viruses are common human pathogens found in this family, and notable animal diseases include rinderpest, a disease of cattle and buffalo (Nanda & Baron, 2006; Baron, 2005), and Newcastle disease, a highly contagious disease among birds (Kite *et al*, 2007). The *Paramyxovirine* subfamily is further divided into several genera (AbuBakar *et al*, 2004). One, called *Henipavirus* consists of two of the newest members of the family, Hendra virus (HeV) and Nipah virus which are classified together due to their common genomic and structural similarities (Harcourt *et al*, 2000). Both of these viruses recently emerged from their natural reservoir and have shown to be deadly to both humans and animals.

1.1.2 Genome, Structure and Virus Replication

Nipah virus, together with another genetically related zoonotic pathogen – Hendra virus, belongs to the Henipavirus genus within the subfamily *Paramyxovirinae,* family of *Paramyxoviridae*. Nipah viruses are enveloped, pleomorphic and variably sized from 40 – 600 nm (Hyatt *et al.,* 2001). The envelope contains a lipid membrane that

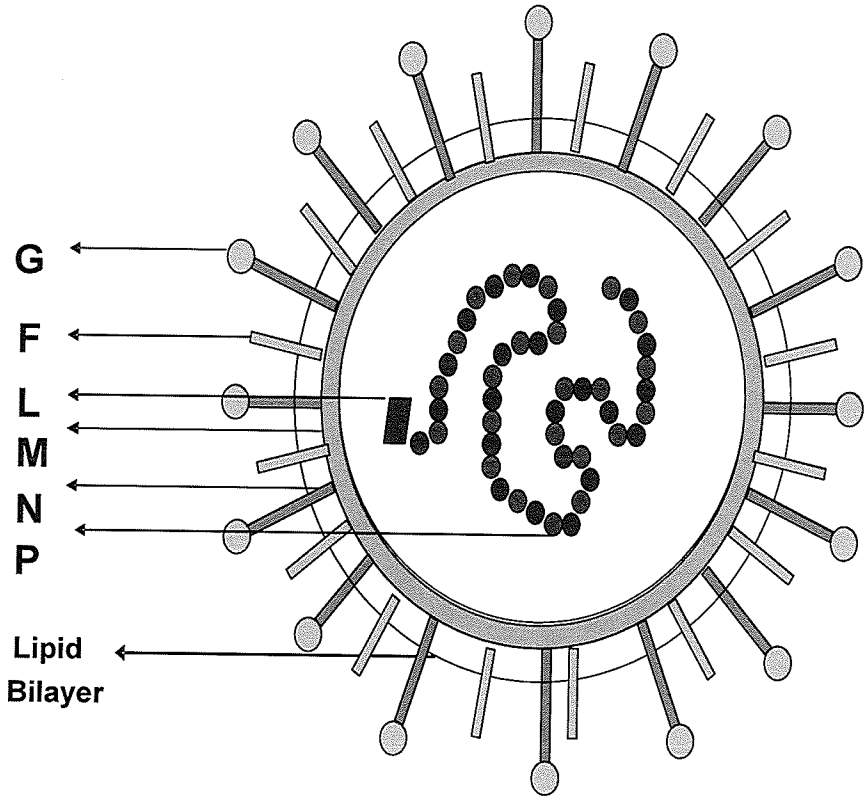


Figure 1a: Schematic diagram of the Nipah Virus structure. The spikes of the virus are made of fusion (F) and attachment (G) glycoprotein and Matrix (M) protein that maintains the virus structure and integrity. The core of the virus is made up of nucleocapsid (N), phosphoprotein (P) and the polymerase (L) protein and RNA.

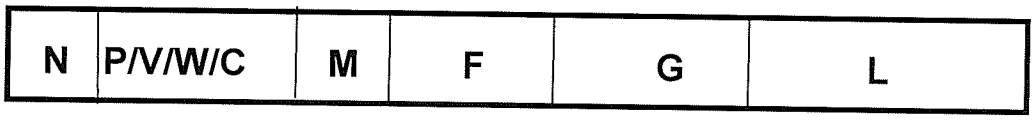


Figure.1b. – Schematic representation of Nipah virus genome

contains spikes of the fusion and attachment glycoproteins overlying a shell of matrix protein. The core of the virus is made up of a single helical strand of genomic RNA tightly bound to nucleocapsid protein with associated P and L proteins.

The genome of Nipah virus is the largest within the *Paramyxoviridae* family and consists of non segmented, single stranded RNA of negative sense that is approximately 18,246 nucleotides long; 12 nucleotides longer than the genome of Hendra virus (Harcourt et al., 2001; Harcourt et al., 2000). Nipah virus, like the majority of viruses within the *Paramyxovirinae* subfamily, has a genome which contains six transcription units encoding six structural proteins: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), attachment glycoprotein (G), and large protein or RNA polymerase (L) (Harcourt et al, 2000; Shaw et al, 2005; Shaw et al, 2004; Chen et al, 2001).

Like other members of its subfamily, Nipah virus encodes multiple non structural proteins (V, W and C) from the P gene using mechanisms such as RNA editing and internal translational initiation. The V and W transcripts are formed as a result of RNA editing in which one (V) or two (W) extra G residues are inserted by the L protein at the RNA editing site during viral transcription by causing a frame shift so that the resulting proteins share the same N-terminal domain with P, but have unique C-terminal domains. C protein is expressed from an alternative open reading frame within 5' end of the P mRNA. All the P gene products have potential interferon antagonist activity.

Very little is known about the replication of Henipaviruses. As far as is known, all aspects of Nipah virus replication occur in the cytoplasm. Glycoprotien G attaches to host cell via ephrin B2, a highly conserved protein that's found in many mammalian cells

(Negrete, *et al.*, 2005; Negrete, *et al.*, 2006; Aguilar et al, 2006; Bonaparte et al, 2005). The F protein fuses the viral membrane with the host cell membrane releasing the virion contents in to the cell. The F protein also causes infected cells to fuse with adjacent non infected cells to form multinucleated syncytia (Lamb and Parks, 2007; Moll et al, 2004).

Transcription begins with a viral RNA-dependent RNA polymerase synthesizing a full-length mRNA which serves as a template for protein synthesis and for transcription of more negative sense RNA. All genes that are transcribed are monocistronic except the P gene (Lamb & Parks, 2007; Murry, 2005; Lamb & Kolakofsky, 1996).

Translation gives rise to proteins that are needed to assemble the virus and allow it to leave the cells. Nucleocapsid proteins assemble with the anti-genome to form ribonucleoproteins (Lamb and Kolakofsky, 1996). Nucleocapsides also associate with the P and L proteins to form the polymerase complex (Lamb and Kolakofsky, 1996). Virus particles assemble at the cell surface by an unknown mechanism. The M proteins have been suggested to move the RNP core to the plasma membrane (Lamb & Parks, 2007). Mature virions exit the cell via budding (Lamb & Parks, 2007).

1.1.3 History of Nipah Virus

The first outbreak of NiV occurred in 1998/99 in Malaysia. The symptoms of this disease were similar to other encephalitic viruses and initially the NiV outbreak was thought to be an outbreak of Japanese encephalitis virus (JEV). JEV outbreaks were common in this region and antibodies against JEV were found in sera of some patients (Chua *et al*, 2002; Field *et al*, 2001).

Nipah virus was initially isolated from cerebro-spinal fluid of a fatal human case in 1999. Concurrent with the human outbreak respiratory disease among pigs was seen. The route of transmission was animal to human where the majority of cases were found among pig farmers or individuals who handled or had direct contact with infected animals (Chua *et al*, 2002; Chew *et al*, 2000; Field *et al*, 2001). Many other animals such as horses, cats, dogs, and goats also showed signs of disease but it was later found that the main cause of infection in these animals was through contact with pigs (Chua, 2003; Hooper & Williamson, 2000). Unlike pigs, these domesticated animals did not further spread the virus and therefore were considered dead end hosts (Chua, 2003).

In early 1999, an outbreak of NiV occurred in Singapore. The appearance of the disease was found to be due to the movement of pigs across the border from Malaysia (Chua, 2003). The outbreak and further spread of the virus was halted when Singapore stopped the import of Malaysian pigs (Chua *et al*., 2000). The first Nipah virus outbreak ended with 256 human cases of viral encephalitis, 105 resulting deaths and the killing of 1.1 million pigs to prevent further spread of the disease (Chua, 2003; Chua *et al*, 2000; Field *et al*, 2001; Tan et al, 1999).

Following the Malaysia/Singapore outbreak, smaller isolated outbreaks of NiV have occurred in the regions of northern India. This outbreak showed symptoms such as fever, vomiting, headaches, and some respiratory symptoms (Chadha *et al*, 2006). Laboratory diagnosis showed anti-NiV antibodies in the serum of patients and real time PCR and virus isolation methods showed that NiV was the agent that caused these symptoms. This outbreak had a mortality rate of 50-70%.

In Bangladesh, there were found to be outbreaks of NiV between 2001 and 2004 with the largest being 2004 (Hossain *et al*, 2008). There was evidence of person to person transmission of the virus without a pig intermediate, and resulted in a higher case fatality rate than the Malaysian outbreak (Chadha *et al*., 2006; Hsu *et al*., 2004). The outbreak resulted in 23 human cases and 17 deaths having a fatality rate in the clinical cases of 74% as compared to 41% in the Malaysian outbreak (Chadha *et al*., 2006; Hsu *et al*., 2004). Infected humans of the Bangladesh outbreak showed similar symptoms of disease as compared to the Malaysian outbreak (Hsu *et al*, 2004). Respiratory disease in humans was seen in the Bangladesh outbreak. This was also seen in Malaysia but in Bangladesh may have been more pronounced because of human to human spread speculated to be through aerosol droplets (Hsu *et al*, 2004; Anonymous. 2004).

The natural reservoir of NiV was found to be fruit bats. The initial suspected route of transmission of NiV from bats to pigs was by contamination of pig feed by infected saliva and feces from bats (Enserink, 2000). It is also believed that direct transmission from bats to humans may have occurred. In particular, island flying foxes and Malayan flying foxes of the *Pteropus* species were found to carry the virus asymptomatically (Chua *et al*, 2002; Field *et al*, 2001; Halpin *et al*, 2004; Olival & Daszak, 2005). Virus was isolated from bat tissue and swabs (Epstein *et al,* 2008). Nipah Virus RNA was found in secretions of the *Pteropus* species of bat, and antibodies against the virus have been present in these animals indicating that fruit bats may in fact be the natural reservoir for the virus (Middleton *et al*., 2002; Harit *et al*., 2006). In addition, within the field, many colonies of the *Pteropus* fruit bat have also found to carry anti-NiV antibodies (Wild, 2008).

Currently there is no known vaccine that is effective in preventing a NiV infection. NiV is considered to be an exotic and highly dangerous agent and is of high risk to an individual working with the agent. This pathogen is classified as a biosafety level 4 virus and a class C bioterrorist agent.

1.1.4 Clinical Manifestations

Nipah virus infection presents itself in humans as severe encephalitis (Chua *et al*, 2003) with symptoms such as fever, headache, malaise and dizziness accompanied by reduced consciousness and seizures (Chua, 2003; Field *et al*, 2001). Some patients have shown relapsed encephalitis where reoccurrence of symptoms have developed months after initial infection. Respiratory disease is also seen, although less common (Chua *et al*, 2002).

In humans, the virus attacks the central nervous system causing severe encephalitis which can result in death (Hooper *et al*, 2001; Amal *et al*, 2000). Nipah virus crosses into the brain blood barrier in humans and causes syncytia and necrosis in cells in vascular tissues. NiV was isolated from cerebrospinal fluid from a fatal patient by tissue culture (Vero 76 cells) (Chua *et al*, 1999; Paton *et al*, 1999; Goh *et al*, 2000) and by electron microscopy (Chow *et al*, 2000).

Infected pigs generally develop respiratory signs and a fraction of infected animals show central nervous system complications (CDC, 1999, Chua *et al*, 2002). Younger pigs (less than 6 months) showed difficulty breathing, barking cough which could be severe, accompanied by haemoptysis and open mouth breathing (CDC, 1999).

Trembling, spasms and trouble walking are an example of neurological signs of the young pigs (Chua, 2003). Older pigs showed respiratory signs that included breathing problems, nasal discharge, and an increase in salivation (CDC, 1999); the neurological signs included seizures or head knocking (Chua, 2003).

Many animals and humans showed sub-clinical infections (Middleton et al, 2000; Tan et al, 1999). From experimentally infected pigs, NiV was recovered from the nose and oropharynx (Middleton et al, 2000). Surveys on individuals in surrounding areas during the Malaysia/Singapore outbreaks found that some individuals had positive serology for NiV indicating that they had a previous subclinical infection (Tan et al, 1999; Chan et al, 2002). Fruit bats also had indication of subclinical infections where NiV was isolated from the urine and serum from experimentally infected animals (Middleton et al, 2007).

The virus spreads mainly to the central nervous system (CNS) and lungs. The virus enters the CNS in pigs by crossing the blood brain barrier or entering via cranial nerves (Weingartl *et al.*, 2005). Comparison of mortality rates of naturally infected pigs and naturally infected humans in the field were interesting. Humans showed 40-70% fatality rate in clinical cases. An infected herd of pigs showed a fatality rate in clinical cases of 5% (Mohd-Nor *et al*, 2000).

1.1.5 Host Response to NiV

Currently, there is little known about the immune response to defend against NiV. In humans infected with NiV, anti- NiV antibodies developed and were found at higher

titres in the patients' serum than in cerebrospinal fluid (Eaton *et al*, 2007). Antibodies such as IgG, were more common than IgM, again in the serum as compared to the CSF of encephalitic patients (Paton *et al*, 1999; Chua *et al*, 1999; Eaton *et al*, 2007). IgM antibodies are used in capture ELISA to diagnose NiV (Paton *et al*, 1999; Wong et al, 2001). Beyond these aspects, to date little has been studied about the adaptive immune response to NiV in humans.

Laboratory experiments have been carried out in a wide variety of animals which show a greater insight to the immune response to NiV. Antibodies against NiV are found to be protective in hamsters (Wang *et al*, 2001), pigs (Weingartl *et al,* 2006), and most recently, cats (Mungall *et al*, 2006).

Similarly to the adaptive immune response, little has been studied about the innate immune response to NiV. However, it is postulated that the innate immune system is activated upon viral entry but the exact mechanism is unknown (Rodriguez *et al*, 2003). The IFN system is thought to play a role early in the infection but may be counter regulated by NiV antagonist proteins (Rodriguez *et al*, 2003).

1.2 Interferon

One of the first natural defences of the innate immune system upon recognition of viral pathogens is the induction of interferon and the antiviral state. Interferon belongs to a family of proteins called cytokines (Janeway *et al*, 2005; Charles *et al*, 2005). Cytokines are proteins that are involved in signal transduction and are critical communication molecules between both innate and adaptive immunity. Cytokines have a

short biological half-life and generate amplification cascades when they are released from cells in response to an invading pathogen.

Human IFN is classified as being the initial response of the innate immune system to a viral infection but can also be induced by other foreign particles such as bacterial lipopolysaccharide or flagella (Perry *et al*, 2005). IFN α is also critical for viral clearance from the host (Perry *et al*, 2005).

Interferon is divided into two groups: type I IFN and type II IFN. This classification is based upon the receptor that each respective IFN binds to (Goodbourn *et al,* 2000). Type II IFN binds to the IFN gamma receptor and this type of IFN is IFN γ (Kunzi & Pitha, 2005). Type II interferon plays a role in control of immune and inflammatory responses. IFN γ is released by T cells following antigen recognition of infected host cells during an infection (Goodbourn *et al*, 2000).

Type I IFN is expressed following viral infection and consists of many groups of IFN: IFN α, β, δ, ε, ζ, ω, τ, and κ. The two most characterized classes are IFN α and IFN β. IFN β is initially released upon infection as part of the innate defence system. IFN β is glycosylated and is released from epithelial cells and fibroblasts (Kunzi & Pitha, 2005). There are two types of IFN β known as IFN-β1 and IFN-β3 (Kunzi & Pitha, 2005). IFN α is released subsequently to IFN β from many cells including dendritic cells and macrophages and is unglycosylated (Korth *et al*, 2005). Human IFN α contains 13 subtypes (Kunzi & Pitha, 2005).

1.2.1 Stages of the Interferon Response

1.2.1.1 Interferon Induction

There are many classes of toll like receptors (TLR), which recognize different pathogens or specific parts of a pathogen. The distribution of TLR varies depending on the cell type. TLRs are also called pathogen recognition receptors (PRR). They can lie in the extracellular surface of a cell or can be found within a cell (Janeway *et al*, 2005; Schroder & Bowie, 2005). For most of the RNA viruses, double stranded RNA is the activator of the antiviral response, through dsRNA binding to TLR3 (Perry *et al*, 2005). Binding of dsRNA activates TLR3 which functions through a toll interleukin-1 receptor (TIR) molecule (See Figure 2a). The TIR associates with the adaptor molecule TRIF and works through a series of kinases to activate interferon regulatory factor 3 (IRF3), (Schroder and Bowie, 2005). IRF3 initiates the transcription of Type I and II IFNs (Perry *et al*, 2005).

1.2.1.2 Interferon Signalling

The induction of IFN α by TLR results in the release of IFN proteins into the extracellular space allowing IFN to bind to neighbouring cells, initiating the antiviral response (Figure 2b). Type I IFN binds to receptors known as the interferon alpha receptor 1 and interferon alpha receptor 2 (IFNAR 1 and IFNAR 2, respectively) which start the signalling process (Goodbourn & Randall, 2000; Pietila *et al*, 2007). IFN γ binds to different receptors to initiate its signal process. When binding of IFN to its receptors occur, the receptors allow for phosphorylation of the Janus and tyrosine 2

kinases (JAK and Tyk2, respectively) within the cytoplasm of a cell (Goodbourn & Randall, 2000).

The signal transducer and activator of transcription protein 1 (STAT1) is phosphorylated by Tyk2 which produces a docking site for another STAT1 protein and allows for its phosphorylation (Goodbourn & Randall, 2000). STAT2 is also phosphorylated and the phosphorylated STAT proteins are translocated into the nucleus and associate with the p48 DNA binding protein (Kunzi & Pitha, 2005). Together the STAT1, STAT2 and p48 complex are called the IFN stimulated gene factor 3 (ISGF 3) (Gotoh *et al*, 2002). This complex binds to the IFN stimulated response element (ISRE) and acts as a promoter for the initiation of transcription of IFN stimulated genes (Gotoh *et al*, 2002). IFN can bind to neighbouring cells and this allows for induction of the antiviral response.

Besides the JAK/STAT pathway, IFN can activate other signalling pathways that have the ability to work independently. An example of this is the p38-MK2 pathway which can be used to induce an antiviral state. Stimulation by IFN causes the p38 molecule to be phosphorylated by mitogen activated protein kinase kinase (MAPKK) (Rincon *et al*, 1998).

The activated p38 interacts with the MAP kinase 2 (MK2) molecules in the nucleus and causes its phosphorylation (Ishida *et al*, 2004). The p38-MK2 complex can phosphorylate transcription factor ATF-2 (Rincon *et al*, 1998) which, when activated, allows for the induction of type I IFN (Goodbourn & Randall, 2000). Previous studies

Figure 2: Three stages to IFN induction and the antiviral response. (a) IFN is induced by binding of dsRNA to TLR3 which activates the TRIF pathway to induce IFN α proteins. (b) IFN α induced in a, bind to neighbouring cells to start signal transduction through the JAK STAT pathway to produce more interferon and induce the antiviral state to regulate foreign viruses (a. Schroder & Bowie, 2005, b. Katze *et al*, 2002).

have implied that the potency of IFN may be enhanced by the activation of more than one antiviral pathway (Ishida *et al*, 2004).

1.2.1.3 Antiviral State

The binding of STAT1/p48 DNA binding protein to the ISRE allows for the mRNA transcription and activation of the antiviral proteins. Many antiviral proteins are induced by IFN α many of which are not well characterized. These proteins have specific ways to downregulate replication of viruses. This is known as the IFN α induced antiviral state. There are between 20-30 different types of proteins, many of which are not well studied and have unknown function in viral inhibition (Goodbourn and Randall, 2000). The more characterized antiviral proteins are protein kinase R (PKR), 2,5 oligoadenylate synthetase (2,5 OAS), and MxA (Katze *et al*, 2002).

PKR, one of the most studied antiviral proteins, functions as a pro-apoptotic protein which is activated through the IFN induction pathway. The activated dimeric form allows for high binding of the dsRNA and reduction of viral replication (Goodbourn & Randall, 2000). This protein also signals for infected cells to undergo cell death and stops ongoing viral replication.

2,5 OAS is an enzyme which polymerizes ATP into 2'-5'-linked oligomers (Katze *et al*, 2002) . These oligomers can now bind to RNase L and activate it (Katze *et al*, 2002). RNase L activation cleaves RNA which results in inhibition translation of mRNA including viral RNA and thus inhibiting virus replication and cellular processes (Goodbourn & Randall, 2000).

MxA is a well studied GTPase which has homology to dynamin, proteins known for their roles in intracellular transport (Goodbourn & Randall, 2000; Katze *et al*, 2002). The MxA protein was first studied in Orthomyxoviruses and its function depends on the virus that has infected the host. For example, when a member of the *Bunyaviridae* family enters the host cell, MxA binds to the nucleocapsid and disrupts proteins of the viral capsid to inhibit replication of the virus (Katze *et al*, 2002). Other antiviral functions of this protein include inhibiting viral polymerase or disrupting viral protein trafficking throughout the cell (Goodbourn & Randall, 2000).

1.3 Viral Evasion of the host response

1.3.1 Interferon Antagonistic mechanisms

While IFN are effective in aiding eliminating viruses such as vesicular stomatitis virus, other viruses have found ways to defer the effect of IFN. Viruses have developed many mechanisms to decrease the threat of IFN. The most common is the production of IFN antagonist proteins which specifically target an area of the IFN system.

Vaccinia Virus is a dsDNA virus and a member of the *Poxviridae* family and of the genus *Orthopoxvirus*. This virus targets IFN right at the beginning of induction in infected cells. It does this by producing two proteins, one which binds and inhibits TLR3 by blocking the active site and the other which binds to TRIF and prevents phosphorylation of downstream proteins (Deng *et al*, 2006).

Influenza, a member of the *Orthomyxoviridae* family, encodes a non-structural protein known as NS1 which is involved in inhibiting the antiviral state (Kochs *et al,*

2007). This protein is involved in shutting down the host response of both IFN α and IFN β. NS1 interacts with PKR to inhibit its activity by directly binding to PKR which blocks PKR activation of Influenza infected cells, which halts its function (Min *et al*, 2007). NS1 also binds to dsRNA and degrades it which prevents establishment of the NF-kappa-B signalling pathway (Lin *et al*, 2007; Min *et al*, 2007), a pathway involved in regulating the immune response to an infection.

Japanese encephalitis virus is classified within the *Flaviviridae* family into the genus *Flavivirus*. This agent can cause fatal encephalitis in animals and humans (Lin *et al,* 2006). This virus can interfere with IFN signalling through the viral NS5 protein (Lin *et al*, 2006). This accessory protein interferes with Tyk2 by preventing its phosphorylation and therefore inhibiting further steps in the JAK-STAT pathway (Lin *et al,* 2006).

Rabies virus a member of the *Rhabdoviridae* family, genus *Lyssavirus* does not produce an accessory protein to interfere with the IFN system. It uses its P protein to bind STAT1 and prevent STAT1 nuclear localization (Brzozka *et al*, 2006). Ebola virus (family *Filovirus*, genus *Ebolavirus*), VP35 protein, binds to the ISRE in the nucleus and blocks its function (Basler *et al*, 2000).

1.3.2 P, V and W Proteins of the *Paramyxoviridae* family

The family *Paramyxoviridae* transcribes the P gene in a unique manner. This family can encode non structural proteins from the P gene by many mechanisms such as RNA editing and internal translation initiation. This gene contains multiple ORF which

have the ability to produce other mRNAs. For example, Sendai virus has eight mRNAs, five of which are transcribed from overlapping reading frames in the P gene (Gotoh et al, 2001). Alternatively, paramyxoviruses transcribe genes as a result of RNA editing. One or two nucleotides are inserted at this point which gives rise to additional mRNAs, V and/or W (Lamb & Parks, 2007). This produces mRNAs with alternative reading frames designated as 0, +1 or +2 reading frames (Lamb & Parks, 2007). The products of the translated mRNAs usually results in accessory proteins and is the most common way that paramyxoviruses produce accessory proteins.

For many viruses in the *Paramyxoviridae* family, V and W are the most common studied accessory proteins as they are proposed to be anti-IFN proteins. The V protein is more commonly found than the W protein among Paramyxoviruses, with only a few members of this family producing W. *Morbilliviruses* such as measles virus and rinderpest, *Respiroviruses* such as Sendai virus and *Henipaviruses* such as Nipah and Hendra virus are examples of viruses that potentially produce both a V and W protein.

NiV potentially produces accessory proteins V and W through RNA editing of the P gene during mRNA synthesis. Many other viruses also produce these proteins and use V and sometimes W to specifically inhibit IFN signalling. An example of this includes mumps virus a member of the *paramyxoviridae* family, genus *Rubulaviruses,* which produces an accessory V protein to target the STAT1 protein for degradation by binding to it (Gotoh *et al,* 2002).

Sendai virus, a member of the genus *Respirovirus* (family *Paramyxoviridae*), was shown to express accessory proteins *in vivo* and the V and C protein here are the main

IFN antagonists (Gotoh *et al*, 2002). Other Paramyxoviruses, including the Rubulavirus and Simian virus 5 were shown to produce the V accessory protein in infected cells (Gotoh *et al*, 2002). Morbilliviruses such as measles virus also displayed expression of an IFN antagonistic protein in infected cells (Gotoh *et al*, 2002; Yokota *et al*, 2003).

In the *Paramyxoviridae* family, accessory proteins produced by different viruses have been expressed in the recombinant system as well as within infected cells (Rodriguez *et al*, 2003). Recently the V and W proteins from Hendra have been proposed to antagonize IFN through the JAK-STAT pathway (Rodriguez *et al*, 2003). Recombinant Hendra V and W proteins were found to bind to STAT1 and STAT2 and sequester these proteins as high molecular weight complexes unable to carry out their normal functioning (Rodriguez *et al*, 2003). The HeV V protein was found in the cellular cytoplasm and the HeV W was found in the nucleus and in these compartments, it was proposed that binding causes inhibition of both IFN α and IFN γ signalling pathways (Rodriguez *et al*, 2003).

1.3.3 Nipah P, V and W proteins

The NiV P protein is approximately 70.9 kDa and has about a 67% homology with the HeV P protein (Wang *et al*, 2001; Wang et al, 1998). One of the functions of this protein is to bind the N and L proteins to form the functional polymerase complex (Kingston *et al*, 2004). The polymerase complex is part of the nucleocapsid of the virus. The phosphoprotein of NiV is phosphorylated at two serine residues (Shiell *et al*, 2003;). This protein has also been found to be associated with inclusion bodies produced by the

virus (Wong *et al*, 2002) which are protein aggregates composed of NiV P and NiV N found in a host cell, during replication of the virus. Two other accessory proteins are believed to be produced through co-transcriptional mRNA editing of the P gene (Wang *et al*, 2001; Wang et al, 1998; Harcourt *et al*, 2000; Shaw *et al*, 2005) (Figure 3).

Within the P gene, there contains an editing site located at nucleotide 1325 (amino acid 407) (Chua *et al*, 2000). At this site, the viral polymerase can add non templated G nucleotides into this editing site (Eaton *et al*, 2006; Harcourt *et al*, 2001; Wang et al, 1998) during transcription. This produces a +1 or +2 frameshift in the 3' end of the mRNA giving rise to two new proteins NiV V and NiV W (Shaw *et al*, 2005; Eaton *et al*, 2006; Harcourt *et al*, 2001; Wang *et al*, 1998). NiV V extends for 47 amino acids beyond the common N terminal end and NiV W for 40 amino acids. The production of the NiV V and NiV W proteins will result in three NiV proteins (V, W and P) with similar N terminals and unique C terminals (Rodriguez & Horvath, 2004) (Figure 3). Phosphorylation sites of the NiV P protein are found on serine residues at positions 257 and 350 (UniProt, 2008). Thus V and W are expected to have two phosphorylation sites.

The NiV C protein is also an accessory protein of NiV. It is not produced in a similar manner as NiV V and W but rather through an alternative reading frame located at the 5' end of

activity but its role is not well studied (Shaw *et al,* 2005).

These three proposed accessory proteins, NiV V, W and C are not well characterized, especially in cells infected with NiV. However, they are suspected to play

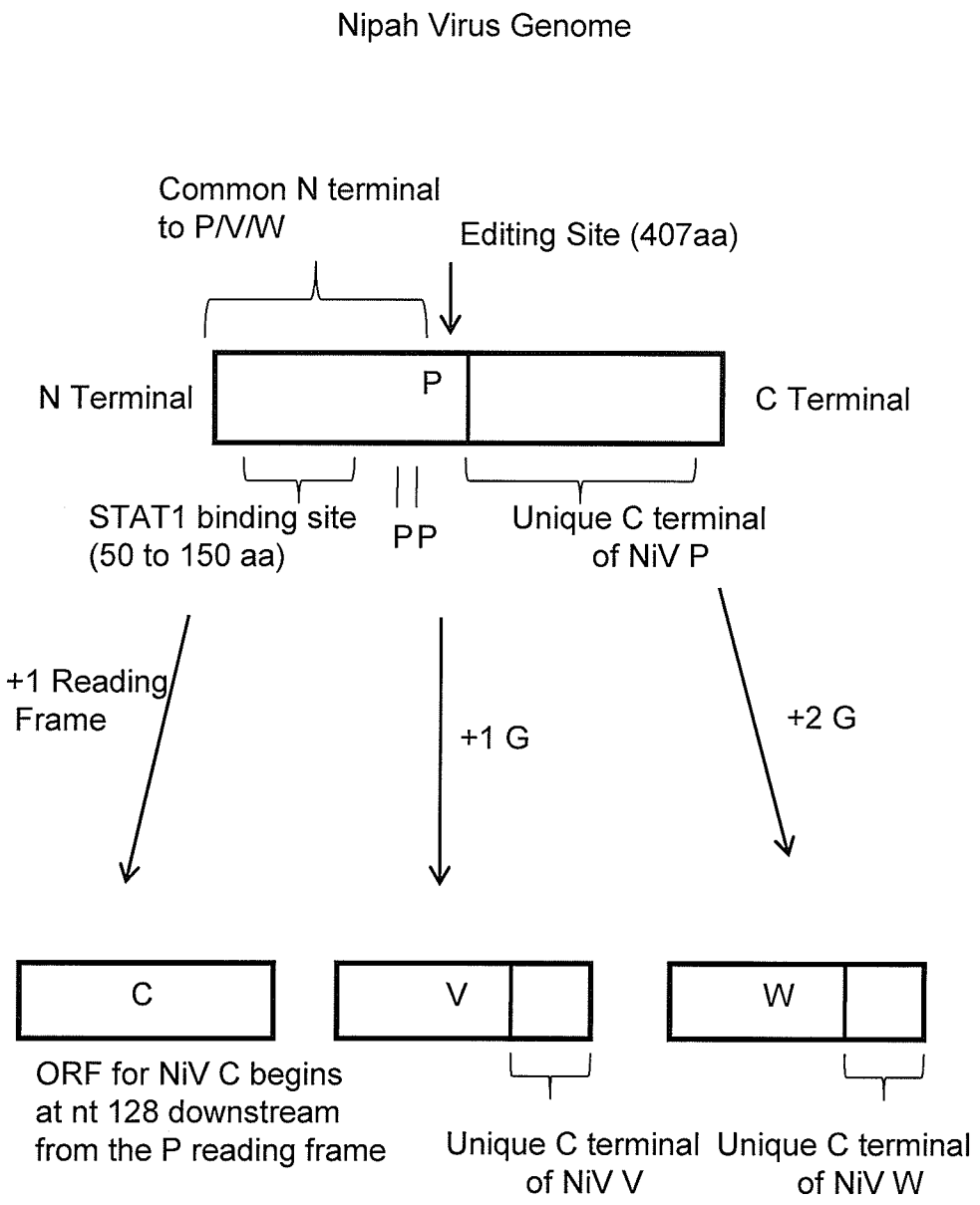


Figure 3: The NiV P gene gives rise to three accessory proteins, NiV V, NiV C and NiV W as well as NiV P. NiV C is produced from an entirely different reading frame within the P gene and NiV V and W are produced from insertion of non templated G's into NiV P reading frame. Each protein shares a common N terminal and a unique C terminal. The STAT1 binding site is located on the N terminal of the protein. The phosphorylation sites for NiV P/V/W are designated as P and located at serines 257 and 350 (UniProt, 2008).

a role in the evasion of the innate immune system by interfering with IFN signalling similar to that proposed for HeV (Shaw *et al*, 2004; Rodriguez *et al*, 2003).

1.3.4 The Proposed Role of NiV V and W

The recombinant NiV V protein was found to co-localize with STAT1 and is suspected to form a NiV V-STAT1 complex (Rodriguez *et al*, 2004; Rodriguez *et al*, 2002). This large complex holds the STAT1 protein in the cytoplasm and prevents downstream steps in the STAT1 signalling pathway (Rodriguez *et al,* 2002; Rodriguez *et al*, 2004). Degradation of STAT1 did not occur (Rodriguez *et al*, 2002). Co-localization with STAT2 also occurred, although it was less pronounced than STAT1 (Shaw *et al*, 2004). It was noted that the predominant method for suppression of IFN signalling was through interaction with STAT1 (Shaw *et al,* 2004).

Later studies focused on the NiV W protein (Shaw *et al*, 2004; Shaw *et al*, 2005), again in a recombinant system. This research showed that NiV W co-localized with STAT1 in the nucleus and it was suggested that the W protein binds to STAT1 in the nucleus that escaped binding by NiV V in the cytoplasm (Shaw *et al,* 2004). This feature was found to be unique to only henipaviruses in the *Paramyxoviridae* family (Eaton *et al*, 2007). Recombinant NiV W also sequestered STAT1 without degrading it (Shaw *et al,* 2005).

The binding site of STAT1 with NiV V and W was looked at and interestingly, it was found that STAT1 bound to a region in the N terminal of the P/V/W proteins. Due to this finding, research was carried out to study if NiV P had any interaction with or inhibition of STAT1. Results from these studies showed that NiV P did show binding to

STAT1 but it was suggested that the interaction may be less significant than that of the proposed accessory proteins (Shaw *et al*, 2005; Shaw *et al,* 2004). Reduced significance of NiV P-STAT1 interaction was proposed to be a result of lower binding that occurred between these two proteins as compared to NiV V/W binding when protein expression levels were quantified (Shaw *et al*, 2005).

Rodriguez and colleagues found that the NiV V protein contained a nuclear export signal (NES) (Rodriguez *et al*, 2004). This signal is found between amino acid positions 174 and 192 (Rodriguez *et al*, 2004). This correlated with their previous data to show that STAT1 was prevented from moving to the nucleus by NiV V. Similar results were seen with recombinant NiV W, where the major difference between V and W was that W contained a nuclear localization signal (NLS) and therefore localized to the nucleus (Shaw *et al*, 2005). This NLS was said to be located in the unique KKAR on the C terminal end of the NiV W protein (Shaw *et al*, 2005).

The site of interaction on STAT1 with NiV V and W (and P) was found to be located between 50 to 150 amino acids on the NiV proteins (Shaw *et al,* 2005). In this area, NiV V and W (and P) could interact with STAT1 and disrupt its nuclear localization needed to initiate the antiviral response.

Hagmaier and colleagues produced constructs which contained mutations to change part of the amino acid sequence in NiV V. These mutations occurred between amino acids 50-150 which normally was used to bind to STAT1. It was stated that these altered NiV V constructs were no longer able to associate with STAT1 in cells from many species including pigs and humans (Hagmaier *et al*, 2006).

1.4 Thesis Approach

Presently, there is no published data on the ability of porcine or human cells to establish an IFN α mediated antiviral state against NiV. Past work on the IFN system with NiV has mainly focused on the human recombinant system and the ability of NiV V and W proteins to co-localize with recombinant STAT1 only in a recombinant system (Shaw *et al,* 2004; Shaw *et al*, 2005; Rodriguez *et al*, 2003). This research focused on human cell lines and the human recombinant system (Shaw *et al,* 2004; Shaw *et al*, 2005; Rodriguez *et al*, 2003).

In vivo work with NiV in hamsters has suggested that the interferon system in hamsters is efficient when induced by Poly I:C (Georges-Courbot *et al,* 2006). Poly I:C was found to induce both IFN α and IFN β and completely block NiV replication (Georges-Courbot *et al,* 2006). This work was shown both in hamsters and in human HeLa cells and suggests that the interferon system may have a significant role in protective activity during an infection. This study demonstrated that type I IFN signalling may be important in both humans and animals as part of the antiviral response (Georges-Courbot *et al,* 2006).

This in vivo work is currently the only study with respect to IFN against NiV. As pigs have lower mortality rate than humans, 5% in contrast to 40-70% (Mohd-Nor *et al*, 2000), we propose that the innate immune system plays a role in the faster recovery of pigs, and that the IFN response in pigs may be more efficient then in humans, including the establishment of an antiviral state. For purposes of this study, we hypothesize that IFN α has a significant role in the recovery of porcine hosts, and that the signalling

pathway of porcine hosts is not compromised to the same degree as it has been found in humans.

The thesis research focused on: 1. The ability of human and porcine cells to develop an exogenous IFN α induced antiviral state against NiV. 2. NiV interference with IFN signalling in infected cells, and whether similar to the recombinant system viral V and W proteins are involved. 3. Determining if co-localization of NiV V and W occurs with STAT1 in human and porcine cells infected with NiV.

1.4.1 Capability of Human and Porcine Cells to Develop an Exogenous IFN α Induced Antiviral State against NiV

1.4.1.1 Objective

To determine the ability of porcine and human cells to develop an induced antiviral state in response to exogenous IFN α, when challenged with NiV.

1.4.1.2 Hypothesis

We hypothesize that the cells are able to establish an IFN α induced antiviral state against NiV, with the possibility that the porcine IFN response is more efficient than the human IFN response to exogenous IFN α.

1.4.1.3 Experimental Design

To compare the ability of porcine and human cells to establish an antiviral state against NiV, first porcine and human cell lines were selected based on the following criteria. Vesicular stomatitis virus (VSV) was used as a control to determine if cells were able to develop an antiviral state using exogenous IFN α in several porcine and human cell lines. Classical antiviral assays were carried out where cells were treated with exogenous IFN α and challenged with VSV. From cell lines capable to enter IFN induced antiviral state, elected porcine and human cells (SJPL and MRC5) which were able to support NiV growth were used in a time course with NiV to determine whether the cell line can establish an exogenous IFN α induced antiviral state against NiV.

1.4.2 The Expression of the NiV V and W Proteins in Human and Porcine Cells Infected with NiV

1.4.2.1 Objectives

To determine and confirm the expression and localization of NiV V and NiV W in NiV infected cells.

1.4.2.2 Hypothesis

Both NiV V and NiV W are expressed in porcine and human cells infected with NiV and the NiV V protein localizes to the cytoplasm and NiV W to the nucleus, analogous to recombinant proteins (Shaw *et al,* 2004; Shaw *et al*, 2005; Rodriguez *et al*,

2003).

1.4.2.3 Experimental Design

Porcine and human cells were infected with NiV and probed with an antibody targeted to the common N terminal of P/V/W proteins to determine the protein expression and localization by immunofluorescence in confocal microscopy. In addition, nuclei and cytoplasm were extracted to detect each protein in the respective compartments. Proteins were visualized on SDS PAGE followed by western blots. To determine the localization of NiV V and NiV W proteins in the infectious system and to compare localization to the recombinant system, NiV V and NiV W genes were cloned into Semliki Forest Expression vector pSCA and transfected into BHK-21 cells to confirm previous published results.

1.4.3 The Co-localization of NiV P/V/W Proteins Produced in Infected Cells with Native Porcine and Human STAT1

1.4.3.1 Objective

To determine the co-localization of STAT1 and NiV V/W/P proteins in infected human and porcine cells.

1.4.3.2 Hypothesis

Based on previous recombinant work (Shaw *et al,* 2004; Shaw *et al*, 2005; Rodriguez *et al*, 2003), STAT1 will co-localize with NiV V in the cytoplasm and with NiV W in the nucleus.

1.4.3.3 Experimental Design

To study the co-localization between STAT1 of human and porcine cells with NiV V and NiV W, immunofluorescence assays and confocal microscopy were carried out to detect STAT1 and the NiV proteins in cells infected with NiV. Results were compared to previous published literature of the recombinant system (Shaw *et al,* 2004; Shaw *et al*, 2005; Rodriguez *et al*, 2003).

2. Materials and Methods

2.1 Viruses and Cells

2.1.1 Cell Maintenance and Preparation

All cell lines were obtained from the American Tissue Culture Collection. The SJPL cells were also obtained from ATCC but with the permission from Dr. Robert G. Webster (St. Jude's Children's Hospital, Memphis, Tennessee). ST (swine testis, fibroblast), HeLa (human cervix, adenocarcinoma, epithelial), PK15 (porcine kidney, epithelial), PT K75 (porcine nasal turbinate mucosa, fibroblast), Vero76 (African green monkey kidney, epithelial), 293, 293T (both human kidney, epithelial), and SJPL (porcine lung, epithelial) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent, Cat No. 319-005CL), supplemented with 10% fetal bovine serum (FBS). MRC5 (human lung, fibroblast), and WISH (Human amnion, epithelial) cells were cultured in Alpha Modified Eagle's medium (αMEM) (Wisent, Cat No 310-011 CL), and Caki-2 cells (human kidney, epithelial) were grown in McCoy's 5a medium (ATCC, Cat No. 30-2007) all supplemented with 10% FBS. BHK-21 (baby hamster kidney, fibroblast) cells were cultured in Glasgow's Modified Eagle's Medium (GMEM) (Wisent, Cat No. 322-005 CL) supplemented with 10% FBS. Cells were passaged using standard procedures: by washing the cells with PBS without magnesium and calcium, followed by trypsinization using 0.25% trypsin-EDTA (Gibco, Cat No. 25200). All cell lines were passaged every 2-3 days to maintain the cultures.

2.1.2 Cultivation of Viruses

Nipah Virus was obtained from Dr. T. Ksiazek and Dr. P. Rollin from the Centre for Disease Control and Prevention in Atlanta Georgia, USA. Vesicular stomatitis virus was obtained from NCFAD. Viral stocks for both viruses were grown in Vero76 cells infected with a multiplicity of infection (MOI) of 0.1. For infection, virus was added to 5ml of DMEM with no supplements and added to the T75 flask (Corning, Cat No. 3276) containing 95-100% confluent cells. After 1 hour of incubation, at 37°C, 5% CO_2, 5ml of DMEM containing 4% FBS (2% final FBS concentration) was added to the flask. These cells were then incubated for 2 days at 37°C, 5% CO_2 and when cytopathic effect (CPE) was observed the virus was harvested. Supernatant was clarified by centrifuging at 3000 g for 20 minutes and was aliquoted and frozen at -70°C before titration of the virus by plaque assay. Control Vero76 cells were treated as described above but no virus was added to the flasks.

2.1.3 Virus Titration (Plaque Assay)

Both VSV and NiV titres were determined on Vero76 cells, and on various porcine and human cell lines. Cells grown to 100% confluency in 12 well plates (Corning, Cat No. 3512) were washed, and 400µl aliquots of virus serial dilutions virus from 10^{-1} to 10^{-7} in DMEM containing no serum or additional supplements were added in duplicate to the wells. Cell controls with media only were used on each plate. Inoculum was removed after one hour incubation and 1.5ml of

carboxymethylcellulose (CMC) (Sigma-Aldrich, Cat No. C-4888) overlay (See Appendix A1) was added to the cells. Cells were then incubated at 37°C, 5% CO_2 for 2-5 days.

Cells in virus titration assays were fixed by addition of 10% PBS buffered formalin (Fisher Scientific, Cat No. SF100-4) which contains 4% paraformaldehyde. Formalin was added directly to cells with CMC still on. Cells with formalin were incubated for 30 minutes to 1 hour at room temperature, CMC/formalin was removed, and fixed cells were gently washed with water. Cells were stained with crystal violet (0.1% crystal violet in milli-Q water) by incubating for 30 minutes and washed again. The titre of the virus was determined as plaque forming units per ml (PFU/ml) (See Apendix A2).

2.1.4 Biosafety and Removal of Infectious Material from Level 4

All infectious NiV manipulations were carried out in biosafety level 4 adhering to standard procedures and following biosafety guidelines. To remove NiV infected material from BSL4, the virus infected material was inactivated and removed from BSL4 following biosafety guidelines using different procedures. To inactivate virus in infected cells, slides or plates were fixed overnight in 10% formalin before removal from level 4. Fractions that were collected for protein analysis were added to 10% SDS and boiled at 95°C before removal following standard biosafety guidelines.

2.2 Antiviral Assays

2.2.1 Interferon Assays with Vesicular Stomatitis Virus

Human IFN α (PBL Biomedical Laboratories, Cat No 11350-1) and porcine IFN α (PBL Biomedical Laboratories, Cat No 17100-1) were used in this assay. SJPL, ST, PTK75, PK15, MRC5, HeLa, WISH, Vero 76, 293 or 293T cells were seeded onto 24 well plates to reach 100% confluency. IFNα was diluted in DMEM containing 2% FBS and two fold serial dilutions were carried out. Concentrations used were 320U, 160U, 80U, 40U, 20U, 10U, and in addition 1U/ml of IFN α. Supernatant from confluent cells was removed, cells were washed 2 times with PBS and 500µl aliquots of IFN α were added in quadruplet at the above dilutions. Cells were incubated for 24 hours at 37°C, 5% CO_2. After incubation, IFN was removed, cells were washed and VSV was added by diluting the virus in DMEM to produce 50 PFU/well. Virus dilutions were added at 200µl/well to all IFN treated wells. Control wells included virus controls, virus back titration to 10^{-2} and cell controls, either untreated or treated with respective dilutions of IFN α (to verify lack of toxicity). All controls were performed in triplicate. Cells were incubated for 1 hour before the virus was removed and CMC was added, then incubated for 2-5 days and fixed and stained as described in 2.1.4. Virus plaque reduction was calculated (see Appendix A3 for explanation and example of calculation) to determine % protection of the cells by porcine or human IFN α against VSV. The number of plaques in the virus control wells was considered to be a 100%.

2.2.2 Interferon Assays with Nipah Virus

IFN α treatment and virus challenge was carried out with NiV using the same protocols as VSV (section 2.2.1) with the following changes: SJPL and MRC5 cells only were used to test respective IFN α activity against NiV. A time course of 3, 6, 12, 18, and 24 hours was conducted for IFN α treatment, before the NiV challenge.

2.3 Cloning of NiV V and NiV W protein coding sequences

Recombinant pCZ (containing a CMV promoter) plasmids containing NiV V and W cDNA were obtained from Dr. Markus Czub's laboratory (Special Pathogens Group, Public Health Agency of Canada). The whole NiV V and W protein coding sequences were re-cloned into Semliki Forest virus based expression vectors pSCA and pSMART2a (additional vector information is provided in appendix B1 and B2) using the following primers:

Primer 1 (Forward): 5' CGCGGATCCATGGATAAATTGGAACTAGTCAATG 3'

Primer 2 (NiV V Reverse): 5' CGCGGATCCTTAACCGCAGTGGAAGCATTC 3'

Primer 3 (NV W Reverse): 5' CGCGAATCCTCAATTTGGACATTCTCCGCATTG 3'

All primers were produced using services provided by Invitrogen Technologies. Two different vectors (pSMART2a and pSCA) were used where the pSMART 2a contained HIS and Flag tags for easier detection. Bam HI restriction

site, (underlined) found within the multiple cloning site of the pSCA and pSMART2a vectors was included in the forward and reverse primers. NiV V and NiV W cDNA was amplified (see appendix B3 and B4 for PCR reaction and master mix) using a high fidelity polymerase, Platinum Pfx kit (Invitrogen Cat No. 11708-013). Following the PCR reaction, 5µl of DNA product was loaded on a 1% agarose gel and visualized by ethidium bromide. A 1kB Plus DNA Ladder (Invitrogen, Cat No. 10488-085) was used to determine the size of the DNA product. The PCR product was cleaned using Wizard SV Gel and PCR Clean Up System according to their provided protocol (Promega, Cat No. A9281).

pSCA/pSMART2a vectors and NiV V and NiV W cDNA were individually digested with Bam HI restriction enzyme (Roche, Cat No. 10567604001) overnight at 37°C (See appendix B5 for restriction enzyme mix). All products were then purified as above and the pSCA/pSMART2a vector was dephosphorylated at 37°C for 1 hour using phosphatase (Roche, Cat No. 11097075001) to prevent self ligation of the vector (see appendix B6 for master mix). Purified inserts and dephosphorylated vector were ligated overnight at 4°C in a 3:1 insert:vector ratio using T4 DNA ligase (Roche, Cat No. 10481220001) according to the calculation provided in appendix B8. For composition of the ligation master mix, see appendix B7.

Ligation mix (a total of 10µl) was transformed into competent DH5 alpha *E.coli* cells (Invitrogen, Cat No 12297-016) by incubating *E.coli* and DNA on ice for 30 minutes, heat shocking at 42°C for 1 minute and incubating at 37°C with shaking for 1 hour after addition of 500µl of SOC media (Invitrogen, Cat No. 15544-034).

The SFV vectors contained the LacZ reporter gene. Cells were plated on LB agar containing the selective marker ampicillin and X-gal/IPTG to produce white or blue colonies. Blue colonies indicated that no insertion occurred and Lac Z was intact allowing this gene to produce β-galactosidase, which when induced by the IPTG cleaves the X-gal producing a blue product. White colonies indicated that inserts were in the vector and were selected to produce overnight bacterial cultures at 37°C with shaking in LB broth containing ampicillin.

Screening for the correct NiV V-pSCA, NiV W-pSCA, NiV V-pSMART2a and NiV W-pSMART2a ligation products was carried out by BamHI restriction analysis. DNA from overnight cultures was isolated following protocols from QIAGEN mini prep kit (Cat No. 27104). DNA was separated on a 1% agarose gel where expected sizes were 12.4 Kb (pSMART2a), 11.5Kb (pSCA), 1.35Kb (NiV W), and 1.37Kb (NiV V). Suspected positive clones after restriction digest screening were sequenced at NCFAD and results were analyzed by Chromas and Vector NTI sequence analysis programs. All four plasmids with sequences that were 100% homologous to literature sources (Chua *et al*, 2000; Harcourt *et al*, 2001) were made into plasmid stocks following the protocol from QIAGEN Maxi Prep Kit (Cat No. 12562).

2.4 Expression of NiV V and W proteins

2.4.1 Cell Transfection with pSMART2a and pSCA expression vectors

BHK-21 cells were grown to confluency between 90-100% in a 6 well plate and transfection of plasmids was carried out using Lipofectamine 2000 transfection reagent (Invitrogen, Cat No. 11668-019). Expression of recombinant P protein from the NiV P pCZ vector (kindly provided by Dr. Markus Czub) was used as positive transfection/expression control in the recombinant expression of NiV V and W. Negative controls included cells transfected as above with pSCA and pSMART-2a empty vectors and untransfected cells. Controls were treated exactly the same as test samples.

Plasmid DNA (4.0 μg), 250μl of OptiMEM and 10μl of Lipofectamine 2000 were used per reaction for one well. Two mixtures were created: A. DNA and optiMEM were mixed together and incubated for 5 minutes at room temperature and B. Lipofectamine and OptiMEM were also incubated together for 5 minutes at room temperature. A and B were then added together and incubated for 20-30 minutes. BHK-21 cells were washed with PBS and 1ml of OptiMEM was added to the cells prior to addition of DNA/Lipofectamine 2000/OptiMEM complex. Cells were incubated for 24 to 48 hours at 37°C, with 5% CO_2. Alternatively, for visualization by immunofluorescence cells were seeded onto glass slides placed in 100mm Petri dishes and transfections were carried out exactly as above, with the following exception of the amount of reagents used (12μg of DNA, 40μl Lipofectamine, 10ml

plating media).

2.4.2 Infection of cells with Nipah Virus

ST, SJPL, HeLa and MRC 5 cells were infected with NiV to study localization and expression of NiV V and W proteins in a natural infectious system. Cells were seeded into either T75 flasks or glass slides for IFAs. The above cells were infected with NiV at an MOI of 0.1 for slides (for IFA) and MOI of 1 for T75 flasks (for western blots). Negative controls included cells prepared as above but no virus was added to the media. Controls were handled in a similar manner to infected cells.

2.4.3 NiV Infection of Cells Transfected with NiV W-pSMART2a

BHK-21 cells were seeded on glass slides and were transfected with NiV W-pSMART2a similar to section 2.4.1. These cells were infected with NiV at an MOI of 0.5 at 24 hours after transfection occurred. Infections were carried out similarly to section 2.1.2. Controls included cells infected with NiV only, cells transfected with NiV W pSMART2a plasmid only, uninfected untransfected cells, uninfected cells transfected with empty pSMART2a vector and infected cells transfected with empty pSMART2a vector.

2.4.4 Preparation of cell lysates

Cells were lysed by addition of 200µl of Laemmli sample buffer (Bio-Rad Cat No. 161-0737) containing 5% β-mercaptoethanol to PBS washed cells. Cells were scraped, centrifuged (3000g for 5 minutes), sonicated for 30 seconds at 2 watts and subsequently boiled at 95°C for 10 minutes before loading onto a SDS PAGE gel.

BHK-21 cells transfected with NiV V/W-pSCA or NiV V/W-pSMART2a plasmids were lysed at 24 or 48 hours post transfection. SJPL, ST, MRC5 and HeLa cells grown to 100% confluency in T75 flasks and infected with NiV were lysed at 24 hours post infection. Respective control cells (see section 2.4.2) were lysed at corresponding time points.

2.4.5 Extraction of Nuclei and Cytoplasm from infected cells

Extraction of nuclei and cytoplasm from cells infected with NiV in T75 flasks and the respective controls (section 2.4.2) were carried out using the Nuclei EZ Prep cell lysate kit (Sigma, Cat No. NUC 101), according to manufacturer's instructions. Extracted nuclei and cytoplasm were either frozen at -70°C or used immediately. Protease inhibitors (Pierce, Cat No. 78415) were used as per manufacturer's instructions to minimize the amount of protein degradation as possible.

Detection of histone proteins was used as a control to confirm integrity of the proteins in lysed material. This was carried out by detection of histones using an anti-histone antibody (see table 1).

2.4.6 Preparation of Nuclei and Cytoplasm for SDS PAGE

A suspension of 400 µl of extracted intact nuclei was pelleted by centrifugation (3000g for 5 minutes). The pellet was washed twice in 1ml of PBS and resuspended in 2 ml of lysis buffer containing 1% Triton X-100 (See appendix C1 for buffer composition). The nuclei suspension (same as above) in PBS (500µl) was passed several times through a 27 gauge syringe and centrifuged at 10,000 x g for 20 minutes to remove membranes and to produce nuclei with no membrane attached. Nuclei with membranes still attached, nuclear fractions with no membranes and cytoplasmic fractions, were aliquoted with an equal amount (500µl) of 2X Laemmli sample buffer. Nuclei with membranes attached were sonicated for 30 seconds. All samples were boiled at 95°C for 10 minutes before loaded on a SDS PAGE gel.

2.4.7 Protein Quantification Assay

To determine the amount of protein in the nuclei and cytoplasmic fractions, protein quantification was carried out by the Bradford method using BioRad Protein Assay Dye Reagent Concentrate (Cat No.500-0006) and following manufacturer's protocol. Bovine serum albumin (BSA) was used as a standard protein to create a standard curve at dilutions of 160µg, 80µg, 40µg, 20µg, 10µg of total protein and optical density was measured at 595nm. Total protein in each sample was determined using the BSA standard curve. All steps were carried out in accordance to manufacturer's protocol. Protein quantification was carried out to ensure that equal amounts of proteins for each sample was used in western blotting.

2.4.8 SDS PAGE and Protein Transfer

SDS PAGE (10% resolving, 5% stacking) gels were used to separate proteins (see appendix for gel compositions C1). Precision Plus Protein Ladder (10µl) (BioRad CatNo. 161-0373), 20µl of cytoplasm and whole cell lysate samples, and 35µl of nuclei samples (see appendix C3, C4 and C5 for protein quantification graphs and table) were loaded into separate lanes. Separation gels were carried out at 150 volts for 60 to 90 minutes and proteins were transferred to a PVDF immune-blot membrane (Bio-Rad Cat No. 162-017) by semi-dry transfer (see appendix C2 for transfer protocol). The membrane was blocked overnight in 5% skim milk (1X TBS-T, see appendix C1 for buffer solution).

2.4.9 Western Blot

Western blots were performed after overnight blocking of the nitrocellulose membrane at 4°C (as per section 2.5.6). Both primary and secondary antibodies were diluted in blocking buffer, and the membranes were washed after each primary and secondary antibody probing, with 1X TBS-T containing 0.1% Tween-20 (see appendix C1 for wash buffer solution). Western blots were developed using Sigma Fast 3,3 Diaminobenzidine tablets (Cat No. D4293). All antibody dilutions and additional information can be found in Table 1. When starting to work with a new lot of antibodies, lysates from transfected, infected and untreated cells were probed with primary antibody only (and substrate) and secondary antibody only (and substrate).

2.4.10 Immunofluorescence Assays and Confocal Microscopy

After 24 or 48 hr transfection/infection of cells, slides were fixed in 10% formalin containing 1% Triton X-100 for 1 hour at 37°C. Slides were then immersed in 1:1 methanol:acetone for 10 minutes at -20°C. Blocking buffer (1% normal goat serum in PBS) was added after removal of methanol:acetone and incubated for 30 minutes at room temperature or 4°C overnight. Proteins were labelled with appropriate primary and secondary antibodies by diluting antibodies in the blocking buffer (see Table 1 for antibody dilutions) and adding antibodies directly to slides. Washing between primary and secondary antibodies was carried out using PBS-Tween (0.1%) three times for 10 minutes. Slides were counterstained with Antifade (Molecular Probes, Cat No. S24636), sealed with coverslips and visualized using an Olympus Fluoview FV300/500/1000c confocal microscope.

2.5 Experimental Design and Controls

2.5.1 Expression of the recombinant V and W proteins

2.5.1.1 Western Blot

Proteins from cell lysates (2.4.4) of transfected cells (2.4.1) were separated by SDS PAGE, transferred onto the PVDF membrane (2.4.8) and probed with anti-NiV P58, anti-guniea pig, anti-HIS and anti-FLAG antibodies. The primary antibody was detected using anti-mouse or guinea pig HRP conjugated antibodies and Sigma Fast 3,3 Diaminobenzidine tablets (Cat No. D4293) were used as a substrate.

Controls used were as follows: (a) Cell lysate from cells transfected with NiV P pCZ expression vector probed with both primary and secondary antibodies; (b) Binary Ethyleneimine (BEI) inactivated NiV probed with both primary and secondary antibodies; (c) Cell lysates from NiV infected cells probed with both primary and secondary antibodies; (d) cell lysates from cells transfected with pSCA and pSMART2a empty vectors and (e) untransfected cells probed with both primary and secondary antibodies.

Probing the above cell lysates with rabbit anti-histone antibodies and secondary antibodies was used as a control for integrity of the cellular proteins.

2.5.1.2 Immunofluorescence and Confocal Microscopy

Slides with transfected cells (2.4.1) were probed with anti-NiV P58 and anti-guinea pig antibodies and the primary antibody was detected using anti-mouse, rabbit or guinea pig alexa fluor conjugated antibodies and examined for presence of specific fluorescence by confocal microscopy.

Controls used included: (a) unlabelled, untransfected cells; (b) untransfected cells probed with both primary and secondary antibodies; (c) untransfected cells probed with secondary antibody (anti-mouse or anti-guinea pig); (d) cells transfected with empty pSCA and labelled with secondary antibody only; (e) transfected NiV P-pCZ probed with anti-NiV guinea pig sera and secondary antibody; (f) transfected NiV P-pCZ probed with NiV P58 and secondary antibody; (g) NiV infected cells

probed with anti-NiV guinea pig sera; (h) NiV infected cells probed with NiV P58 and secondary antibody.

Cells were also probed with primary antibodies only, when a new lot was being used.

2.5.2 Expression of NiV V and W proteins and STAT1 in infected cells

2.5.2.1 Western Blot

Proteins from cell lysates (2.4.4) of infected cells (2.4.2) were separated by SDS PAGE, transferred onto the PVDF membrane (2.4.8), and probed with anti-NiV P58, anti-guinea pig, anti-STAT1 primary antibodies. The primary antibody was detected using anti-mouse, rabbit or guinea pig HRP conjugated secondary antibodies respectively and Sigma Fast 3,3 Diaminobenzidine tablets (Cat No. D4293) were used as a substrate (Table 1).

Controls used were: (a) Binary Ethyleneimine (BEI) inactivated NiV probed with primary and secondary antibody; (b) uninfected cell lysate probed with primary and secondary antibody; (c) infected cell lysate probed with secondary antibody only.

2.5.2.2 Immunofluorescence assay and Confocal Microscopy

Slides with infected cells (2.4.2) were probed with anti-NiV P58, anti-NiV guinea pig sera; anti-STAT1 primary antibody, detected by anti-mouse, anti-guinea

pig or anti-rabbit Alexa Fluor secondary antibodies, respectyively (2.4.10) (Table 1), and examined for presence of specific fluorescence by confocal microscope.

Controls included: (a) unlabelled, uninfected cells; (b) uninfected cells probed with both primary and secondary antibodies; (c) uninfected cells probed with secondary antibody only; (d) NiV infected cells probed with secondary antibody only; (e) NiV infected cells probed with anti-NiV guinea pig sera and secondary antibody; (f) NiV infected cells probed with NiV P58 and secondary antibody. Cells were also probed with primary antibodies only, when a new lot was being used.

2.5.3 Intracellular localization of the V and W proteins in NiV infected cells

2.5.3.1 Western Blot

Proteins from cytoplasm and extracted nuclei (both with and without nuclear membrane) (2.4.5, 2.4.6) of the infected cells (2.4.2) were separated on the SDS PAGE, transferred onto the PVDF membrane (2.4.8), and probed with anti-NiV P58, anti-STAT1 and anti-histone antibodies. The primary antibody was detected using anti-mouse or anti-rabbit HRP conjugated secondary antibodies and Sigma Fast 3,3 Diaminobenzidine tablets (Cat No. D4293) were used as a substrate (Table 1).

Control included: (a) BEI inactivated NiV probed with primary and secondary antibodies; (b) NiV infected whole cell lysates probed with primary and secondary antibodies; (c) cytoplasm and nuclei fractions probed rabbit anti-histone antibodies and secondary antibodies was used as a control for nuclei extraction, and protein

integrity. (Histone H3 is found associated with the nucleus) These antibodies were tested and were found to cross react with both porcine and human cells; (d) nuclei and cytoplasm from uninfected cells and probed with NiV P58 only; (e) nuclei and cytoplasm from uninfected cells probed with histone antibodies and secondary antibodies; (f) uninfected cell lysate probed with primary and secondary antibody; (g) infected cell lysate probed with secondary antibody only.

2.5.4 Co-localization of NiV P, V and W with STAT1 in cells infected with NiV

2.5.4.1 Immunofluorescence assays and Confocal Microscopy

Slides with infected cells (2.4.2) were probed with anti-NiV P58, anti-NiV guinea pig sera; anti-STAT1 primary antibody, detected by anti-mouse, anti-guinea pig or anti-rabbit Alexa Fluor secondary antibodies (2.4.10) (Table 1), and examined for presence of specific fluorescence by confocal microscope.

Controls used in localization of NiV V and W in cells infected with NiV and co-localization of NiV V and W with STAT1 included: (a) infected cells detected with anti-NiV guinea pig serum; (b) transfected cells with NiV V and W detected by NiV P58 mAB; (c) Negative controls included uninfected cells labelled with NiV P58 mAB anti-

NiV guinea pig serum or anti-STAT1 secondary antibody only; (d) Infected cells labelled with secondary antibody only.

Table 1: Antibodies and dilutions used for western blots and IFAs

Antibody	Species	Type	Source	Dilutions and Incubation time and temp	
				Western Blot	IFA
NiV P58	Mouse	Monoclonal	Produced by NCFAD	1:100 1 hour at room temperature	1:100 1 hour at room temperature or overnight at 4°C
Guinea Pig Sera	Guinea Pig	Polyclonal	From infected animals (NCFAD)	1:200 1 hour at room temperature	1:250 1 hour at room temperature or overnight at 4°C
Anti-STAT1	Rabbit	Polyclonal	Santa Cruz Biotechnology Cat No. Sc-346	1:500 1 hour at room temperature	1:200 1 hour at room temperature or overnight at 4°C
Anti-Histone, H3, CT, Pan, Clone A3S	Rabbit	Polyclonal	Upstate Cell Signalling Cat No. 05-928	1:500 1 hour at room temperature	N/A
Alexa Fluor 594 Anti-mouse	Goat	Fluorescent Red	Molecular Probes Cat No A11005	N/A	1:500 1 hour at room temperature
Alexa Fluor 488 Anti-rabbit	Goat	Fluorescent Green	Molecular Probes Cat No A11070	N/A	1:500 1 hour at room temperature
Anti-mouse	Goat	HRP	Southern Biotech Cat No. 103005	1:1000 1 hour at room temperature	N/A
Anti-Guinea Pig	Donkey	HRP	Chemicon International, AP193P	1:1000 1 hour at room temperature	N/A
Anti-rabbit	Goat	HRP	Imagenex, Cat No 20301	1:1000 1 hour at room temperature	N/A
Alexa Fluor 488 Anti-mouse	Goat	Fluorescent Green	Molecular probes Cat No A11017	N/A	1:1000 1 hour at room temperature
Alexa Fluor 594 Anti-guinea pig	Goat	Fluorescent Red	Molecular Probes Cat No A11076	N/A	1:1000 1 hour at room temperature
His-Tag Antibody	Mouse	Monoclonal	Novagen Cat No 70796	1:1000 1 hour at room temperature	1:2000 1 hour at room temperature
Anti-Flag M5 Antibody	Mouse	Monoclonal	Sigma, F4042	1:250 1 hour at room temperature	1:500 1 hour at room temperature

3. Results

3.1 The Capability of Human and Porcine Cells to Develop an Exogenous IFN α Induced Antiviral State against Nipah Virus

To determine the ability of porcine and human cells to develop an antiviral state to NiV using VSV was used as a control virus. VSV is sensitive to IFN (Vogel *et al,* 2001) and would determine if the cells can enter an antiviral state.

3.1.1 The Induction of an Exogenous IFN α Induced Antiviral State in Human and Porcine Cells against Vesicular Stomatitis Virus

In order to determine the ability of the studied cell lines to establish an antiviral state, and to select cells which would be used in further studies, VSV was used as a control virus. VSV was first titrated in each cell line. All cell lines which could support the growth of VSV (Table 2) were then used in a classical antiviral assay to test the cells' ability to establish an IFN α induced antiviral state.

Confluent cells of porcine or human origin were treated with the respective exogenous leukocyte IFN α and then challenged with VSV to determine how well IFN protects them against the virus. Cells were treated for 24 hours with IFN α in concentrations between 1U/ml and 320U/ml of IFN α. VSV reduction was calculated based on the number of plaques in each well as compared to the virus control.

Table 2 – VSV titres on porcine, human and non human primate cell types. Assays were carried out on 12 well plates and VSV dilutions (10^0 to 10^{-7}) were added at 400µl aliquots in duplicate wells to three individual plates per cell line (four wells total). Plates were analyzed between 2-5 days after infection.

Cell Type	Origin of Cells	Species	Titre (PFU/ml)
PK15	Kidney	Porcine	$1.23x10^7$
PT K75	Nasal Turbinate Mucosa	Porcine	$1.84x10^7$
Vero 76	Kidney	Non Human Primate	$3.79x10^7$
293	Kidney	Human	$4.39x10^7$
293T	Kidney	Human	$1.83x10^8$
MRC 5	Lung	Human	$5.75x10^7$
SJPL	Lung	Porcine	$4.93x10^7$
Caki-2	Kidney	Human	$3.53x10^5$
HeLa	Cervix	Human	$3.05x10^7$
WISH	Amnion	Human	$5.45x10^4$
ST	Testis	Porcine	$3.95x10^7$

Controls used in this assay included: (a) cell controls to test for cell growth, (b) cell-IFN controls where no virus and only IFN at different concentrations was added to cells to test for IFN toxicity to the cells and (c) virus back titrations to test the actual virus titre in comparison to the original calculated titre. Results of controls in these assays showed cell controls with no contamination of any type (bacterial, viral etc), cell-IFN controls with no toxic effects of IFN to cells and virus back titrations within range of expected titres. Controls were set up for each cell line used and the assay would be repeated if any of these controls had unexpected or suspicious results.

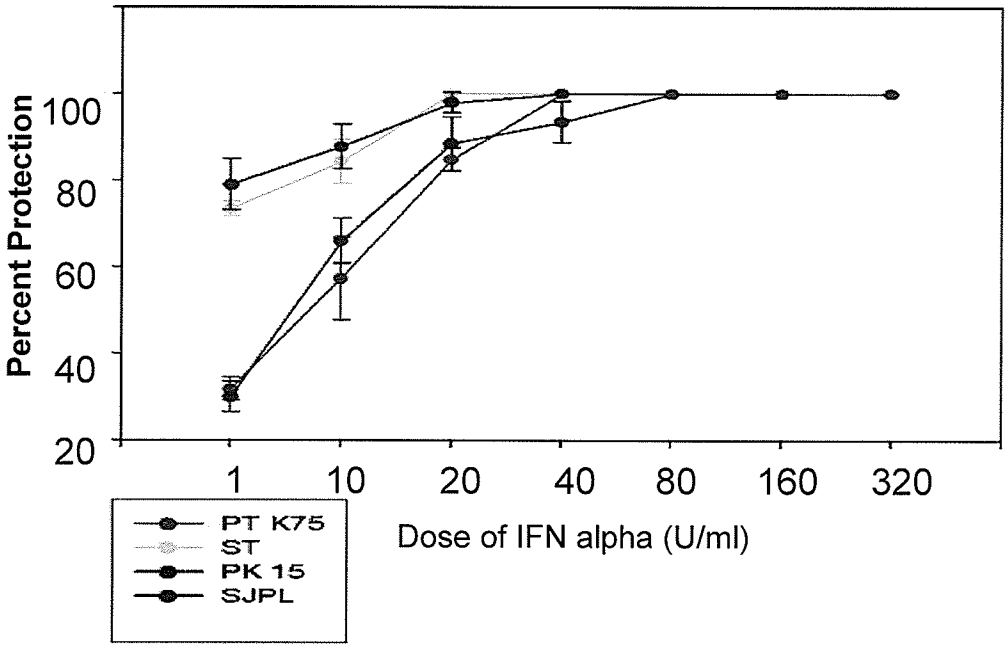
IFN α protection against VSV in various porcine and human cells is given in Figure 4 and mean and standard deviation of plaques produced is given in Table 3. These results have shown that of the porcine cells tested (Figure 4.A; Table 3), ST and PK15 cells needed the lowest amount of IFN to reach complete antiviral state at 20U/ml used, and SJPL cells needed a higher amount, 80U/ml to establish a 100% viral plaque reduction. PT K75 were able to establish an antiviral state at 40U/ml. Although these porcine cell lines differed in the amount of IFN α required to achieve an antiviral state, all four lines tested were able to do so against VSV at 24 hours using 320U of IFN.

Human cell lines (Figure 4B; Table 3) showed a different case where 293T were not able to establish an antiviral state, even at the highest concentration of IFN used (See Figure 4b). However, 293 and 293T were previously noted to have deficiencies in their ability to response to exogenous IFN and develop an antiviral state (Sarkis *et al*, 2006). MRC 5 cells established the antiviral state using 10U/ml and HeLa and WISH cells used a higher amount of IFN at 20U/ml and 80U/ml respectively.

Table 3: Mean and standard deviation of viral plaques produced for porcine, human and non human primate cell lines. Cells were treated with IFN α for 24 hours and challenged with VSV, after 24 hours. Results represent quadruplicate wells of three plates performed at three different times (12 wells in total).

		Vesicular Stomatitis Virus (VSV)															
		Plaque Number															
IFN α	**Cells**	**1U**		**10U**		**20U**		**40U**		**80U**		**160U**		**320U**		**Control**	
		Mean	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**
Porcine	PT K75	75.6	16.7	47.25	12.6	17.4	7.6	0	0	0	0	0	0	0	0	106	12.8
	ST	28.6	2.90	14.33	3.3	0	0	0	0	0	0	0	0	0	0	102	5.6
	PK 15	13.1	7.02	6.83	3.9	0.6	1.2	0	0.3	0	0	0	0	0	0	57.9	38.4
	SJPL	67.0	7.78	33.67	7.4	12.0	4.1	4.6	2.2	0	0	0	0	0	0	106	11.7
Human	MRC5	3.19	2.35	0.45	0.7	0	0	0	0	0	0	0	0	0	0	23.6	4.2
	WISH	78.2	3.39	63.83	3.5	35.3	6.3	8.6	3.7	0.3	0.6	0	0	0	0	106	8.7
	293	19.5	5.74	17.92	4.0	18.5	3.9	12.7	4.9	7.8	4.1	7.1	3.8	4.4	2.1	22.5	3.9
	HeLa	35.7	3.52	26.17	4.5	0.5	1.2	0	0	0	0	0	0	0	0	124	9.4
	293T	125	5.89	125.3	4.2	117	3.1	115	4.7	107	6.0	101	3.2	97.3	2.5	138	8.2
Porcine	Vero76	45.6	8.50	35.17	5.7	24.6	8.3	15.5	9.1	10.0	6.4	6.8	5.6	0	0	68.9	7.0
Human	Vero76	181	10.6	70.00	12.2	12.8	3.4	0.7	1.5	0	0	0	0	0	0	302	21.7

a) **Porcine cells treated with IFN alpha and challenged with VSV**



b) **Human cells treated with IFN alpha and challenged with VSV**

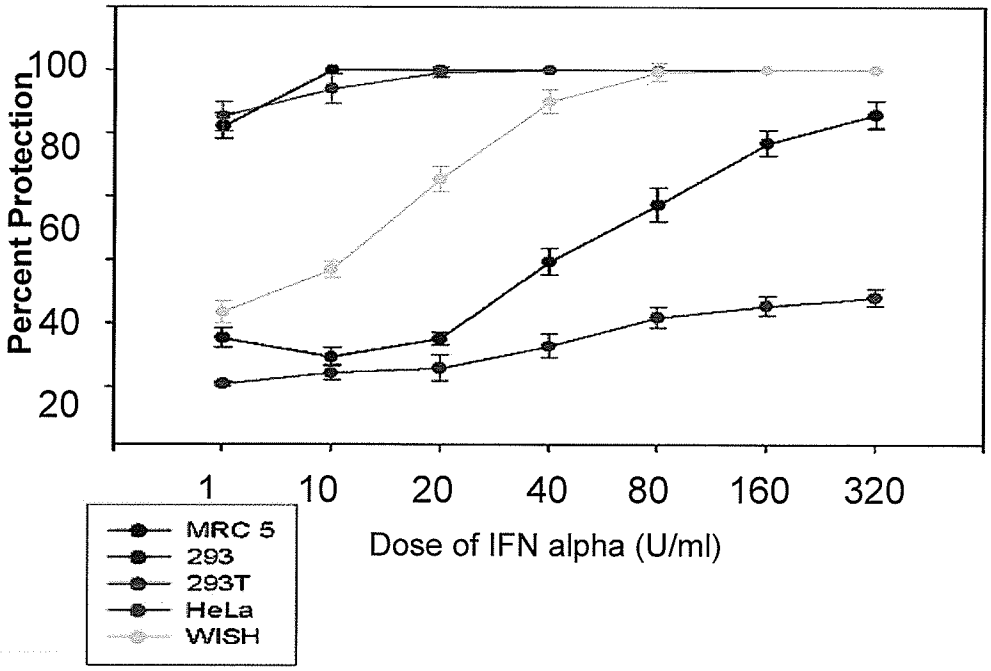


Figure 4: Selected porcine (a) and human (b) cells treated with exogenous IFN α for 24 hours and challenged with approximately 50 PFU/ml of VSV. Graphs show the mean percent protection of cells (from 12 replicates, 3 individual experiments) with increasing doses of IFN for human and porcine cell lines.

Human cells and porcine cells showed little difference between the need for exogenous IFN α to drive viral reduction. These results overall, indicated, no significant differences between cells of porcine and human host origins and allowed for a comparison of cells between hosts and organ types. From the tested cell lines, SJPL and MRC 5 derived from lung cells were chosen to determine if they can support the growth of NiV. If they would support the growth of NiV, they would be used in further NiV studies.

3.1.2 Establishment of an Exogenous IFN α Induced Antiviral State against NiV

To study the difference between porcine and human cell lines in the ability to establish an exogenous IFN α induced antiviral state against NiV, SJPL and MRC5 cells, which are both lung cells from pigs and humans, respectively, were selected based on the results in section 3.1.1. To determine if these cells could support the growth of NiV, the virus was plaque titrated on both cell lines. Only once virus growth was observed in both cell lines was the assay with NiV performed. This study was done in a similar manner to section 3.1.1 except that a time course was carried out to look specifically at the length of time of treatment and amount of IFN it would take to protect against NiV. Cells were treated with IFN at 3, 6, 12, 18, and 24 hours before virus challenge.

Controls used were exactly as in section 3.1.1. Again, if controls did not perform as expected the experiments were repeated.

Tables 4 and 5 show percent of viral protection for all time points, and IFN α concentrations used for induction of the antiviral state in SJPL and MRC5 cells. Figures 5 and 6 display the extreme and most important time points and IFN α doses used. Figure

Table 4 – Time course results for porcine IFN α protection against Nipah Virus on porcine SJPL cells at different concentrations of IFN by classical IFN assays. Virus was added to cells at a concentration to produce 50PFU/ml. Viral reduction was based on a virus control with no IFN added. Results were based on 12 replicates (Four wells per plate, three plates per cell line) and reduction was a mean percentage of NiV PFU reduction compared to the virus control.

Time (hrs)	Mean virus titre reduction						
	1U	10U	20U	40U	80U	160U	320U
3	11.49	13.62	29.79	31.49	34.47	28.69	48.92
6	5.12	24.19	28.37	40.47	51.63	53.24	81.21
12	15.68	53.51	49.73	63.24	69.73	74.30	85.32
18	19.20	38.95	53.10	55.60	63.54	92.11	100.0
24	22.15	38.95	42.21	70.03	69.21	86.82	100.0

Table 5 - Time course results for human IFN α protection against Nipah Virus on human MRC5 cells at difference concentrations of IFN α by classical IFN assays. Virus was added to cells at a concentration to produce 50PFU/ml. Virus reduction was based on a virus control with no IFN added. Results were based on 12 replicates (Four wells per plate, three plates per cell line) and reduction was a mean percentage of NiV reduction compared to the virus control.

Time (hrs)	Mean virus titre reduction						
	1U	10U	20U	40U	80U	160U	320U
3	+9.3	12.79	19.77	30.2	17.44	15.81	14.32
6	7.9	30.69	35.64	50.50	43.56	48.11	47.72
12	1.45	8.70	17.39	21.74	40.12	48.79	59.48
18	11.97	40.46	44.21	54.11	53.27	57.71	64.56
24	17.15	18.43	46.41	50.37	74.08	79.48	100

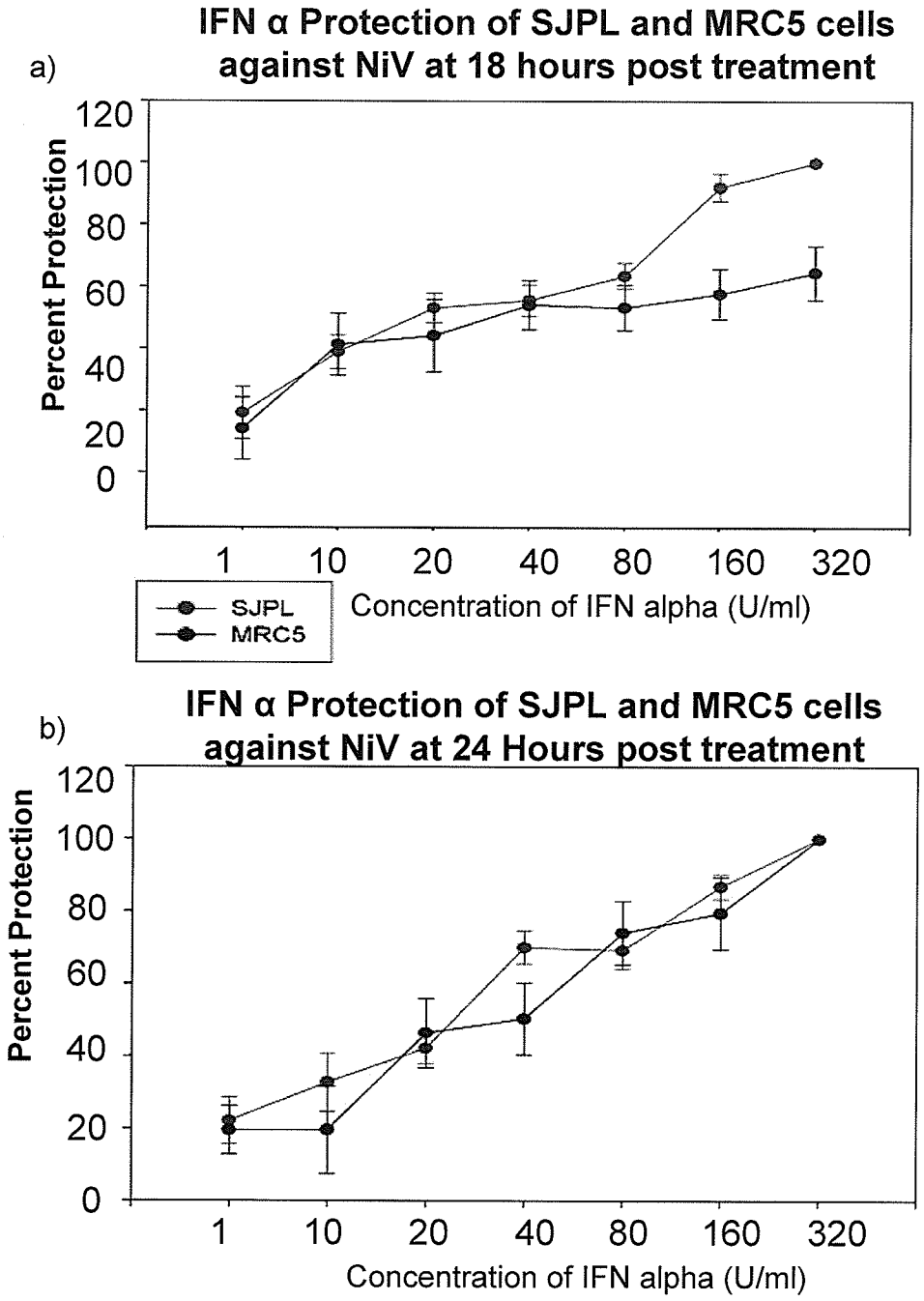


Figure 5: Mean protection of SJPL and MRC5 cells by IFN α against Nipah Virus at concentrations between 1 U/ml to 320U /ml of IFN at 18 hours (a) and 24 hours (b) of IFN α treatment. Results are based on 12 replicate wells for each cell line. (3 experiments). Virus was added at a concentration to produce approximately 50 PFU/ml.

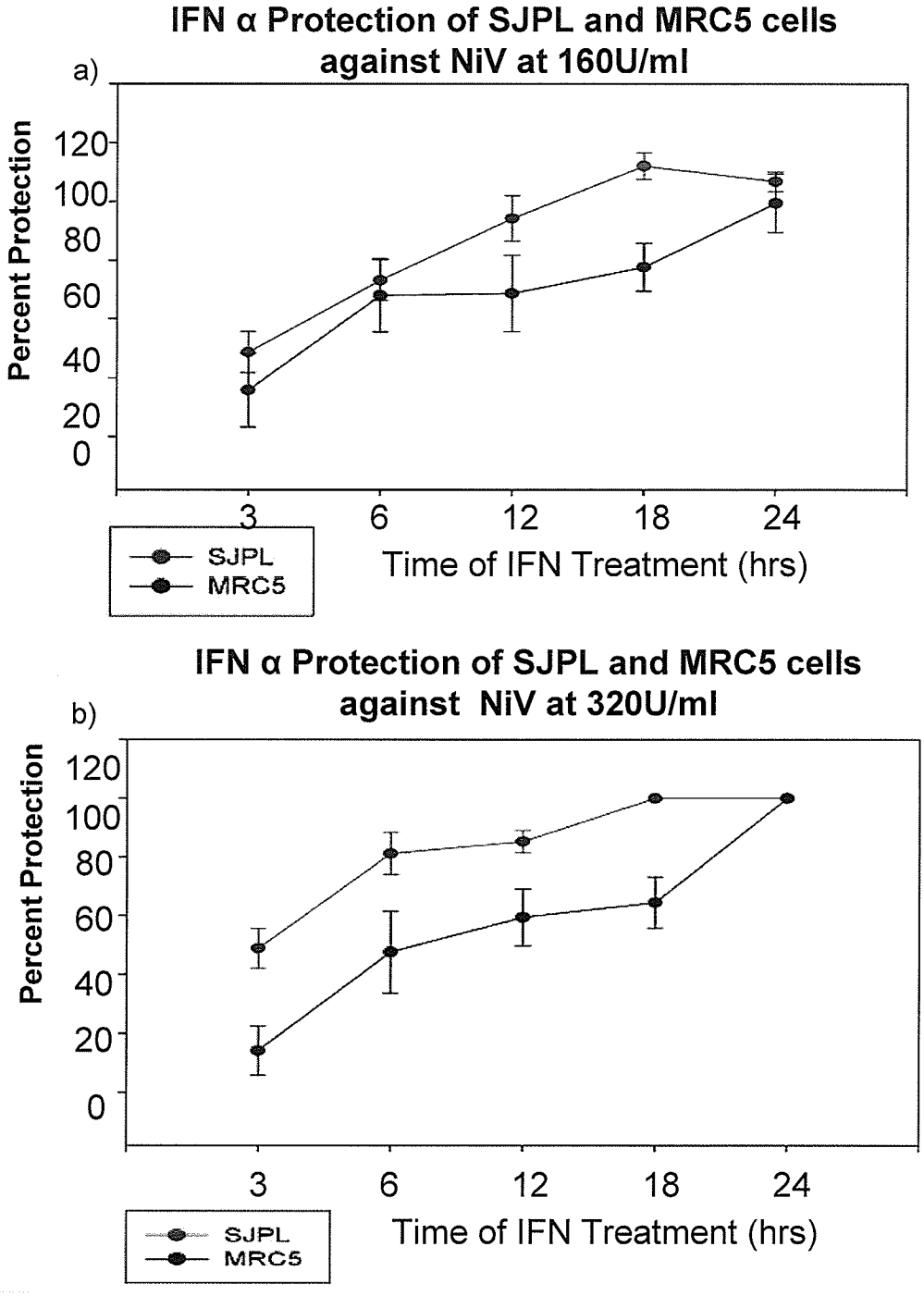


Figure 6: Mean protection of SJPL and MRC5 cells by IFN α against Nipah Virus at 3, 6, 12, 18 and 24 hours after treatment when 160 U/ml (a) and 320 U/ml (b) of IFN α was used. Results are based on 12 replicate wells for each cell line. Virus was added at a concentration to produce approximately 50 PFU/ml.

5 and 6 indicate that SJPL cells show beginning signs of an antiviral state as early as 3 hours after treatment (Figure 6) and virus reduction for all IFN doses was always higher than MRC 5 cells (Figure 5). SJPL cells were able to achieve 92% viral reduction at 160U/ml of IFN used (18 hours post treatment with IFN α) where MRC5 cells were unable to enter a complete antiviral state until 320U/ml of IFN were used for 24 hours prior to virus challenge. These results have shown that SJPL cells were quicker at establishing an antiviral state at both an earlier time and a lower dose of IFN as compared to MRC 5 cells.

As compared to VSV results, the MRC5 cells established viral reduction faster against VSV than NiV. SJPL cells showed similar results where the cells treated with IFN α had quicker reduction of VSV as compared to NiV. These results show that NiV may be a more difficult virus to eliminate early, as compared to VSV. The biological significance cannot be defined here as only small difference in time and IFN concentration was detected between the two cell lines studied.

3.2 The Expression of the NiV V and W Proteins

Previous work on the NiV V and W proteins in the recombinant system using human cells has shown that NiV V localizes to the cytoplasm and NiV W localizes to the nucleus (Shaw *et al*, 2004, 2005, Rodriguez *et al*, 2004). However, no studies on NiV V and W expression and localization using live NiV have been published to date.

3.2.1 Recombinant Expression and Localization of NiV V and NiV W Proteins

The recombinant system was used in our work as a reference to the published work (Rodriguez *et al*, 2004; Shaw *et al*, 2005) In addition this system was used to determine whether the P58 monoclonal antibody produced in our laboratory which recognizes NiV P protein, will also detect the V and W proteins.

3.2.1.1 Cloning of NiV V and NiV W Coding Sequences

NiV V and NiV W were PCR cloned from the pCZ vector (Figure 7) into pSCA and pSMART2a, Semliki Forest Virus (SFV) based expression vector (Figures 8 and 9, respectively). The SFV based vectors were used because they can have high expression of the cloned protein in the cytoplasm, and the pSMART2a vector contains N-terminal HIS and Flag tags for protein identification. The obtained PCR products had expected sizes of 1368 (NiV V) and 1360 (NiV W) bp, in agreement with previously published data, (Figure 7).

Figure 8 shows the results of the restriction analysis of the pSCA vector with the inserted NiV V cDNA and the pSMART2a vector with the inserted NiV V cDNA. All plasmids screened were digested with the restriction enzyme Bam HI and positive clones would show band sizes of 1.37Kb and 11.2Kb for NiV V inserted into pSCA, and 1.37Kb and 12.4Kb for pSMART2a. Controls used were pSCA and pSMART2a vectors, where the vector gave sizes of 11.2Kb (pSCA) and 12.4 (pSMART2a) when digested. NiV W

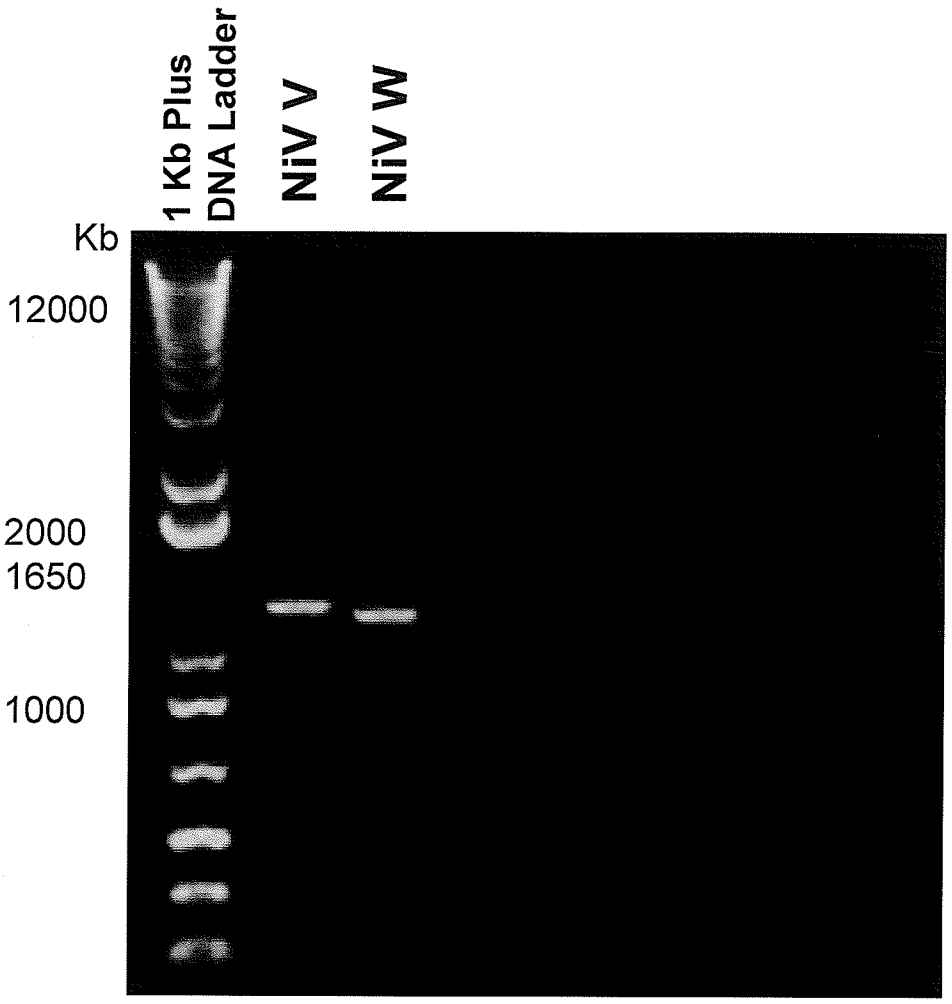


Figure 7: NiV V and NiV W PCR amplified cDNA products from pCZ cloning vector. Products are visualized on a 1% agarose gel with ethidium bromide. Expected band sizes are 1.37 Kb for NiV V and 1.35 Kb for NiV W which is compared to a 1 KB Plus DNA ladder (lane 1).

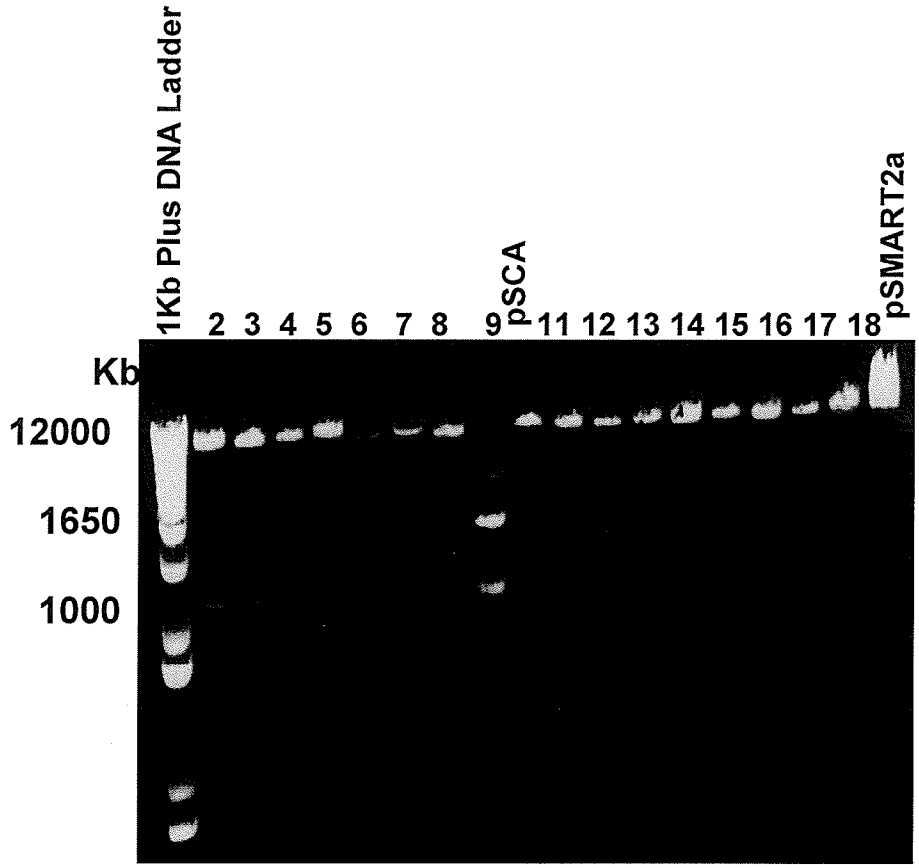


Figure 8: Restriction digest analysis of the pSCA vector with the inserted NiV V cDNA (lanes 2-9) and the pSMART2a vector with inserted NiV V cDNA (lanes 11-18). Plasmids were cut with Bam HI and expected band sizes were 1.37 Kb and 11.2 Kb (pSCA) and 1.37 Kb and 12.4 Kb (pSMART2a). Positive clones are found in lanes 2, 3 and 8 (pSCA) and lanes 12, 16 and 18 (pSMART2a).

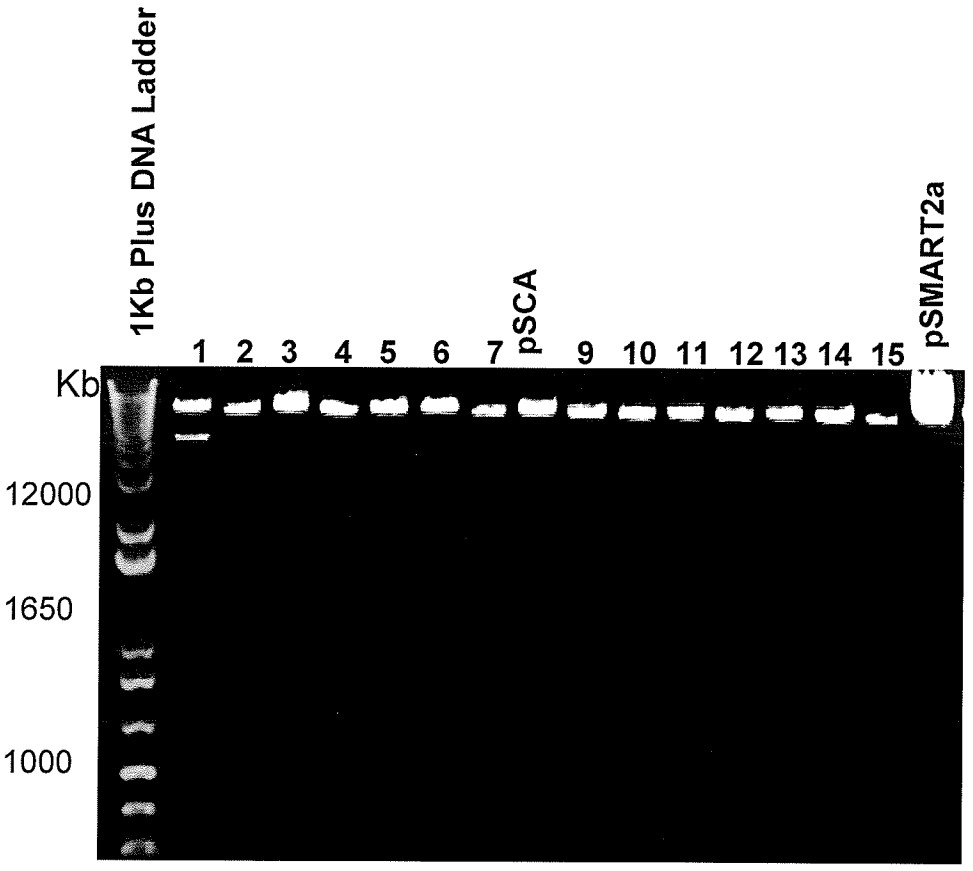


Figure 9: Restriction digest analysis of the pSCA vector with the inserted NiV W cDNA lanes 1-7 and pSMART2a vector with the inserted NiV W cDNA (9-15) . Plasmids were cut with Bam HI and expected band sizes were 1.35Kb and 11.2Kb (pSCA) and 1.35Kb and 12.4Kb (pSMART2a). Positive clones are found in lane 4 (pSCA) and lanes 9 and 14 (pSMART2a).

plasmids were screened similarly to NiV V (including controls) and results after digestion with Bam HI can be seen in Figure 9. These results show that putative positive clones are seen in lane 4 (pSCA) and 9 and 14 (pSMART2a) where band sizes when digested with Bam HI are 1.35Kb and 11.2Kb. Sequencing of these genes were carried out at NCFAD and the obtained sequences for these clones showed 100% homology with published NiV gene nucleotide sequences found in NCIB (Chua *et al*, 2000; Harcourt *et al*, 2001, accession number AY988601).

3.2.1.2 Expression of NiV V and NiV W Proteins

The obtained expression vectors were used in further experiments where DNA was transfected into cells and translated recombinant proteins were detected using anti-NiV P58, anti-NiV guinea pig polyclonal serum or anti-HIS or FLAG tagged antibodies by Western blot and IFA . The IFA was at the same time used in localization of the expressed recombinant proteins by confocal microscopy (section 3.2.1.3.)

To determine the reactivity of the NiV P58 MAb with NiV V and NiV W proteins, as well as to determine the expression of NiV V and W from the pSCA and pSMART2a vectors, BHK-21 cells were transfected with each plasmid individually and cell lysates were harvested at 24 and 48 hours. Controls used were NiV P-pCZ (positive control) and transfected empty pSCA or pSMART2 vectors and untransfected cells. Anti-HIS and anti-FLAG antibodies were also tested for their reactivity to NiV V/W in the pSMART2a vector. The lysates were loaded onto an SDS PAGE gel and analyzed for protein expression and P58 reactivity by western blot (Figure 10).

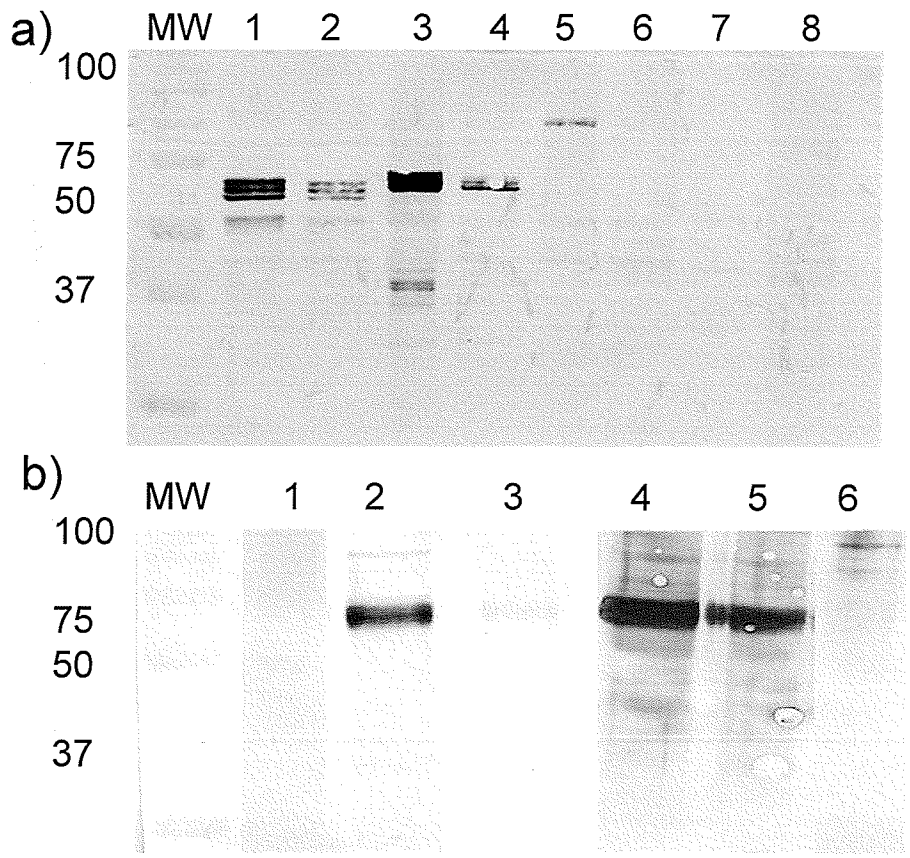


Figure 10: Western blot detecting expression of NiV V and W proteins from the pSCA (a) and pSMART2a (b) vectors separated on a 10% gel. NiV W Plasmids were transfected (4μg) into BHK-21 cells and analyzed for expression at 24 hours and 48 hours (pSCA only) post transfection. Order of gel (a) NiV V-pSCA, 24 hrs (lane 1), NiV W-pSCA, 48 hrs (lane 2), NiV V-pSMART2a, 24 hrs (lane 3), NiV W-pSMART2a, 48 hrs (lane 4), NiV P-pCZ (lane 5), pSCA vector (lane 6), pCZ vector (lane 7), untransfected BHK-21 cells (lane 8). B) pSMART2a vector (lane 1), NiV W-pSMART2a detected by anti-HIS (lane 2), NiV W-pSMART2a detected by anti-FLAG (lane 3), NiV W-pSMART2a detected by P58 (lane 4), NiV V-pSMART2a detected by P58 (lane 5), BEI inactivated NiV (lane 6). NiV Proteins were detected by P58 MAb (1:100), anti-His antibody (1:1000) or anti-FLAG antibody (1:250) (NiV W-pSMART2a only). Presence of three bands detected by the antibody would be consistent with identification of two phosporylaiton sites on the P/V/W proteins (UniProt, 2008). NiV V-pSCA Assay was performed in duplicate where each was carried out separately at different times.

NiV P58 detected a protein band at ~70kDa (lane 6) and untransfected and vector control lanes did not show any protein expressed that was detected by NiV P58. Bands are seen at approximately 45.6 kDa and 45 kDa which represent NiV V and NiV W, respectively. This western blot confirms that NiV V and NiV W are expressed from the SFV expression vectors and are detectable by NiV P58, as well as by both the anti-HIS and anti-FLAG tag antibodies (pSMART2a vector). The above results show that the P58 monoclonal antibody can be used for NiV V/W detection in further experiments.

3.2.1.3 Localization of Recombinant NiV V and NiV W Proteins

Previous studies have shown that recombinant, NiV V and W proteins are found within the cytoplasm and nucleus, respectively (Shaw *et al*, 2005). To confirm these findings, NiV V-pSCA and NiV W-pSCA plasmids were transfected into BHK-21 cells seeded on glass microscope slides. For this assay BHK-21 cells were chosen because they are highly transfectable cells and can easily be visualized by confocal microscopy. Cells were transfected with 12μg of DNA using Lipofectamine 2000, harvested and labelled at 24 and 48 hours post transfection. Localization was determined by immunofluorescence assays and confocal microscopy. Negative controls (see section 2.5.1) used in this experiment were negative as expected. Positive controls used, were (a) transfected NiV P-pCZ detected by anti-NiV guinea pig sera; (b) transfected NiV P-pCZ detected by NiV P58. Positive controls (a) and (b) can be seen in Figure 12. Positive controls (a) and (b) can be seen in Figure 12. Recombinant NiV P was localized solely in

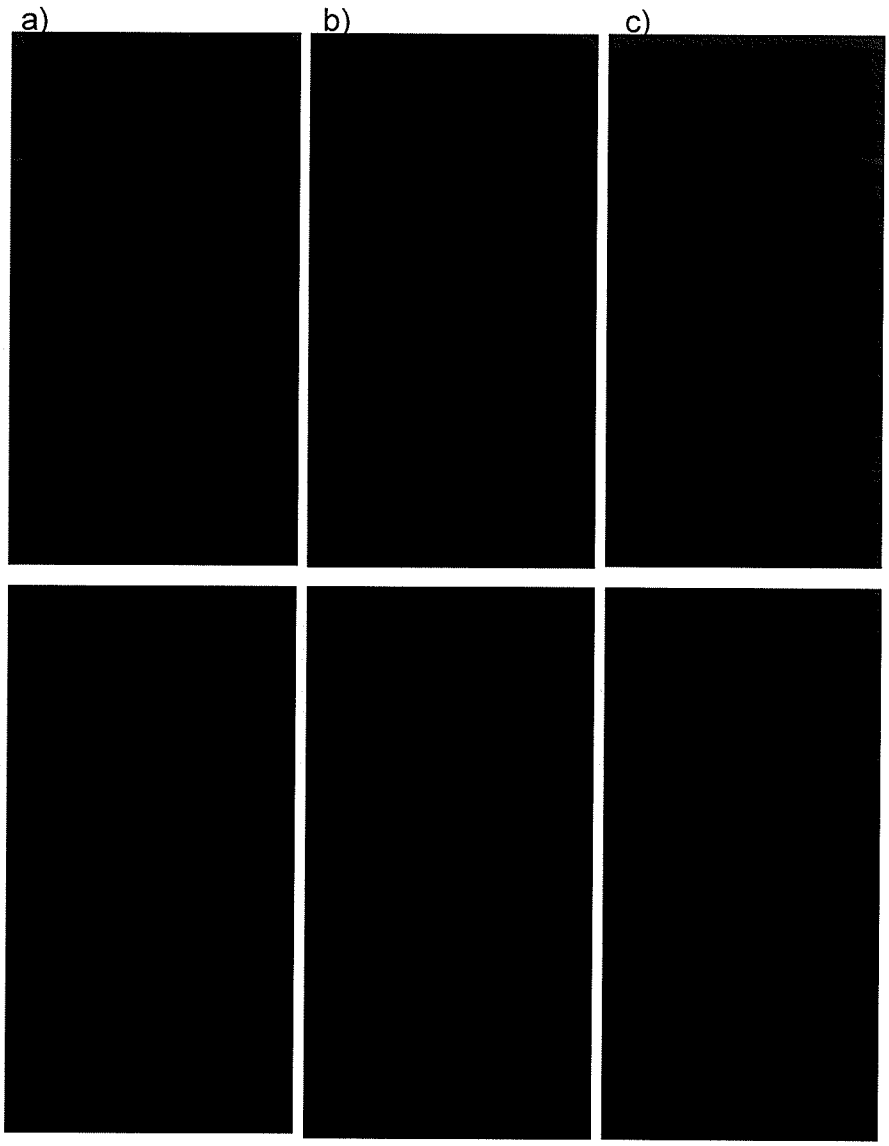


Figure 11: Localization of NiV V (bottom panels) and NiV W (top panels) as visualized by confocal microscopy using the recombinant system (pSCA vector). NiV plasmids were transfected at 12μg into BHK-21 cells and stained at 24 hours (a,c) and 48 hours (b) and detected with NiV P58 MAb (1:100) (a,b) and anti-NiV guinea pig serum (1:200) (c). Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. All images are 30X Magnification.

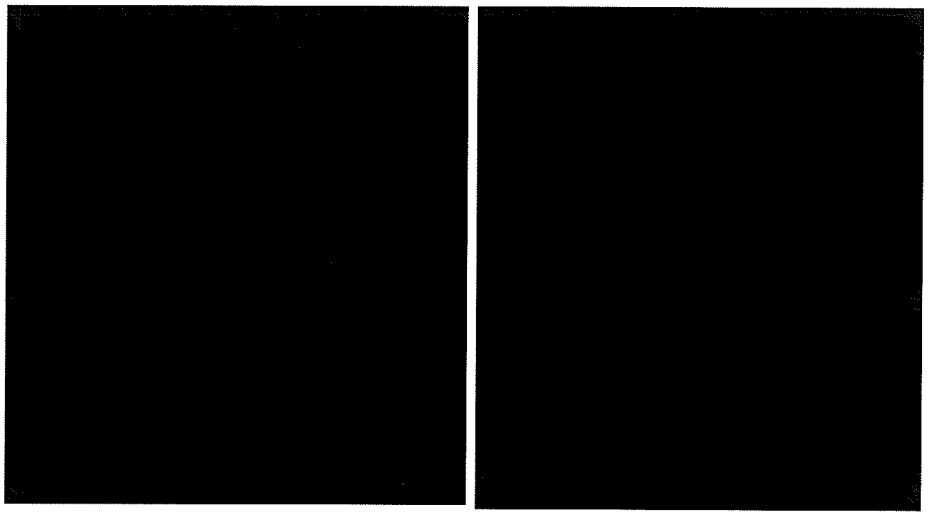


Figure 12: Localization of NiV P as visualized by confocal microscopy using the recombinant system. BHK-21 cells were transfected with NiV P-pCZ (12μg of DNA) and labelled with NiV P58 (1:100) (right) MAb and anti-NiV Guinea Pig polyclonal antibody (left) at 24 hours. Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. Magnifications: left 10X, right 20X.

the cytoplasm as expected based on published data (Roderiguez *et al,* 2003; Shaw *et al,* 2005). Figure 11 shows the IFAs of BHK-21 cells transfected with NiV W-pSCA and NiV V-pSCA. Recombinant NiV W protein was detected by both the guinea pig serum and NiV P58 MAb within the nucleus only. The NiV V-pSCA expressed protein was found in the cytoplasm of transfected BHK-21 cells confirming work by Shaw and colleagues (2004). There was no difference in the localization of NiV V and NiV W recombinant proteins between 24 and 48 hour time points.

3.2.1.4 Expression and Localization of NiV V and NiV W Proteins in Porcine and Human Cells Infected with NiV

To date localization and expression of the Nipah virus accessory proteins V and W (and C) have not been looked at within an infectious system with live virus. As mentioned, recombinant systems are not always accurate. Due to this it was necessary to look at the expression and localization of NiV V and W proteins in porcine and human cells infected with NiV.

The expression and localization of NiV V and W within an infectious system was studied under BSL 4 conditions to determine if these two proteins are being expressed during an infection and whether there are differences between human and porcine cells. Since the only available monoclonal antibody, the mouse monoclonal antibody P58, recognized three proteins derived from the P gene: P, V, and W, the expression of the proteins had to be studied in conjunction with their localization. Based on the recombinant system data NiV V and P proteins were expected to localize to the cytoplasm, while the W protein would localize to the nucleus (Shaw *et al*, 2005,

Rodriguez *et al*, 2004). NiV V and W are almost the same size (around 45kD) and cannot be distinguished based on apparent molecular size while the P protein is significantly larger (around 70kD).

We hypothesized, that western blot performed on cytoplasm and nuclei separately would allow to determine expression of all the proteins: 45 kD W protein in nucleus, 45kD V protein in cytoplasm, and 70kD V protein in cytoplasm. Immunofluorescence and confocal microscopy would confirm expression/localization of the W protein in the nucleus, and localization of the V and P proteins in the cytoplasm, although these cytoplasmic proteins would not be differentiated from each other in an IFA.

3.2.1.4a. Immunofluorescence

SJPL, ST, HeLa and MRC5 cells grown on microscopic slides were infected with NiV at an MOI of 0.1. The cells were probed with P58 MAb to visualize proteins in the cytoplasm and nucleus of the above cell lines.

Controls used in these experiments included positive controls: transfected cells with NiV V and W detected by NiV P58 Mab. Negative controls included uninfected cells labelled with NiV P58 mAB or anti-NiV guinea pig serum or secondary antibody only and infected cells labelled with secondary antibody only. Negative controls showed no fluorescence, and positive controls had fluorescence for transfected NiV V (cytoplasmic) and NiV W (nucleus). Infected cells probed with anti-NiV guinea pig serum had signal throughout the cytoplasm of the cell, but none in the nucleus.

Results for IFAs can be seen in Figure 13 and 14. The results obtained from these experiments were unexpected. There was no localization of NiV proteins recognized by P58 in the nucleus for any porcine or human cell line, and the immunostaining in all the cell lines suggested cytoplasmic localization only of the proteins recognized by P58 monoclonal antibody. Counterstaining of nuclei was not completed here as this option was not available at the time this experiment was carried out. Instead a western blot was perfomed on nuclear and cytoplasmic fractions from lysed NiV infected cells.

3.1.2.4b. Western Blot

Confluent SJPL, ST, HeLa and MRC5 cells were infected with NiV at an MOI of 1, and the nuclei and cytoplasm were extracted after 1 dpi. A portion of the nuclei fractions were passed through a syringe in an attempt to extract nuclear membranes. This was done to eliminate any possible nuclear membrane association of these proteins. Samples were run on an SDS PAGE gel and western blots were carried out to detect proteins in each compartment and to differentiate proteins based on size. NiV P58 antibodies were again used to detect the NiV V/W/P proteins. In addition rabbit anti-histone H3 antibodies were used as a positive extraction control for the nucleus.

Protein assays were also performed to quantify total protein in each compartment as compared to a BSA standard to ensure that an equal amount of protein was loaded for each fraction. The results for protein quantity can be found in appendix C3-C5. For all other controls see section 2.5.3.

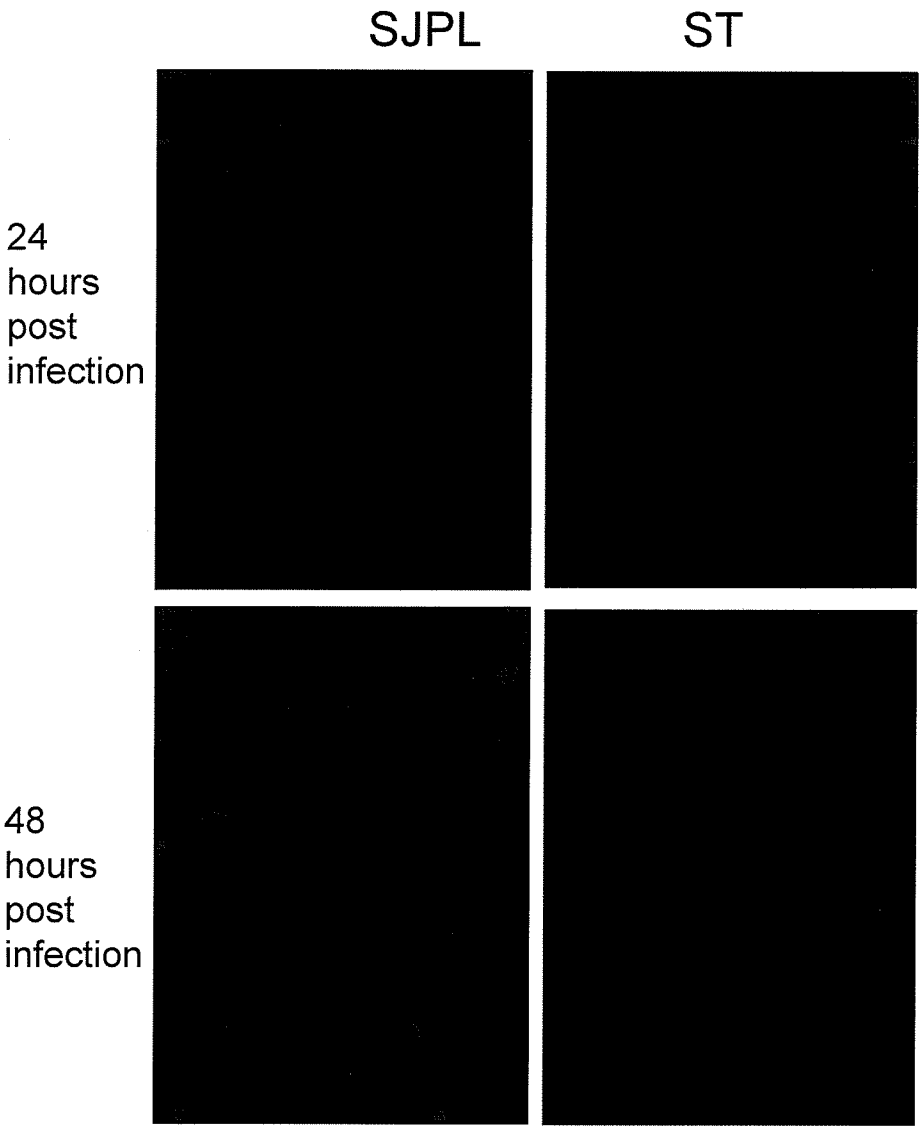


Figure 13: Nipah Virus infected porcine SJPL (left) and ST (right) cells (MOI of 0.1) at 24 (top) and 48 (bottom) hours after infection with NiV. The Nipah virus proteins were detected with the NiV P58 Mab antibody (1:100). The secondary antibody used for detection was an Alexa Fluor 594 which emits a red fluorescence when excited. Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. SJPL and ST 24 hours are shown in 20X magnification and ST 48 hours are in 10X magnification.

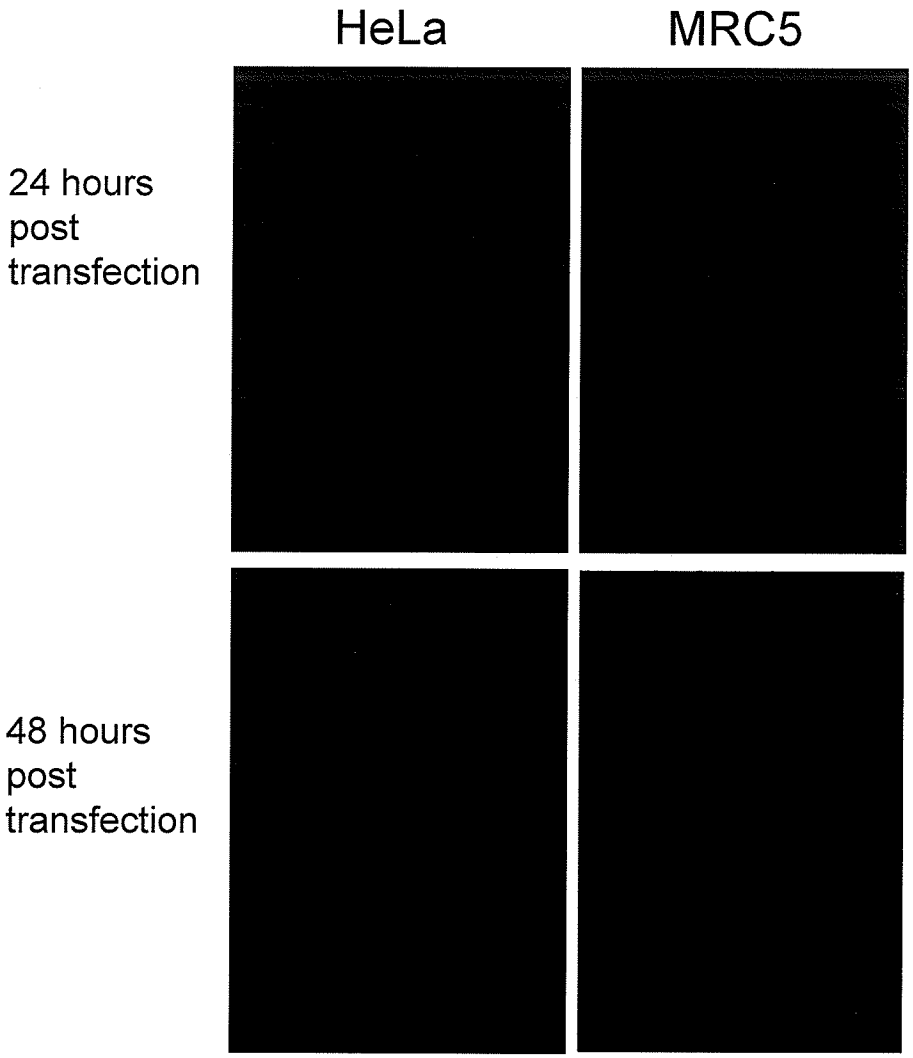


Figure 14: Nipah Virus infected human HeLa (left) and MRC5 (right) cells (MOI of 0.1) at 24 (top) and 48 (bottom) hours after infection with NiV. The Nipah Virus proteins were detected with the NiV P58 Mab antibody (1:100). The secondary antibody used for detection was an Alexa Fluor 594 which emits a red fluorescence when excited. Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. MRC5 24 hour slides are shown in 10X magnification, all other are 20X.

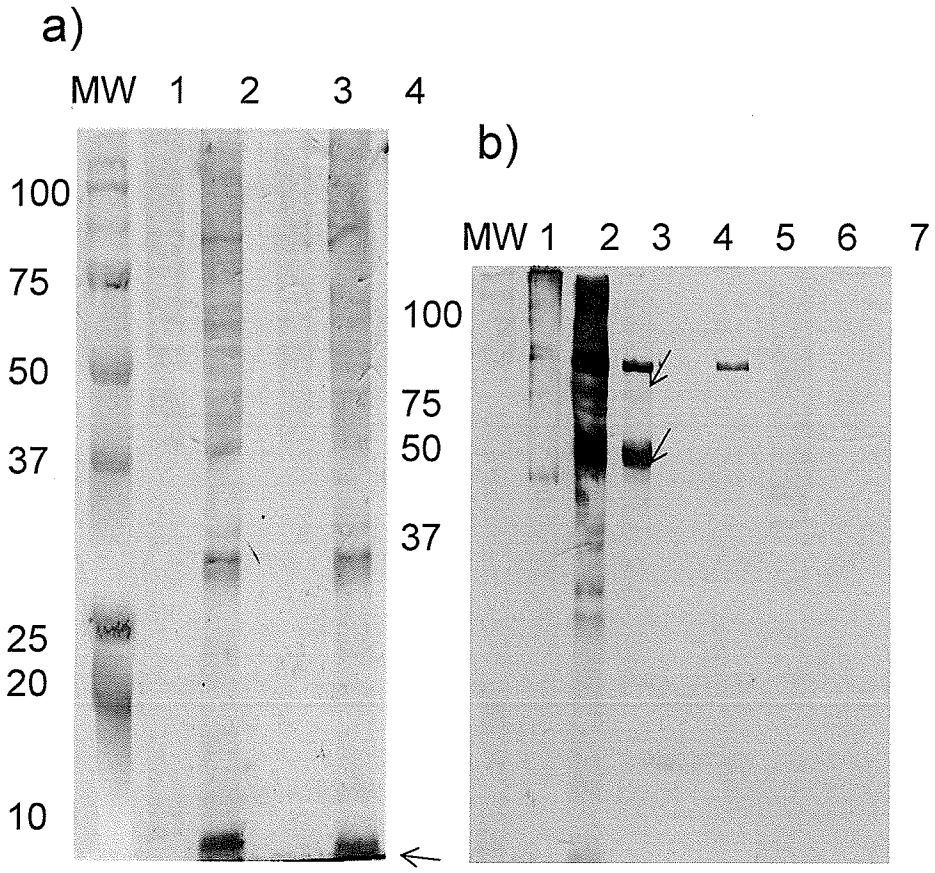


Figure 15: Western blot of extracted nucleolar and cytoplasm proteins from HeLa cells from uninfected or infected . Cells (MOI of 1) with NiV separated on a 10% resolving gel. (a) Nuclei (lanes 2 and 4) and cytoplasm (lane 1 and 3) of infected (lanes 1 and 2) and uninfected cells (lanes 3 and 4) were probed with rabbit anti-histone H3 (1:500) antibodies (b) Nuclei containing membranes (lane 5), nuclei with no membranes (lane 4) and Cytoplasm (lane 3) from NiV infected cells after 1dpi and probed with mouse monoclonal antibody NiV P58 (1:100). Controls: BEI inactivated NiV (lane 1), Infected whole cell lysate (lane 2), uninfected nuclei (lane 6), and uninfected cytoplasm (lane 7). Precision Plus Protein Ladder (Bio-Rad) indicate Molecular weight (MW) at the left of each gel. Blots were prepared in duplicate for each experiment. Arrows indicate proteins of interest (a) Histone (b) NiV P and NiV V/W.

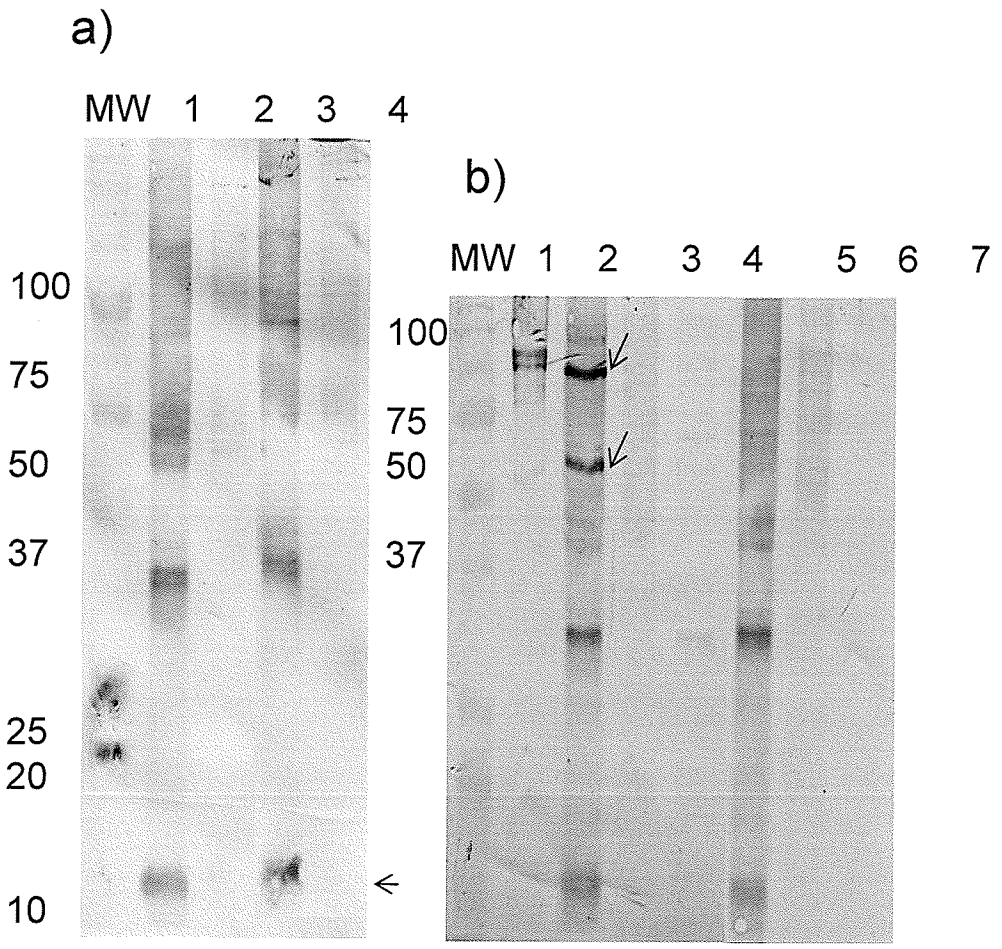


Figure 16: Western blot of extracted nucleolar and cytoplasm proteins from MRC5 cells from uninfected or infected Cells (MOI of 1) with NiV separated on a 10% resolving gel. (a) Nuclei (lanes 1 and 3) and cytoplasm (lane 2 and 4) of infected (lanes 1 and 2) and uninfected cells (lanes 3 and 4) were probed with rabbit anti-histone H3 (1:500) (b) Nuclei containing membranes (lane 3), nuclei with no membranes (lane 4) and Cytoplasm (lane 2) from NiV infected cells after 1dpi and probed with mouse monoclonal antibody NiV P58 (1:100). Controls: Infected whole cell lysate (lane 1), uninfected whole cell lysate (lane 5), uninfected cytoplasm (lane 6) and uninfected nuclei (lane 7). Precision Plus Protein Ladder (Bio-Rad) indicate Molecular weight (MW) at the left of each gel. Blots were prepared in duplicate for each experiment. Arrows indicate proteins of interest (a) Histone (b) NiV P and NiV V/W.

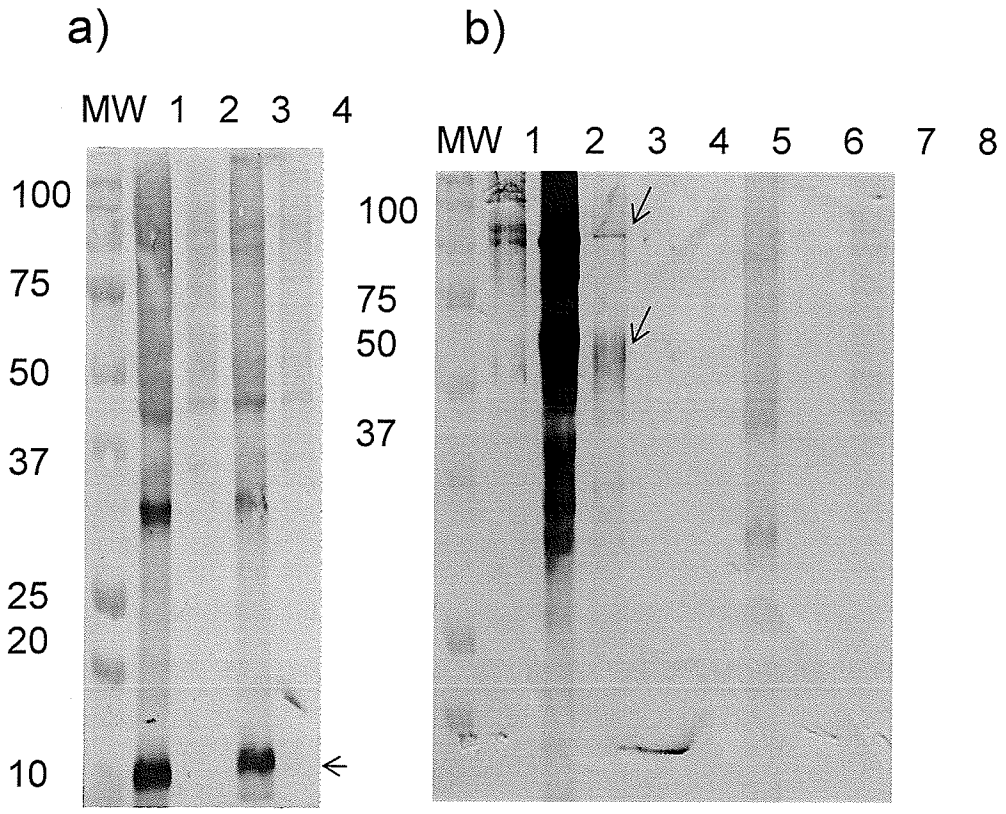


Figure 17: Western blot of extracted nucleolar and cytoplasm proteins from SJPL cells from uninfected or infected Cells (MOI of 1) with NiV separated on a 10% resolving gel. (a) Nuclei (lanes 1 and 3) and cytoplasm (lane 2 and 4) of infected (lanes 1 and 2) and uninfected cells (lanes 3 and 4)were probed with rabbit anti-histone H3 (1:500) antibodies (b) Nuclei containing membranes (lane 5), nuclei with no membranes (lane 4) and cytoplasm (lane 3) from NiV infected cells after 1 dpi and probed with mouse monoclonal antibody NiV P58 (1:100). Controls: BEI inactivated NiV (lane 1), Infected whole cell lysate (lane 2), uninfected whole cell lysate (lane 6), uninfected nuclei (lane 7), and uninfected cytoplasm (lane 8). Precision Plus Protein Ladder (Bio-Rad) indicate Molecular weight (MW) at the left of each gel. Blots were prepared in duplicate for each experiment. Arrows indicate proteins of interest (a) Histone (b) NiV P and NiV V/W.

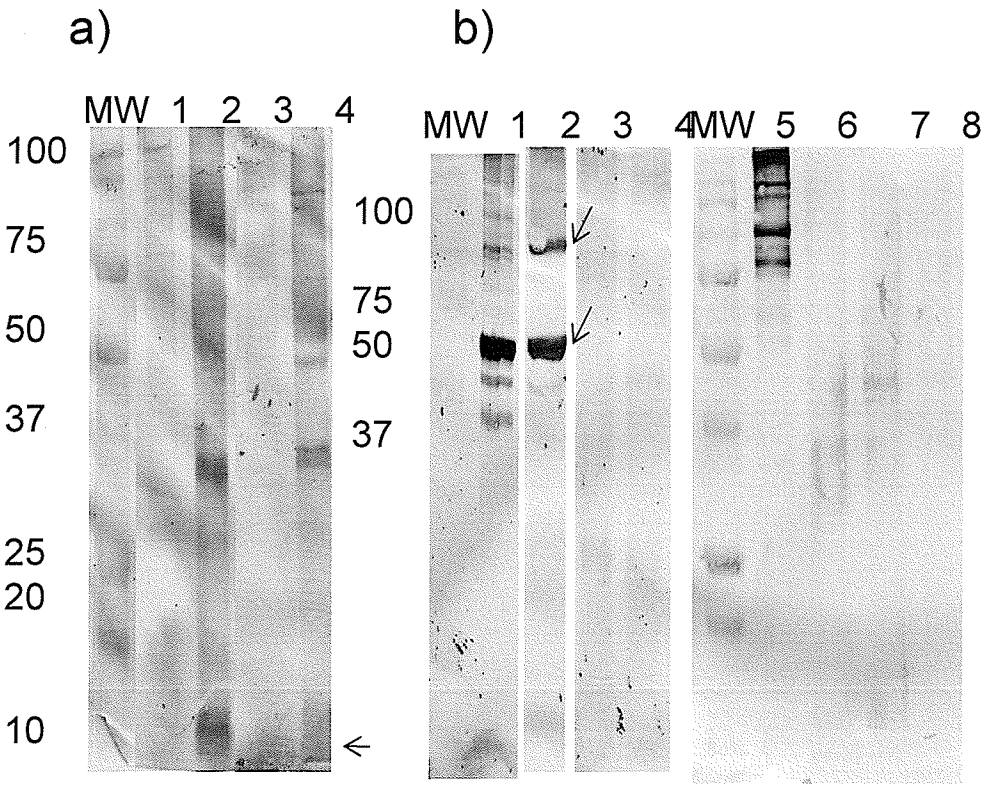


Figure 18: Western blot of extracted nucleolar and cytoplasm proteins from ST cells from uninfected or infected Cells (MOI of 1) with NiV separated on a 10% resolving gel. (a) Nuclei (lanes 2 and 4) and cytoplasm (lane 1 and 3) of infected (lanes 1 and 2) and uninfected cells (lanes 3 and 4)were probed with rabbit anti-histone H3 (1:500) (b) Nuclei containing membranes (lane 3), nuclei with no membranes (lane 4) and Cytoplasm (lane 2) from NiV infected cells after 1 dpi and probed with mouse monoclonal antibody NiV P58 (1:100). Controls: BEI inactivated NiV (lane 5), Infected whole cell lysate (lane 1), uninfected whole cell lysate (lane 6), uninfected nuclei (lane 7), and uninfected cytoplasm (lane 8). Precision Plus Protein Ladder (Bio-Rad) indicate Molecular weight (MW) at the left of each gel. Blots were prepared in duplicate for each experiment. Arrows indicate proteins of interest (a) Histone (b) NiV P and NiV V/W.

Results from the nuclei and cytoplasmic extraction western blots can be found in figures 15 for the HeLa cells, 16 for the MRC5 cells, 17 for the SJPL cells and 18 for the ST cells. The histone probed western blots confirmed that separation of these compartments was successful (arrow at approximately 10kDa band in sections (a) of Figures 15 – 18). Other bands seen in these fractions indicate phosphorylation of the histone proteins (Mochizuki *et al*, 2008). The blots probed with P58 showed that in all cell lines, the only proteins detected were in the cytoplasm: bands in all cell lines represented NiV P protein (approximately 70.9kDa) and bands were also seen with in the region of about 40 to 45 kDa which would represent NiV V and/or NiV W (arrows in the section (b) of Figures 15 – 18). None of these bands were seen in either nuclei fraction (those with membrane and those without membranes), for any cell line used. The only exception that was seen was that for HeLa

cells where in the fraction that contained the nuclei membrane, a band representing NiV P was seen.

The non specific bands were differentiated from the specific ones based on the uninfected fraction controls. These results cofirmed the IFA results that NiV P/V/W proteins are not found in the nuclei and only in the cytoplasm, contrary to published recombinant work (Shaw *et al*, 2005; Rodriguez *et al*, 2003). NiV V and NiV W are very close in amino acid size (456 amino acids for NiV V and 450 amino acids for NiV W). Due to this, it is difficult to determine if the positively stained 45kDa cytoplasmic proteins are either NiV Vor NiV W protein or both.

In summary, IFAs and western blots showed that there is no localization of NiV P/V/W proteins in the nucleus as positive immunostaining was detected in cytoplasm only. Western blots show positively stained proteins at approximately 70kDa and 45kDa in the cytoplasm, corresponding to the NiV P protein and V/W proteins, respectively.

3.2.1.5 Localization of Transfected NiV W in cells Infected with NiV

NiV W was not found localized in the nucleus in cells infected with virus. There are many reasons that this could occur, one being the influence of NiV viral proteins preventing transfer of NiV W to the nucleus. To determine if another NiV protein has the ability to influence the localization of NiV W in a natural infection, NiV W-pSMART2a was transfected into BHK-21 cells for 24 hours and infected with NiV at an MOI of 0.5.

The NiV P58 Mab could not be used in this assay. It would not be able to differentiate between transfected NiV W and naturally produced NiV P/V/W. To account for this, cells were

transfected with NiV W-pSMART2a, which yields HIS-tagged W protein. To detect transfected NiV W, anti-HIS tag antibodies were used and to detect NiV viral proteins, anti-NiV polyclonal guinea pig serum was used.Controls used in this experiment included, negative controls similar to those used in section 3.1.2.4a for IFA negative controls and section 3.2.1.3 for negative transfection controls. Positive controls included NiV W transfected cells only stained with anti-HIS only (Figure 19b); and NiV infected cells only stained with guinea pig polyclonal antibodies (Figure 19aI).

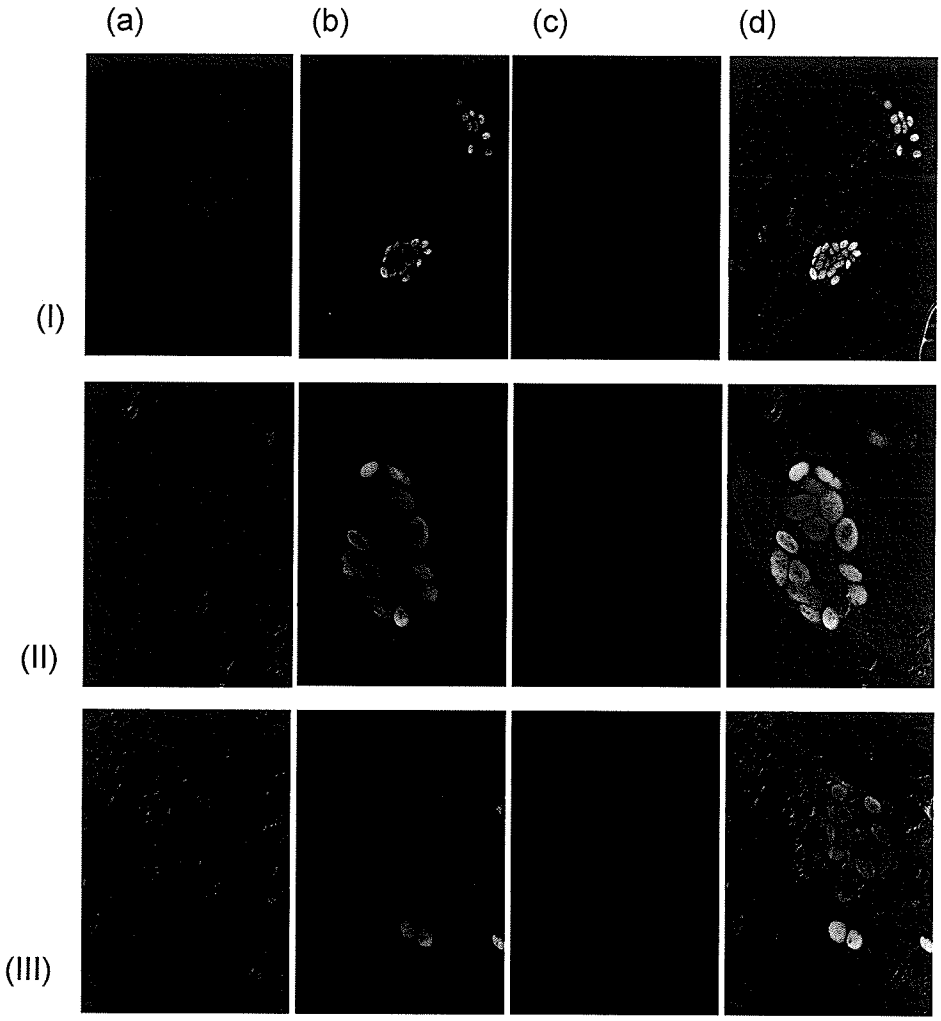


Figure 19: Immunofluorescence assays of transfected NiV W-pSMART2a (4μg of DNA) BHK-21 cells infected with NiV at an MOI of 0.5. Cells were labelled with anti-HIS antibody (1:1000) to detect transfected NiV W and anti-NiV polyclonal serum (1:200) to detect NiV viral proteins. (aI) Positive control, cells infected with NiV showing no nuclear localization of any NiV protein.. Probed with anti-NiV polyclonal serum. (b), (c) and (d) cells transfected with NiV W and infected with NiV: (b) localization of recombinant W protein within nuclei (detected with anti-HIS antibody). (c) Localization of NiV proteins (infected and transfected) detected with anti-NiV guinea pig polyclonal serum. Nuclear staining of this antibody is a results of detection of the transfected NiV W protein. (d) Colocalization. (aII) and (aIII) cells shown with no staining. Arrow indicates nuclei. Magnifications: I - 20X, II - 40X, III - 30X. Experiment was performed three times and repeated twice.

No IF signal was detected in the negative controls indicating that non-specific binding did not occur (Results not shown). Results from positive controls showed NiV infected cells (Figure 19aI), displaying fluorescence only in the cytoplasm. Transfected NiV W displayed fluorescence in nucleus (Figure 19b) and transfected NiV W cells also infected with virus showed detection of proteins in both the cytoplasm and nucleus by guinea pig polyclonal serum (Figure 19c). The anti-His tag antibody only showed detection of recombinant NiV W in the nucleus.

It is difficult to draw any conclusions from this experiment, since all the transfected W proteins could had been already localized to the nucleus by the time native viral proteins were produced during the infection (Figure 19(b)).

There is also a possibility the HIS tag in the pSMART2a vector may create problems with localization or function or the NiV W protein. This may result in the inability of the NiV proteins to prevent its transport into the nucleus.

These results suggest that there are no NiV proteins that act on NiV W when it is already in the nucleus. However, time course experiments would be needed to determine if proteins produced by the virus initially act on NiV W to prevent its movement into the nucleus. If this is true, then NiV W may not at all move into the nucleus of infected cells.

3.3 Co-localization of NiV P/V/W Proteins Produced during an Infection with Native Porcine and Human STAT1

Previous studies have shown that recombinant NiV V, NiV W and NiV P co-localize with STAT1, in both the cytoplasm and nuclei of transfected cells (Shaw *et al*,

2004, 2005). The co-localization of NiV V/W or P with STAT1 has never been studied in a system with live virus.

3.3.1 Localization of Porcine and Human STAT1

Initially, to observe NiV V/W co-localization with the STAT1 protein, attempts were made to clone and express porcine STAT1 and double transfect NiV V-pSCA or W-pSCA with porcine STAT1 and develop antibodies against porcine STAT1. Cloning was carried out; however, products resulted in the final DNA products containing point mutations which did not allow expression of the protein. Due to time constraints, mRNA was sent to a commercial company but their attempts to clone this gene were unsuccessful. Concurrently, different rabbit polyclonal STAT1 antibodies were tested by western blot to determine whether antibodies against human STAT1 would cross-react with porcine STAT1 as there are no commercially available anti-porcine STAT1 antibodies. These antibodies were tested on native porcine and human STAT1 in cell lysates from two porcine cell lines and one human cell line (Figure 20a). The rabbit anti-STAT1 antibody was able to detect both porcine and human STAT1

(approximately 75kDa) in both cell lines and therefore was considered suitable for use in further experiments.

human STAT1 in cell lysates from two porcine cell lines and one human cell line (Figure 20a). The rabbit anti-STAT1 antibody was able to detect both porcine and human STAT1

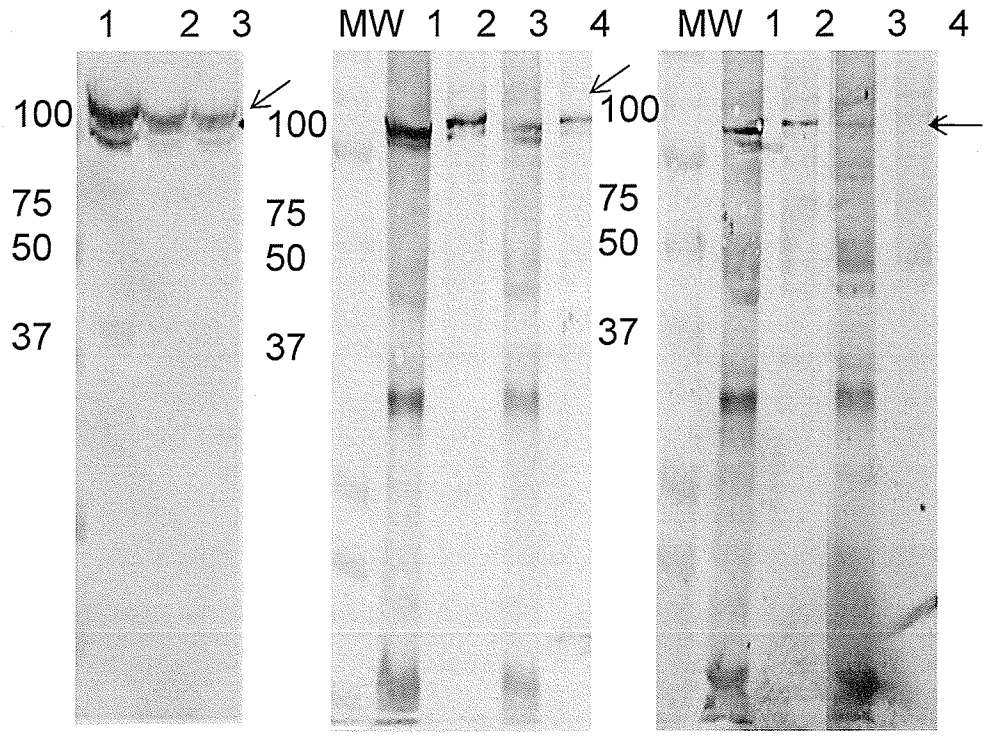


Figure 20: Western blot results for the expression and localization of porcine and human STAT1 of uninfected or infected cells (MOI of 1) with live NiV separated on a 10% gel. (a) Whole cell lysates of uninfected MRC5 cells (lane 1), ST (lane 2) and SJPL (lane 3). Expected sizes of both porcine and human STAT1 is approx. 75kDa. Cytoplasmic and nuclear fractions of ST cells (b) and MRC5 (c). Cytoplasm of infected cells (b,c lane 1), nuclei of infected cells (b,c lane 2), uninfected Cytoplasm (b,c lane 3) and uninfected nuclei (b,c, lane 4) for both cell types are seen here. Experiment was carried out in duplicate at different times. Lower bands seen in (b) and (c) are the phosphorylation bands of STAT1 (Pietila *et al*, 2007). Arrows indicate protein of interest, STAT1. Precision Plus Protein Ladder (Bio-Rad) indicate Molecular weight (MW) at the left of each gel.

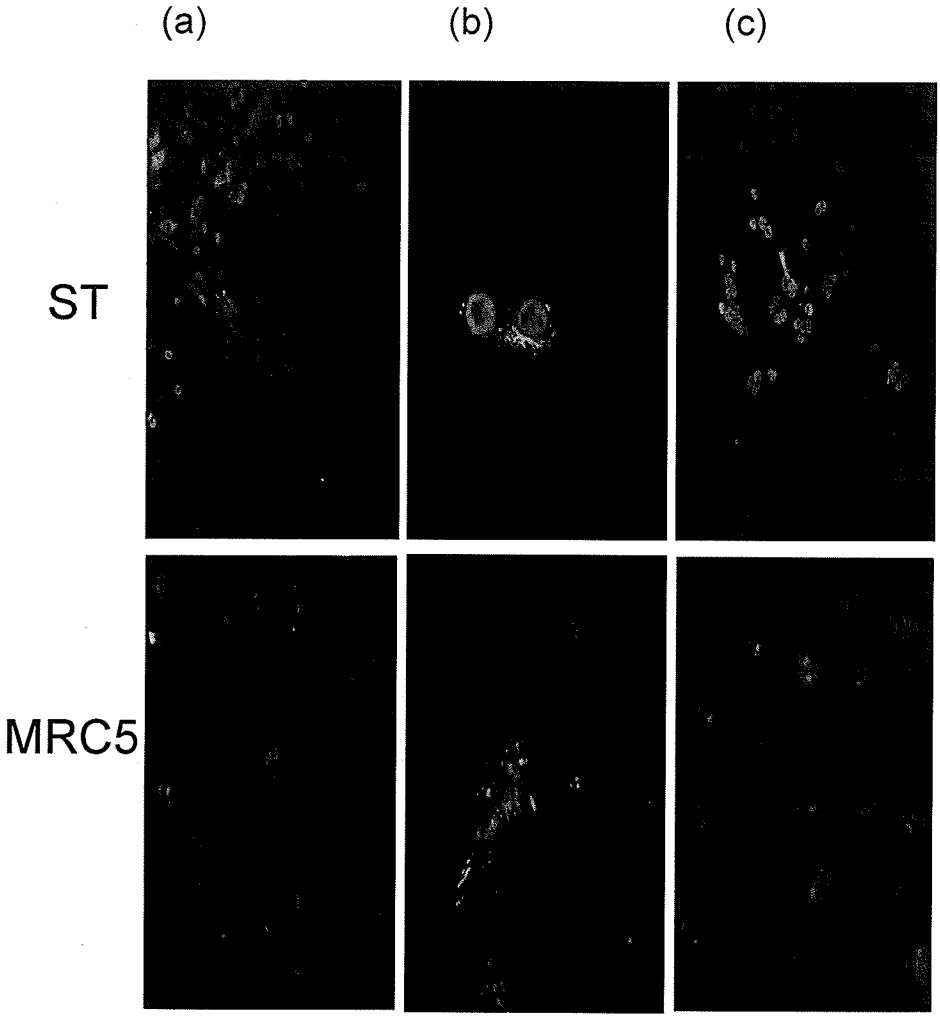


Figure 21: Localization of STAT1 in uninfected and infected ST cells (top panels) and MRC5 cells (bottom panels) with NiV. Infections were carried out at an MOI of 0.1. Cells were grown to confluence and STAT1 was detected with rabbit anti STAT1 polyclonal antibody (1:200) for uninfected (a), infected at 24 hours (b) and infected for 48 hours (c). The secondary antibody used for detection was an Alexa Fluor 488 which emits green fluorescence when excited. In ST cells, panel b, there appears to be increased staining intensity of the STAT-1 antibody in the nuclei of infected cells. Syncytia is also present in cells that are infected with NiV.Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. Magnifications: ST (a) and (c) 10X, ST (b) 40X, MRC5 (a) and (c) 40X, MRC5 (b) 20X.

(approximately 75kDa) in both cell lines and therefore was considered suitable for use in further experiments.

Localization of STAT1 in one porcine cell line (ST) and one human cell line (MRC5) was determined using the antibody. Cytoplasm and nuclei of infected and uninfected ST and MRC5 cells were extracted and western blot using the above antibody carried out to determine the presence and localization of STAT1 within these different cells types. Figure 20b shows the results from the porcine cells and Figure 20c shows the results in the human cells and these blots show that porcine cells have STAT1 in the cytoplasm and nucleus in both uninfected and infected cells. MRC5 showed similar results in the infected cells where both the nucleus and the cytoplasm had STAT1. In the uninfected nuclei STAT1 was also present.

To visualize STAT1 in the cytoplasm and nucleus and to confirm the western blot results, IFAs on infected and uninfected ST and MRC5 cells was carried out. A negative control using both infected and uninfected cells stained with secondary antibody (anti-rabbit) only was used to ensure that non-specific binding of the antibody did not occur. Results from this control showed that there was no nonspecific binding occurring with the antibodies.

Figure 21 shows native STAT1 in uninfected and infected cells at 24 and 48 hours post infection for both human and porcine cell lines. These results show that STAT1 is found in both the nucleus and cytoplasm of uninfected cells and NiV infected cells at both 24 and 48 hours post infection. MRC5 cells showed that STAT1 was

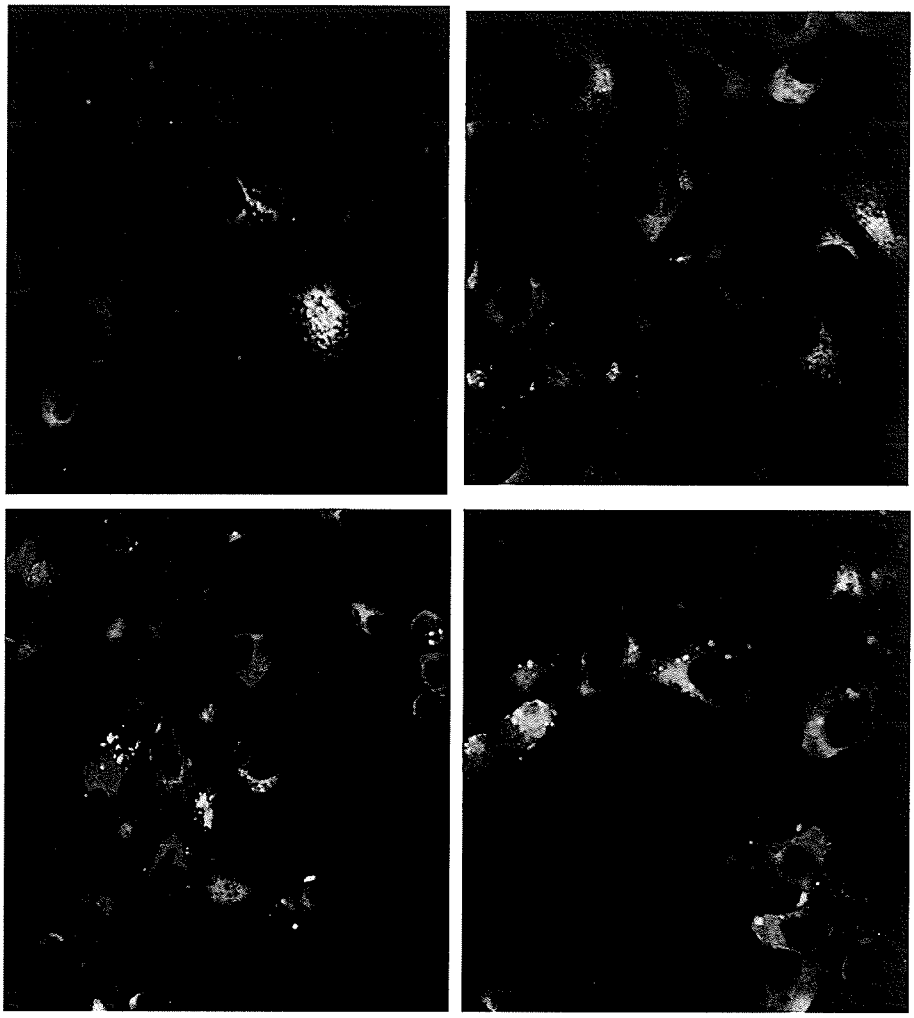


Figure 22: Colocalization of porcine STAT1 and NiV P/V/W proteins within SJPL Cells (porcine cells) infected with live NiV at an MOI of 0.1. Localization of these Proteins at 24 hours (top panels) and 48 hours (bottom panels) can be only seen within the cytoplasm. Goat anti-mouse Alexa Fluor 594 (red-detecting NiV P/V/W) and goat anti-rabbit Alexa Fluor 488 (green – detecting native porcine STAT1) were used to detect primary anti-STAT1 and anti-NiV P58 antibodies. Co-localization of NiV proteins with STAT1 is visualized by yellow. Slides were prepared in duplicate for each experiment. One of two representative experiments is shown. Magnifications: bottom left: 30X, all others 40X.

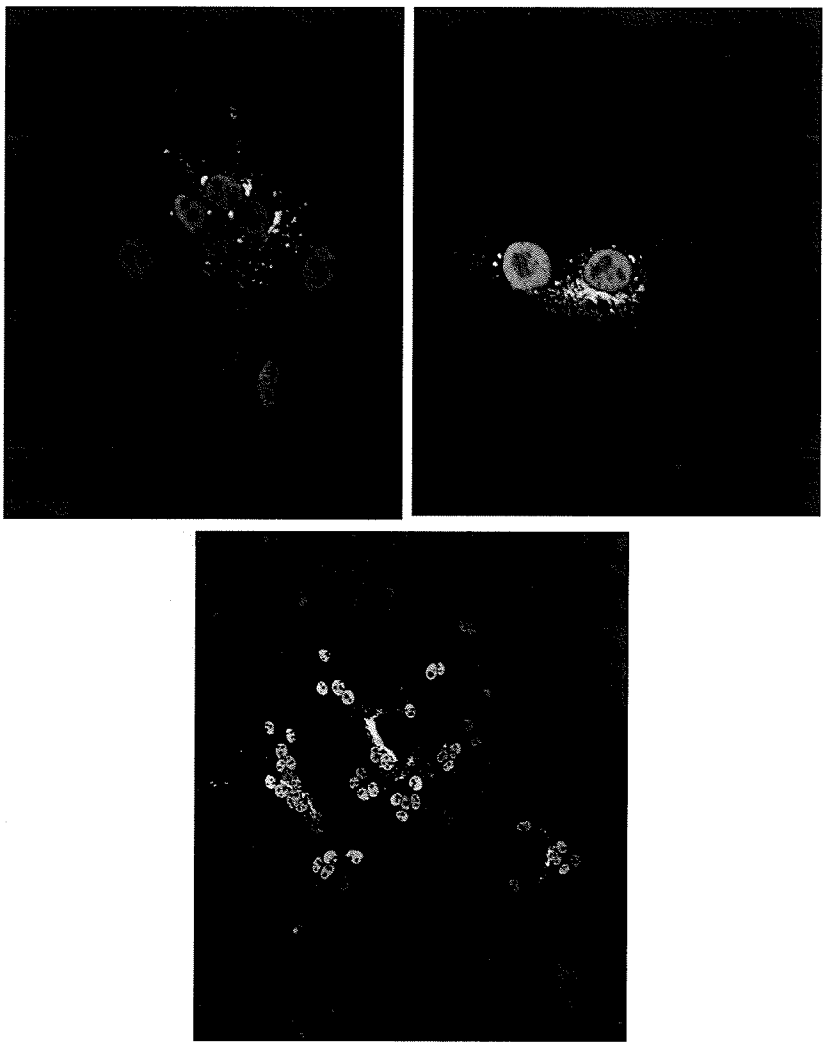


Figure 23: Colocalization of porcine STAT1 and NiV P/V/W proteins within ST cells (porcine cells) infected with live NiV at an MOI of 0.1. Localization of these proteins at 24 hours (top panels) and 48 hours (bottom panel) can be only seen within the cytoplasm. Goat anti-mouse Alexa Fluor 594 (red- detecting NiV P/V/W) and goat anti-rabbit Alexa Fluor 488 (green- detecting native porcine STAT1) were used to detect primary anti-STAT1 and anti-NiV P58 antibodies. Co-localization of NiV proteins with STAT1 is visualized by yellow. Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. Top left panel is shown in 40X magnification, Top right 30X and bottom is 10X.

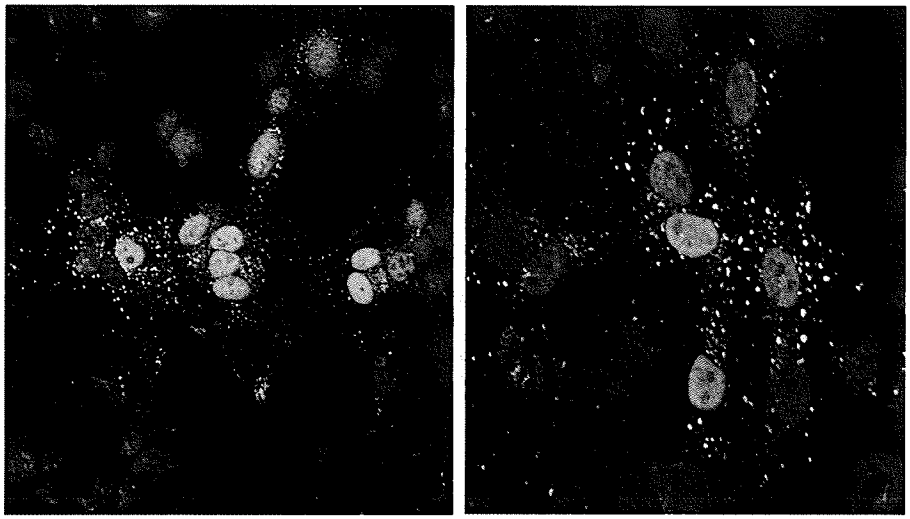


Figure 24: Colocalization and interaction of porcine STAT1 and NiV P/V/W proteins within HeLa cells (human cells) infected with live NiV at an MOI of 0.1. Localization of these proteins at 24 hours (left panel) and 48 hours (right panel) can be only seen within the cytoplasm. Goat anti-mouse Alexa Fluor 594 (red- detecting NiV P/V/W) and goat anti-rabbit Alexa Fluor 488 (green- detecting native human STAT1) were used to detect primary anti-STAT1 and anti-NiV P58 antibodies. Co-localization of NiV proteins with STAT1 is visualized by yellow. Slides were prepared in duplicate for each experiment. One of two representative experiments are shown. Magnifications: left: 40X, right: 30X.

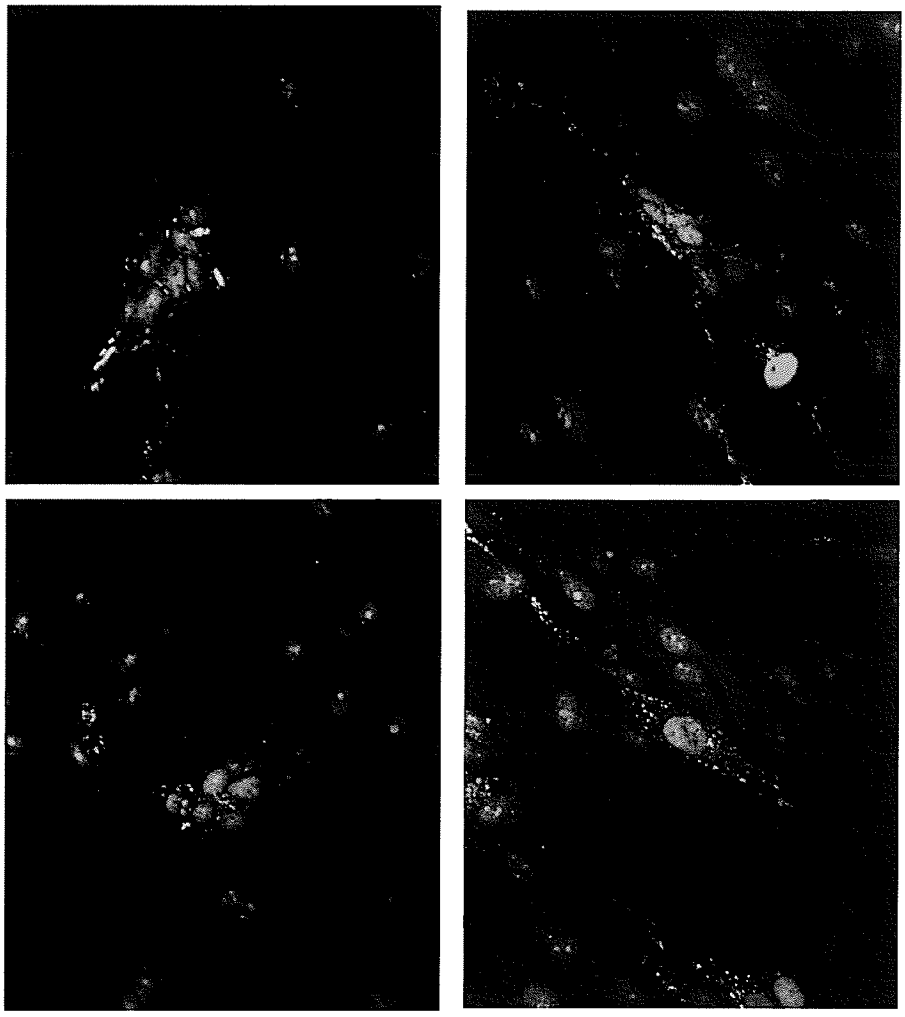


Figure 25: Colocalization of porcine STAT1 and NiV P/V/W proteins within MRC5 cells (human cells) infected with live NiV at an MOI of 0.1. Localization of these proteins at 24 hours (top panels) and 48 hours (bottom panels) can be only seen within the cytoplasm. Goat anti-mouse Alexa Fluor 594 (red- detecting NiV P/V/W proteins) and goat anti-rabbit Alexa Fluor 488 (green- detecting native human STAT1) were used to detect primary anti-STAT1 and anti-NiV P58 antibodies. Co-localization of NiV proteins with STAT1 is visualized by yellow. Slides were prepared in duplicate for. Each experiment. One of two representative experiments are shown.. Magnifications: Right images: 30X, left images: 20X.

present in uninfected nuclei suggesting that immunofluorescence may be more sensitive than western blot.

3.3.2 Colocalization of NiV V and NiV W in Infected Cells with Native Porcine and Human STAT1

Figures 22 to 25 show the confocal microscopy pictures from infected SJPL, HeLa, MRC5, and ST cells with NiV and stained with anti NiV P58 MAb (red fluorescence) and anti STAT1 (green fluorescence) antibodies at 24 and 48 hours post infection.

Controls used in this study included, positive controls (a) NiV infected porcine or human cells stained with anti-NiV guinea pig serum; (b) infected cells stained with only NiV P58; (c) infected cells stained only with anti-STAT1 antibodies and (d) NiV V/W transfected cells detected with NiV P58. Negative controls were similar to those used in section 3.2.1.3 with the addition of uninfected cells labelled with anti-rabbit secondary antibody only. Similar to all sections, standards for controls were met before the results from the experiment was considered accurate.

Similar results were seen for human and porcine cells where all four of the infected cell lines had only cytoplasmic localization of NiV P/V/W proteins and not nuclear. Only STAT1 was located in the nucleus but no protein produced by NiV at either 24 or 48 hours had nuclear localization.

Interestingly, SJPL cells did not show STAT1 in the nucleus to the same degree as the 3 other cell lines at either 24 or 48 hours, although the cells were able to

successfully establish an antiviral state against both VSV and NiV using exogenous IFN. However, it is found that STAT1 can be detected in the nucleus and cytoplasm of SJPL cells after treatment with IFN α (Oral communication by Beata Stachowiak). This may be a reason why more IFN was needed for SJPL cells to reach an antiviral state as compared to other porcine cells.

Results from these experiments have shown that co-localization between native porcine STAT1 and NiV P/V/W only occurs within the cytoplasm and not the nucleus. There appears to be no qualitative difference between porcine and human cells in the co-localization of STAT1 and NiV P/V/W proteins.

4. DISCUSSION

Throughout the first outbreak, the porcine host had a lower case fatality ratio and greater recovery rates than the human hosts (Field *et al*, 2001). As the innate immune response and IFN α may be involved in the quick recovery and better survival of porcine hosts, we focused on the activity of IFN α in human and porcine hosts during a NiV infection, namely the ability to establish an antiviral state and the role of the V and W proteins (putative IFN antagonists).

IFN response in viral infections plays an important role in host defence not only during the early phases of infection by limiting virus replication, but also later on by assisting in virus clearance.

4.1 The Ability of Exogenous IFN α to Induce an Antiviral State in Human and Porcine Cells

To date, the only work on the effect of IFN during a NiV infection was concerned with the protective effect of IFN in a hamster model. This study used an in vitro model with human HeLa cells and it was found that when poly(I)-poly(C_{12}U) was used to induce IFN α and IFN β, NiV replication was blocked completely (Georges-Courbot *et al,* 2006). In a hamster model, again poly(I)-poly(C_{12}U) was used to study IFN protective effects. Daily injection of poly(I)-poly(C_{12}U) over 10 days showed protection of the animals from NiV infections (Georges-Courbot *et al*, 2006). These results were consistent with those from the HeLa cells and suggested that type I IFN may have an important role in protecting against NiV infections.

The IFN response in humans and pigs has never previously been characterized for NiV. The only work that has been studied in humans has been done in vitro (Georges-Courbot et al, 2006) and for porcine hosts no studies have been conducted in vivo or in vitro to study the IFN response. This thesis focused on the in vitro systems for both humans and pigs and looked at the ability of exogenous IFN α to induce an antiviral state in porcine and human cells.

To begin studying the NiV-IFN system in porcine and human cells, the ability of porcine and human cells to establish an antiviral state against NiV was examined. Selected cells to test the antiviral state were SJPL and MRC5 cells. Our goal in cell selection was to obtain cells which could be used in comparison to each other. These cells are both lung cells, they differ in morphology which is a limitation in our assay.

Unfortunately, no other cells were available so these were used for the antiviral assays, as the cells had to be susceptible to NiV infection and to be able to enter an antiviral state by treatment with exogenous IFN α (using VSV as a control virus).

Data obtained in this experiment showed that porcine cells were able to establish an IFN α induced antiviral state quicker than human cells, at 18 hours post treatment and with 160U/ml of exogenous IFN used (Figure 4). Human cells needed 24 hours and 320U/ml of IFN before 95-100% protection was observed (Figure 4). The results were statistically significant, but it is not known whether that indicates also biological significance.

These experiments indicated that both human and porcine cells were able to use exogenous IFN for their advantage to aid inhibition of NiV. Results with exogenous IFN

α confirm that IFN α likely plays a role in inhibiting viral invasion (Georges-Courbot et al, 2006), however, IFN α is probably not the sole contributor to porcine hosts recovering faster or more successfully than human hosts (Darwich *et al*, 2003; Shimizu *et al*, 1996).

To further investigate the IFN pathway, we studied the STAT1-IFN signalling pathway (JAK-STAT1) to determine if in, *in vitro* infected cells, NiV replication can interfere with establishment of the antiviral state, as suggested by the recombinant studies (Shaw et al, 2005, Rodriguez et al, 2003).

4.2 The Expression and Localization of NiV V and NiV W

Nipah virus was initially thought to produce a V protein as other members of the paramyxovirus family also produced these proteins including Hendra virus (Chua *et al*, 2000, Harcourt *et al,* 2000). The NiV V protein was first studied in detail in 2000 (Harcourt et al, 2000), when the gene was sequenced and compared to HeV. It was found that NiV V protein was three amino acids shorter than HeV V protein and had 81.1% homology (Harcourt *et al*, 2000). The W protein of NiV was also discovered during this time (Harcourt *et al*, 2000) but was less studied.

After discovery and characterization of NiV V, studies began to determine the function of the protein. One of the first was conducted by Rodriguez and colleagues in 2002 which suggested that NiV V evades IFN signalling by preventing STAT1 functioning (Rodriguez *et al,* 2002). The NiV W protein was first studied in detail in 2004 and similarly to NiV V was found to have similar functions to V, however would

act in the nucleus instead of the cytoplasm (Shaw *et al,* 2004, Shaw *et al*, 2005). Further studies on this protein found similar results but were carried out solely in the human recombinant system.

Due to high biosafety requirements of NiV for work with live virus, the expression and localization of the NiV V and W proteins in the infectious system was not characterized. Nor has the activity of these proteins been studied in a porcine system.

The establishment of an antiviral state induced by exogenous IFN α did not show significant differences between porcine and human cells. Due to this we decided to investigate if there are differences in a step preceding the establishment of the antiviral state -the IFN-signalling in porcine and human hosts.

As NCFAD has BSL4 laboratories, we were able to look at the localization of NiV V and W in infected cells and determine if there is any co-localization with native STAT1. This was carried out in both human and porcine cells of origin. Recombinant experiments were carried out in parallel to compare results to published data and to the data obtained in the infectious live virus system. The recombinant expression system was also used to verify that the anti-P58 recognizes V and W proteins.

This thesis research excluded the study of NiV C as there is a lack of tools available to study this protein (no antibodies) within the time frame allotted for this project. In addition, previous work by Shaw and colleagues has stated that in the recombinant system NiV C had little role in STAT1 co-localization (2005).

To study NiV V and W expression and localization we first confirmed the expression of these proteins in the recombinant system and also used the recombinant system to test the NiV P58 antibody for NiV V and W specificity.

4.2.1 Recombinant System

NiV V and W coding sequences were cloned into Semliki Forest Expression Vectors either pSCA or pSMART2a. The pSMART2a contains FLAG and HIS tags. This vector was used for the detection of the recombinant proteins where the NiV P58 MAb could not be used. This antibody was produced against NiV P and would be also detect NiV V and W by the common N terminal. It was found that these proteins were detected at 45kDa and 45.6kDa representing NiV W and NiV V, respectively.

Expression and localization of recombinant NiV P, V and W was studied and we confirmed reports in literature (Shaw *et al*, 2005, Rodriguez *et al,* 2003) showing that recombinant NiV V is located in the cytoplasm, recombinant NiV W is located in the nucleus (Figure 12) and recombinant NiV P (Figure 11) is also located in the cytoplasm. These results were seen by IFA where BHK-21 cells were transfected with plasmids containing NiV V, W or P genes.

4.2.2 Live Virus System

NiV V and W protein have never been studied in a system using infected cells and to study the expression and localization of NiV V and W in a system using live virus, two

porcine and two human cell lines were infected with NiV. NiV P58 MAb was used for detection of the P/V/W proteins. Since this antibody was not able to differentiate between the NiV P/V/W proteins as such, it was used to detect expression based on localization and size of the proteins.

Both, western blot of the nuclear and cytoplasmic proteins and the IFAs showed that when both human and porcine cells infected with NiV had proteins recognized by the P58 MAb only in the cytoplasm. In all cell lines (porcine and human) no signal was detected in the nuclei. These results were quite surprising for NiV W, as it is expected, based on recombinant system results, that this protein can be detected in the nucleus.

The western blots confirmed that the NiV P protein was expressed in the cytoplasm. In addition, the western blot showed that a second protein(s) of a size corresponding to 45kDa was expressed. It is not possible confirm the identity of this protein (Figures 16 to 19) using the P58 mAB as NiV V and W have only a 6 amino acid difference in length. Use of antibodies specific for NiV W or V only, attempts to detect respective mRNA or 2D gel electrophoresis followed by western blot would be methods to determine if both the NiV V and W proteins are expressed.

The band at 45kDa may solely be NiV V which would correlate with recombinant results and show that NiV W is not at all expressed. The band could also represent NiV W alone which would indicate cytoplasmic localization and no expression of NiV V; or the 45 kDa protein could also represent both NiV V and NiV W, both having cytoplasmic localization.

Many Paramyxoviruses express accessory proteins in the form of V and/or W similar to NiV (Gotoh *et al*, 2002). The majority of Paramyxoviruses that do this express

only V and only a few viruses in this family express a W protein (Gotoh *et al*, 2002). If NiV does express only one accessory protein it most likely would be NiV V as no other Paramyxoviruses express only a W protein. If only NiV W is found to be expressed, and not NiV V, NiV would be the first paramyxovirus to solely express a W protein and not a V protein.

It is also possible that both of these proteins are produced but do not localize in a similar manner to the recombinant system. The 45 kDa band found produced in the cytoplasm of all cell lines studied, may be a combination of both NiV V and NiV W, where NiV W would localize to the cytoplasm and not the nucleus. If these results prove to be true, this would be an interesting discovery as previously a nuclear localization signal was published for the recombinant W (Shaw *et al*, 2005). If NiV W is found in the cytoplasm, this would also suggest that another mechanism (cellular, viral or both) plays a role in retaining the protein in the cytoplasm or removing it from the nucleus. Recombinant systems at times do not mimic that of in vivo or in vitro situations and this may be the case here.

It has been found that the structure of proteins is important for localization (Harty & Palese, 1995). For example, the conformation of measles virus P protein is important for its binding to the N protein and this influences its localization (Harty & Palese, 1995). Similarly, this could be an event that occurs with NiV where structure of the proteins and their interaction to others could be a direct influence of their localization. It is possible that NiV W protein must be in a specific conformation to localize to the nucleus, which is found in the recombinant system. In reality this protein may be in a conformation which does not allow its localization to the nucleus.

There is also the possibility that NiV W is not detected by this antibody due to low protein expression. It is known that the paramyxoviruses that do express a W protein, do so at low amounts as it is a secondary protein to V proteins in carrying out STAT1 binding activities (Gotoh *et al*, 2002). If this is correct, and NiV expresses this protein at very low amounts, it may not be able to be detected by NiV P58. To solve this problem, monoclonal antibodies directed to the NiV W would have to be produced to allow for stronger detection. If it is shown that NiV W is not expressed at all, it would show that NiV and possibly Henipaviruses, only have two accessory proteins, NiV V and C (although NiV C has not yet been studied in an infectious system). This would contradict the recombinant system results and show that NiV may produce only one accessory protein that may have significance in STAT1 interference.

Our results, for the first time, have shown the expression and localization of NiV V/W proteins in a system using live NiV in both porcine and human cell lines with no nuclear localization of the W protein. We have decided to briefly examine the possible influence of other Nipah viral proteins on the localization of the NiV W protein by using a combination of the infectious and recombinant system.

4.3 Influence of NiV Proteins on the Localization of NiV W

NiV W was transfected into BHK-21 cells and these transfected cells were infected with NiV. The transfected NiV W was detected with an anti-HIS antibody and NiV infection was detected with anti-NiV guinea pig serum.

These results showed that there is no qualitative movement of the NiV W protein out of the nucleus (Figure 20) when the above experiment was carried out. The NiV proteins seemed to have no effect on the localization of the already expressed recombinant NiV W protein. While these results have suggested that there is no effect of native NiV proteins on the recombinant NiV W protein localization, they remain inconclusive. To determine if the NiV W protein is held back in the cytoplasm by another NiV protein, cells would have to be infected with NiV first before transfected with NiV W. Transfection would have to be carried out by a gradient to control possible over expression of transfected NiV W.

We have seen from the expression of NiV W in the previous section that the recombinant system does not mimic a natural infection as expected. If other NiV proteins do have an effect on the localization of NiV W, similar assumptions can be made here, as the natural infection may not have the same effect on the transfected proteins, especially if they are tagged.

4.4 Co-localization of Porcine and Human STAT1 with NiV V and W

The NiV V and W proteins co-localize with STAT1 in the recombinant system and binding is implied by this co-localization (Shaw *et al*, 2005, Rodriguez *et al*, 2003). The co-localization of STAT1 and NiV V and W in cells infected with the virus was studied here.

As a prerequisite to studying co-localization of NiV P/V/W with porcine and human STAT1, the presence of STAT1 in these cell lines was determined by western blot and IFA.

The STAT1 was found in both nuclei and cytoplasmic compartments in infected and uninfected cells except for SJPL cells. It was found that STAT1 was found in the nucleus of SJPL cells only after treatment of IFN α (Oral communication by Beata Stachowiak). STAT1 is found active only when phosphorylatyed. In the recombinant system it was found that NiV V and W co-localize with STAT1 and implied that binding occurred (Shaw *et al,* 2005, Rodriguez *et al*, 2004). It has not thoroughly been studied if NiV P/V/W proteins change the form of active STAT1 but has been suggested that they bind to and hold the protein in the cytoplasm (Shaw *et al*, 2005). Based on this work, these cells may be already in an antiviral state as STAT1 is found in the nucleus in uninfected cells. A recent study has showed that STAT1 was found in both the nucleus and cytoplasm of uninfected cells and was dependent upon the cell type (Meyer et al, 2007). As STAT1 is not found in the nucleus of SJPL cells, these cells may not be in an antiviral state and only when treated with IFN does this process begin. This may be the reason that more IFN was needed to allow these cells to enter an antiviral state as compared to the other porcine cells.

Since the epitope for the P58 antibody does not overlap with the STAT1 protein binding site on the P/V/W proteins, we used this antibody for the co-localization studies by IFA in NiV infected SJPL, ST, MRC5 and HeLa cells. Confocal microscopy results have detected co-localization of the NiV P/V/W proteins with the STAT1 protein only in the cytoplasm and not the nucleus supporting our observation on expression and

localization of native NiV proteins.. These results suggest STAT1-NiV P/V/W binding in that cell compartment.

Although NiV P/V/W co-localizes with STAT1 in the cytoplasm, a proportion of the STAT1 may still escape for the NiV proteins and be able to move into the nucleus, potentially stimulating the antiviral state. Poly(I)-poly($C_{12}U$) was found to be protective in both hamsters and HeLa cells and was suggested that the NiV IFN antagonistic proteins alone are not enough to fully disrupt IFN signalling (Georges-Courbot *et al*, 2006) In addition with the aid of exogenous IFN α a greater amount of STAT1 is stimulated as compared to cells that are not treated with exogenous IFN and which normally do not survive the NiV challenge.

4.5 Summary and Conclusions

This study focused on an investigation of the NiV-IFN system in porcine and human hosts. We looked at two sections of the IFN system, the establishment of an antiviral state induced by exogenous IFN α and the IFN signalling via the JAK-STAT1 signalling pathway.

We looked at the difference between porcine and human cells to establish an exogenous IFN α induced antiviral state when challenged with NiV and have found that porcine cells are able to develop a slightly faster antiviral state than human cells, however it is difficult to say whether this difference may have biological significance in the infected host.

NiV has two putative proteins, V and W which have been reported to interfere with STAT1 in the recombinant systems (Shaw *et al*, 2005; Rodriguez *et al,* 2003). This thesis studied the expression and localization of the putative V and W proteins in a system using live NiV and our results suggested that expression only occurred in the cytoplasm of the infected cells. These results indicated that either only NiV V or W is expressed, or both proteins are expressed, but localize only to the cytoplasm. In agreement with this finding, detectable levels of co-localization with the STAT1 protein occurred only within the cytoplasm. These results have shown that there was no qualitative difference seen between porcine and human cells infected with NiV. These results suggest that a difference between STAT1 signalling is not a contributing factor to the differences seen between humans and pigs, where pigs recover from a NiV infection faster than humans.

We for the first time have shown in vitro, that NiV is sensitive to the antiviral action of IFN α. This work has shown the cytoplasmic co-localization of native STAT1 with NiV P/V/W in NiV infected cells.

These IFN studies were a novel aspect in NiV research and the results could contribute to a better understanding of NiV pathogenesis.

5. REFERENCES

AbuBakar, S., Chang, L.-Y., Ali, A. M., Sharifah, S., Yusoff, K., & Zamrod, Z. (2004). Isolation and Molecular Identification of Nipah Virus from Pigs. *Emerging Infectious Diseases* , 10:2228-2230.

Aguilar, H.C., Matreyek, K.A., Filone, C.M., Hashimi, S.T., Levroney, E.L., Negrete, O.A., Bertolotti-Ciarlet, A., Choi, D.Y., McHardy, I., Fulcher, J.A., Su, S.V., Wolf, M.C., Kohatsu, L., Baum, L.G., & Lee, B. 2006. N-Glycans on Nipah Virus Fusion Protein Protect Against Neutralization but Reduce Membrane Fusion and Viral Entry. *Journal of Virology.* 80: 4878-4889.

Amal, N.M, Lye, M.S., Ksiazek, T.G. Kitsutani, P.D., Hanjeet, K.S., Kamaluddin, M.A., Ong, F., Devi, S., Stockton, P.C., Ghazali, O &., Zainab, R. 2000. Risk Factors for Nipah Virus Transmission, Port Dickson, Negeri Sembilan, Malaysia: Results from a Hospital-Based-Case-Control-Study. *Southeast Asian Journal of Tropical Medicine and Public Health.* 31:301-306.

Anonymous 2004. Person-to-person transmission of Nipah Virus during Outbreak in Faridpur District. HSB 2:5-9.

Baron, M.D. 2005. Wild-Type Rinderpest Virus uses SLAM (CD150) as its Receptor. *Journal of General Virology.* 86:1753-1757.

Basler C.F., Wang, X., Mühlberger, E., Volchkov, V., Paragas, J., Klenk, H.D., García-Sastre, A., and Palese, P. (2000). The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proceedings of the National Academy of Sciences of the United States of America.* 97: 12289-12294.

Bonaparte, M.I., Dimitrov, A.S., Bossart, K.N., Crameri, G., Mungall, B.A., Bishop, K.A., Choudhry, V., Dimitrov, D.S., Wang, L.F., Eaton, B.T., and Broder, C.C. 2005. Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. Proc. *Natl. Acad. Sci. U.S.A.* 102:10652-10657.

Brooks, G. F., Butel, J. S., & Ornston, L. N. (1995a). Paramyxoviruses and Rubella Virus. In G. F. Brooks, J. S. Butel, & L. N. Ornston, *Medical Microbiology* (pp. 460-478). East Norwalk, Connecticut: Simon and Schuster.

Brooks, G. F., S.Butel, J., & Ornston, L. N. (1995b). General Properties of Viruses. In G. F. Brooks, J. S. Butel, & L. Ornston, *Medical Microbiology* (pp. 303-325). East Norwalk, Connecticut: Simon and Schuster.

Brzozka, K., Finke, S., & Conzelmann, K.-K. (2006). Inhibition of Interferon Signalling by Rabies Virus Phosphoprotein P: Activation-Dependent Binding of STAT1 and STAT2. *Journal of Virology* , 80:2675-2683.

Centre for Disease Control and Prevention: Outbreak of Hendra like Virus-Malaysia and Singapore 1998-1999. (1999). *MMWR Morbidity and Mortality Weekly* , 48:265-269.

Chadha, M., Comer, J., Lowe, L., Rota, P., Rollin, P., Bellini, W., *et al.* (2006). Nipah Virus Associated Encephalitis Outbreak, Siliguri, India. *Emerging infectious Disease* , 12:235-240.

Chakalova, L., Debrand, E., Mitchell, J.A., Osborne, C.S., and Fraser, P. 2005. Replication and transcription: Shaping the Landscape for the Genome. *Nature Reviews Genetics*. 6:669-677.

Chan KP, Rollin PE, Ksiazek TG, Leo YS, Goh KT, Paton NI, Sng EH, Ling AE. 2002. A survey of Nipah virus infection among various risk groups in Singapore. *Epidemiology and Infection*, 128:193-198.

Chan, Y.P., Koh, C.L., Lam, S.K., and Wang, L-F. (2004). Mapping of Domains Responsible for Nucleocapsid Protein-Phosphoprotein Interaction of Henipaviruses. *Journal of General Virology,* 85:1675-1684.

Charles A. Janeway, J., Travers, P., Walport, M., & Shlomchik, M. J. (2005). Innate Immunity. In C. A. Jr, P. Travers, M. Walport, & M. J. Shlomchik, *Immunobiology* (pp. 37-95). New York: Garland Science Publishing.

Chen, Y. P., Chua, K. B., Koh, C., Lim, M., & Lam, S. (2001). Complete Nucleotide Sequences of Nipah Virus Isolates from Malaysia. *Journal of General Virology* , 82:2151-2155.

Chew, M., Arguin, P., Shay, D., Goh, K.-T., Rollin, P., Shieh, W.-J., *et al.* (2000). Risk Factors for Nipah Virus Infection among Abattoir Workers in Singapore. *Journal of Infectious Diseases* , 181:1760-1763.

Chow, V.T., Tambyah, P.A., Yeo, W.M., Phoon, M.C., & Howe J. 2000. Diagnosis of Nipah Virus Encephalitis by electron Microscopy of Cerebrospinal Fluid. *Journal of Clinical Virology.* 3:143-147.

Chua, K. B. (2003). Nipah Virus Outbreak in Malaysia. *Journal of Clinical Virology* , 26:265-275.

Chua, K. B., Koh, C. L., Hooi, P. S., Wee, K. F., Khong, J. H., Chua, B. H., *et al.* (2002). Isolation of Nipah Virus from Malaysiam Island Flying Foxes. *Microbes and Infection* , 4:145-151.

Chua, K., Bellini, W., Rota, P., Harcourt, B., Tamin, A., Lam, S., *et al.* (2000). Nipah Virus: A Recently Emergent Deadly Paramyxovirus. *Science* , 288:1432-1435.

Chua, K., Goh, K., Wong, K., Kumarulzaman, A., Tan, P., Ksiazek, T., *et al.* (1999). Fatal Encephalitis Due to Nipah Virus among Pig Farmers in Malaysia. *Lancet* , 354:1257-1259.

Darwich L., Baleisch, M., Plana-Duran, J., Segates, J., Domingo, M., and Mateu, E. (2003). Cytokine Profiles of Peripheral Blood Mononuclear Cells from Pigs with Postweaning Multisystemic Wasting Syndrome in Response to Mitogen Superantigen or Recall Viral Antigens. *Journal of General Virology*, 84: 3453-3457.

Deng, L., Dai, P., Ding, W., Granstein, R. D., & Shuman, S. (2006). Vaccinia Virus Infection Attenuates Innate Immune Responses and Antigen Presentation by Epidermal Dendritic Cells. *Journal of Virology* , 80:9977-9987.

DiCiommo, D.P., and Bremner, R. (1998). Rapid, High Level Protein Production Usin DNA-Based Semliki Forest Virus Vectors. *Journal of Biological Chemistry*, 273: 18060-18066.

DiCiommo D.P., Duckett, A., Burcescul, I., Bremner, R., and Gallie, B.L. (2004). Retinoblastoma Protein Purification and Transduction of Retinal and Retinablastoma Cells Using Improved Alphavirus Vectors. *Investigative Ophthalmology and Visual Science*, 45: 3320-3329.

Didcock, L., Young, D.F., Goodbourn, S., & Randall, R.E. (1999). The V Protein of Simian Virus 5 Inhibits Interferon Signalling by Targeting STAT1 for Proteasome-Mediated Degradation. *Journal of Virology*, 73: 9928-9933.

Eaton, B. T., Mackenzie, J. S., & Wang, L.-F. (2007). Henipaviruses. In D. m. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, *et al.*, *Fields Virology* (pp. 1587-1600). Lippincott Williams and Wilkins.

Eaton, B., Broder, C., Middleton, D., & Wang, L.-F. (2006). Hendra and Nipah Viruses: Different and Dangerous. *Nature Reviews Microbiology* , 4:23-35.

Epstein, J.H., Prakash, V., Smith, C.S., Daszak, P., McLaughlin, A.B., Meehan, G., Field, H.E., and Cunningham, A.A. (2008). *Henipavirus* Infection in Fruit Bats (*Pteropus giganteus*), India. *Emerging Infectious Disease.* 14: 1309-1311.

Enserink, M. (2000). Emerging Diseases: Malaysian Researchers Trace Nipah Virus Outbreak to Bats. *Science* , 289:518-519.

Field, H., Young, P., Yob, J. M., Mills, J., Hall, L., & Mackenzie, J. (2001). The Natrual History of Hendra and Nipah Viruses. *Microbes and Infection*, 3:307-314.

Fukuhara N, Huang C, Kiyotani K, Yoshida T, & Sakaguchi T. (2002). Mutational analysis of the Sendai virus V protein: importance of the conserved residues for Zn binding, virus pathogenesis, and efficient RNA editing. Virology, 299: 172-181.

Georges-Courbot, M.C., Contamin, H., Faure, C., Loth, P., Baize, S., Leyssen, P., Neyts, J., Deubel. 2006. Poly (I)-(Poly $C_{12}U$) but not Ribavirin Prevents Death in a Hamster Model of Nipah Virus Infection. *Antimicrobial Agents and Chemotherapy*, 50: 1768-1772.

Goh K.J., Tan, C.T., Chew, N.K., Tan, P.S., Kamarulzaman, A., Sarji, S.A., Wong, K.T., Abdullah, B.J., Chua, K.B., and Lam, S.K. 2000. Clinical Features of Nipah Virus Encephalitis among Pig Farmers in Malaysia. *New England Journal of Medicine,* 27:1229-1235.

Goodbourn, S., L, D., & Randall, R. (2000). Interferons: Cell Signalling, Immune Modulation, Antiviral Responses and Virus Countermeasures. *Journal of General Virology*, 81:2341-2364.

Gotoh, B., Komatsu, T., Takeuchi, K., and Yokoo, J. (2001). Paramyxovirus Accessory Proteins as Interferon Antagonists. *Microbiology and Immunology*, 45: 787-800.

Gotoh, B., Komatsu, T., Takeuchi, K., & Yokoo, J. (2002). Paramyxovirus strategies for Evading the Interferon Response. *Reviews in Medical Virology*, 12:337-357.

Hagmaier, K., Stock,N., Goodbourn, S., Wang, L-F., & Randall, R. (2006). A Single Amino Acid Substitiution in the V Protein of Nipah Virus Alters its Ability to Block Interferon Signalling in Cels from Different Species. *Journal of General Virology*, 87: 3649-3653.

Halpin, K., Bankamp, B., Harcourt, B., Bellini, W., & Rota, P. (2004). Nipah Virus Conforms to the Rule of Six in a Mingenome Replication Assay. *Journal of General Virology* , 85:701-707.

Harcourt, B. H., Tamin, A., Ksiazek, T. G., Rollin, P. E., Anderson, L. J., Bellini, W. J., *et al.* (2000). Molecular Characterization of Nipah Virus, a Newly Emergent Paramyxovirus. *Virology*, 271:334-349.

Harcourt, B., Tamin, A., Halpin, K., Ksiazek, T., Rollin, P., Bellini, W., *et al.* (2001). Molecular Characterization of the Polymerase Gene and genomic termini of Nipah Virus. *Virology* , 287:192-201.

Harit, A., Ichhpujani, R., Gupta, S., Gill, K., Lal, S., Ganguly, N., *et al.* (2006). Nipah/Hendra Virus Outbreak in Siliguri, West Bengal, India in 2001. *Indian Journal of Medical Research* , 123:553-560.

Harty, R.N. & Palese, P. 1995. Measles Virus Phosphoprotein (P) Requires the NH2-COOH-Terminal Domains for Interactions with the Nucleoprotein (N) but only the COOH Terminus for Interactions with Itself. *Journal of General Virology.* 76:2863-2867.

Hooper, P., & Williamson, M. (2000). Hendra and Nipah Virus Infections. *Veterinary Clinics of North America: Equine Practice* , 16:597-603.

Hooper, P., Zaki, S., Daniels, P., & Middleton, D. 2001. Comparative Pathology of the Diseases Caused by Hendra and Nipah Viruses. *Microbes and Infection.* 3:315-322.

Hossain, M.J., Gurley, E.S., Montgomery, J.M., Bell, M., Carroll, D.S., Hsu, V.P., Formenty, P., Croisier, A., Bertherat, E., Faiz, M.A., Azad, A.K., Islam, R., Molla, M.A., Ksiazek, T.G., Rota, P.A., Comer, J.A., Rollin, P.E., Luby, S.P., Breiman, R.F. (2008). Clinical presentation of nipah virus infection in Bangladesh. Clinical Infectious Diseases. 46: 977-984.

Hsu, V., Hossain, M., Parashar, U., Ali, M., Ksiazek, T., Kuzmin, I., *et al.* (2004). Nipah Virus Encephalitis Reemergence, Bangladesh. *Emerging Infectious Disease* , 10:2082-2087.

Hyatt A.D., Zaki S.R., Goldsmith C.S., Wise T.G., and Hengstberger S.G. (2001). Ultrastructure of Hendra virus and Nipah virus within cultured cells and host animals. *Microbes and Infection.* 3: 297-306.

Ishida, H., Ohkawa, K., Hosui, A., Hiramatsu, N., Kanto, T., Ueda, K., *et al.* (2004). Involvement of p38 Signalling Pathway in Interferon alpha Mediated Antiviral Activity Toward Hepatitis C Virus. *Biochemical and Biophysical Research Communications* , 321:722-727.

Janeway, C. A., Travers, P., Walport, M., & Shlomchik, M. J. (2005). Adaptive Immune Response. In C. A. Janeway, P. Travers, M. Walport, & M. J. Shlomchik, *Immunobiology* (pp. 319-446). New York: Garland Science Publishing.

Katze, M. G., He, Y., & Gale, M. (2002). Viruses and Interferon: A Fight for Supremacy. *Nature*, 2:675-690.

Kingston, R. L., Hamel, D. J., Gay, L. S., Dahlquist, F. W., & Matthews, B. W. (2004). Structural Basis for the Attachment of a Paramyxoviral Polymerase to its Template. *PNAS* , 101:8301-8306.

Kite, V., Boyle, D., Heine, H., Pritchard, I., Garner, M., & East, I. (2007). A Serological and Virological Survey for Evidence of Infection with Newcastle Disease Virus in Australian Chicken farms. *Production Animals* , 85:236-242.

Kiyotani K, Sakaguchi T, Kato A, Nagai Y, & Yoshida T. (2007). Paramyxovirus Sendai virus V protein counteracts innate virus clearance through IRF-3 activation, but not via interferon, in mice. *Virology*, 359: 82-91.

Kochs, G., Garcia-Sastre, A., & Martinez-Sobrido, L. (2007). Multiple Anti-Interferon Actions of the Influenza A Virus NS1 Protein. *Journal of Virology* , 81:7011-7021.

Korth, M. J., Kash, J. C., Furlong, J. C., & Katze, M. G. (2005). Virus Infection and the Interferon Response: A Global View through Functional Genomics. In D. J. Carr, *Interferon Methods and Protocols* (pp. 37-56). Oklahoma City. Oklahoma: Humana Press.

Kunzi, M. S., & Pitha, P. M. (2005). Interferon Research: A Brief History. In D. J. Carr, *Interferon Methods and Protocols* (pp. 25-36). Oklahoma City, Oklahoma: Humana Press.

Lamb, R. A., & Parks, G. D. (2007). Paramyxoviridae: The Viruses and their Replication. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, *et al., Fields Virology* (pp. 1449-1497). Lippioncott Williams and Wilkins.

Lin, D., Lan, J., & Zhang, Z. (2007). Structure and Function of the NS1 Protein of Influenza A Virus. *Acta Biochimica et Biophysica Sinica* , 39:155-162.

Lin, R.-J., Chang, B.-L., Yu, H.-P., Liao, C.-L., & Lin, Y.-L. (2006). Blocking of Interferon-Induced JAK-STAT Singalling by Japanese Encephalitis Virus NS5 through a Protein Tyrosine Phosphatase Mediated Mechanism. *Journal of Virology* , 80:5908-5918.

Mayo, M. (2002). A Summary of Taxonomic Changes Recently Aproved by ICTV. *Archaic Virology* , 147:1655-1663.

Meyer, T., Begitt, A., and Vinkemeier, U. (2007). Green Fluorescent Protien-Tagging Resuces the Nucleocytoplasmic Shuttling Specifically of Unphosphorylation STAT1. *FEBS Journal*. 274: 815-826.

Middleton, D., Westbury, H., Morrissy, C., Heide, B. v., Russell, G., Braun, M., *et al.* (2002). Experimental Nipah Virus Infection in Pigs and Cats. *Journal of Comparitive Pathology* , 126:124-136.

Middleton, D.J., Morrissy, C.J., van der Heide, B.M., Russell,G.M., Braun, M.A., Westbury, H.A., Halpin, K., and Daniels, P.W. (2007). Experimental Nipah Virus Infection in Pteropid Bats (Pteropid poliocephalus). *Journal of Comparative Pathology.* 136:266-272.

Min, J.-Y., Li, S., Sen, G. C., & Krug, R. M. (2007). A Site on the Influenza A Virus NS1 Protein Mediates both Inhibition of PKR Activation and Temporal Regulation of Viral RNA Synthesis . *Virology* , 363:236-243.

Mochizuki, K., Takabe, S., and Goda, T. (2008). Changes on Histone H3 modifications on the GLUT5 gene and its expression in Caco-2 cells co-treated with p44/42 MAPK inhibitor and glucocorticoid hormone. *Biochemical and Biophysical Research Communications.* 371: 324-327.

Mohd-Nor, M., Gan, C., & Ong, B. (2000). Nipah Virus Infection of Pigs in Peninsualr Malaysia. *Revus Scientifique et Technique*, 19:160-165.

Moll, M., Kaufmann, A., and Maisner, A. (2004). Influence of N-glycans on Processing and Biological Activity of the Nipah Virus Fusion Protein. *Journal of Virology*, 78:7274-7278.

Mungall, B.A., Middleton, D., Crameri, G., Bingham, J., Halpin, K., Russell, G., Green, D., McEachern, J., Pritchard,I.L., Eaton,B.T., Wang,L-F.,. Bossart,K.N., and Broder, C.C. (2006). Feline Model of Acute Nipah Virus Infection and Protection with a Soluble Glycoprotein-Based Subunit Vaccine. *Journal of Virology,* 80(24): 12293–12302.

Murry, P. (2005). Paramyxoviruses. In P. Murry, *Medical Microbiology* (pp. 597-609). Bethesda, Maryland: Northeastern Ohio Universities College of Medicine.

Nanda, S. K., & Baron, M. D. (2006). Rinderpest Virus Blocks Type I and Type II Interferon Action: Role of Structural and Nonstructural Proteins. *Journal of Virology* , 80:7555-7568.

Narin, R. (1995). Immunology. In G. F. Brooks, J. S. Butel, & L. Ornston, *Medical Microbiology* (pp. 105-127). East Norwalk, Connecuit: Simon and Schuster.

Negrete, O., Levroney, E., Aguilar, H., Bertolotti-Ciarlet, A., Nazarian, R., Tajyar, S., *et al.* (2005). EphrinB2 is the Entry Receptor for Nipah Virus, An Emergent Deadly Paramyxovirus. *Nature* , 436:401-405.

Negrete, O., Wolf, M., Aguilar, H., Enterlien, S., Wang, W., Muhlberger, E., *et al.* (2006). Two key Residues in EphrinB3 are Critical for its Use as an Alternative Receptor for Nipah Virus. *PLoS Pathogens* , 2:e7.

Olival, K., & Daszak, P. (2005). the Ecology of Emerging Neutropic Viruses. *Journal of Neurovirology* , 11:441-446.

Paton, N., Leo, Y., Zaki, S., Auchus, A., Lee, K., Ling, A., *et al.* (1999). Outbreak of Nipah Virus InfectionAmong abattoir Workers in Singapore. *Lancet* , 354:1253-1256.

Perry, A. K., Chen, G., Zheng, D., Tang, H., & Cheng, G. (2005). The Host Type I Interferon Response to Viral and Bacterial Infections. *Cell Research* , 15:407-422.

Pietila, T.E.,Veckman, V., Lehtonen A., Lin, R., Hiscott, J., & Julkunen, I. 2007. Multiple NF-kB and IFN Regulatory Factor Family Transcription Factors Regulate CCL19 Gene Expression in Human Monocyte-Derived Dendritic Cells. *Journal of Immunology.* 178:253-261.

Rincon, M., Enslen, H., Raingeaud, J., Recht, M., Zapton, T., Su, M. S.-S., *et al.* (1998). Interferon-gamma Expression by Th1 Effector T Cells Mediated by the p38 MAP Kinase Signalling Pathway. *The EMBO Journal* , 17:2817-2829.

Rodriguez, J. J., & Horvath, C. M. (2004). Host Evasion by Emerging Paramyxoviruses: Hendra Virus and Nipah Virus V Proteins Inhibit Interferon Signalling. *Viral Immunology* , 17:210-219.

Rodriguez, J. J., Cruz, C. D., & Horvath, C. M. (2004). Identification of the Nuclear Export Signal and STAT Binding Domains of the Nipah Virus V Protein Reveals Mechanisms Underlying Interferon Evasion. *Journal of Virology* , 78:5358-5367.

Rodriguez, J. J., Parisien, J.-P., & Horvath, C. M. (2002). Nipah Virus V Protein Evades Alpha and Gamma Interferons by Preventing STAT1 and STAT2 Activation and Nuclear Accumulation. *Journal of Virology* , 76:11476-11483.

Rodriguez, J. J., Wang, L.-F., & Horvath, C. M. (2003). Hendra Virus V Protein Inhibits Interferon Signalling by Preventing STAT1 and STAT2 nuclear Accumulation. *Journal of Virology* , 77:11842-11845.

Rose, J.M., Crowley, C.K., & Fleishmann, W.R, Jr., Evidence that IFN alpha/beta Induces Two Antiviral States Active against Different Viruses. 1985. *Journal of General Virology.* Pt5:1153-1158.

Sarkis, P.T., Ying, S., Xu, R., Yu, X.F., STAT1-Independent Cell Type-Specific Regulation of Antiviral APOBEC3G by IFN-alpha. 2006. *Journal of Immunology.* 177:4530-4540.

Schroder, M., & Bowie, A. G. (2005). TLR3 in Antiviral Immunity: Key Playeror Bystander. *TRENDS in Immunology* , 26:462-468.

Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P., & Basler, C. F. (2005). Nuclear Localization of the Nipah Virus W Protein Allows for Inhibition of both Virus and Toll-Like receptor-3 Triggered Signalling Pathways. *Journal of Virology* , 79:6078-6088.

Shaw, M. L., Garcia-Sastre, A., Palese, P., & Basler, C. F. (2004). Nipah Virus V and W Proteins have a Common STAT1-Binding Domain yet Inhibit STAT1 Activation from the Cytoplasmic and Nuclear Compartments, Respectively. *Journal of Virology* , 78:5633-5641.

Shiell, B., Gardner, D., Crameri, G., Eaton, B., & Michalski, W. (2003). Sites of Phosphorylation of P and V Proteins from Hendra and Nipah Viruses: Newly Energed Members of Paramyxoviridae. *Virus Research* , 92:55-65.

Shimizu, M., Yamada, S., Kawashima, K., Ohashi, S., Shimizu, S., Ogawa, T. (1996). Changes of Lymphocyte Subpopulations in Pigs Infected with Porcine Reproductive and Respiratory Syndrome (PRRS) Virus. *Veterinary Immunology and Immunopathology.* 50: 19-27.

Tan, K.S., Tan, C.T., and Goh, K. J. 1999. Epidemiological Aspects of Nipah Virus Infection. *Neurol. J. Southeast Asia.* 4:77-81.

UniProtKB (2008). Non Structural Protein V. Retrieved February 18, 2009 from http://www.uniprot.org/uniport/Q997F2.

Vidal, S., Curran, J.& kolakofsky, D. 1990. A Stuttering Model for Paramyxovirus P mRNA Editing. *EMBO Journal.* 9:2017-2022.

Vogel, S.N, Friedman R.M., and Hogan MM. (2001). Measurement of antiviral activity induced by interferons alpha, beta, and gamma. *Current Protocols in Immunology.* Chapter 6. Unit 6.9.

Wang L.F, Michalski W,P., Yu M., Pritchard L.I., Crameri G, Shiell B, and Eaton B.T. (1998). A novel P/V/C gene in a new member of the Paramyxoviridae family, which causes lethal infection in humans, horses, and other animals. *Journal of Virology,* 72:1482-1490.

Wang, L.-F., Harcourt, B. H., Yu, M., Tamin, A., Rota, P. A., Bellini, W. J., *et al.* (2001). Molecular Biology of Hendra and Nipah Viruses. *Microbes and Infection* , 3:279-287.

Weingartl, H., Czub, S., Copps, J., Berhane, Y., Middleton, D., Marszal, P., *et al.* (2005). Invasion of the Central Nervous System in a Porcien Host by Nipah Virus. *Journal of Virology* , 79:7528-7534.

Wild, T.F. (2008). Henipaviruses: A new family of emerging Paramyxoviruses. *Pathologie Biologie.* Epub ahead of print.

Wong, K., Shieh, W., Kumar, S., Norain, K., Abdullah, W., Guarner, J., *et al.* (2002). Nipah Virus Infection: Pathology and Pathogenesis of an Emerging Paramyxoviral Zoonosis. *American Journal of Pathology* , 161:2153-2167.

Yokota, S.-i., Saito, H., Kunota, T., Yokosawa, N., Amano, K.-i., & Fujii, N. (2003). Measles Virus Suppresses Interferon-alpha Signalling Pathway, Suppression of JAk1 Accessory Proteins C and V with Interferon alpha receptor Complex. *Virology* , 306:135-146.

APPENDIX A

1. CMC Preparation

Preparation of	1	x CMC overlay
	100	mL required
	2.00	% final

1 Put magnetic stirring bar in a bottle
2 Add 76.57 mL mQ water
3 Heat to almost boiling point
4 Add slowly 2.00 g of CMC
5 Stir on a magnetic stirrer until CMC is dissolved
6 Heat again if necessary. Do not boil
7 Autoclave at 121C for 15'
8 Cool to 37C
9 Add (mL):

10.00	10X DMEM
2.00	FBS
4.93	7.5% NaHCO3
2.50	1M HEPES
1.00	0.4 g/L Folic Acid (100x)
1.00	200 mM L-Glu
1.00	100mM (11.0 mg/mL) Sodium Pyruvate
1.00	100x Pen/Strep

10 Mix on magnetic stirrer in 37C incubator until ready to use

2. PFU/ml Calculation

$$\frac{\text{Average number of plaques* x dilution factor}}{\text{Volume plated}} = \text{PFU/ml}$$

*Plaques must be visible and countable (between 30-300)

3. Viral Plaque Reduction Assay Calculation

The virus control well with no IFN added is considered to have 100% viral plaques. IFN treated wells of the same concentration are averaged and compared to the virus control well to determine the reduction of plaques.

Example of Calculation:

Number of plaques in virus control well = 50

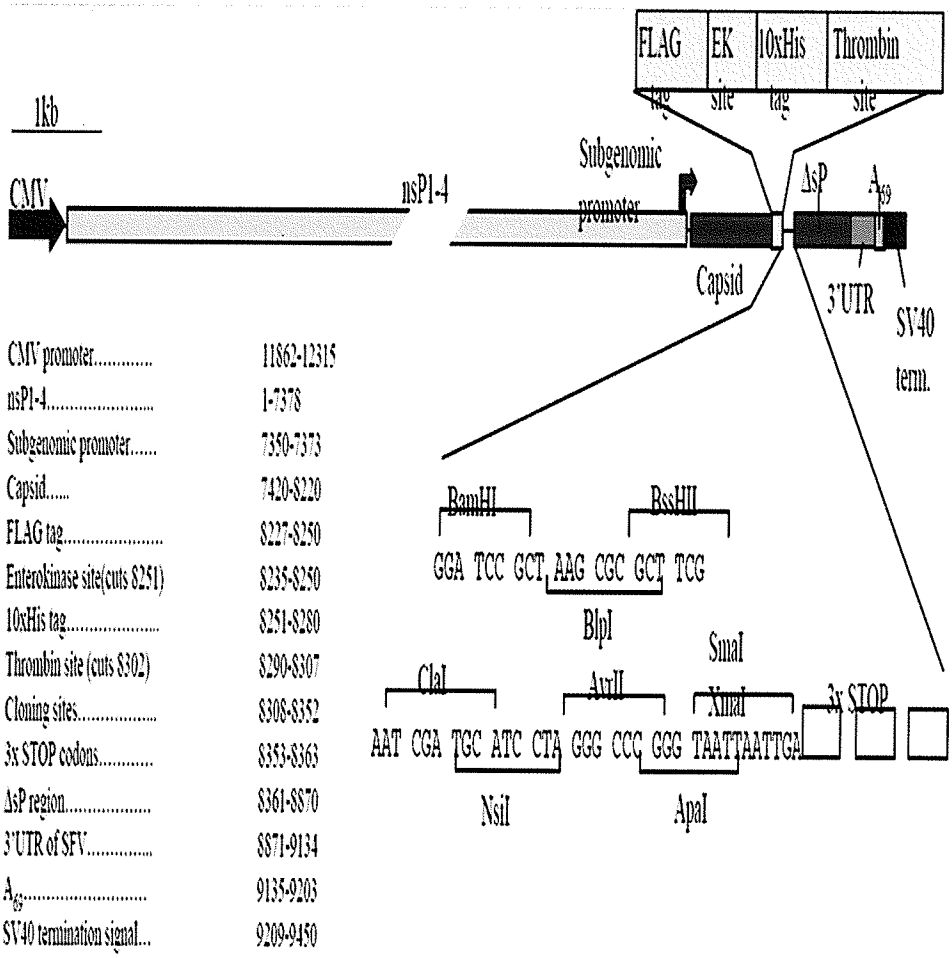
Average number of plaques in IFN treated well = 30

100- (30/50 x 100) = 40 of plaques are reduced (from 100% viral infection). At the concentration used, the cells are protected by IFN at 40%.

APPENDIX B

1.

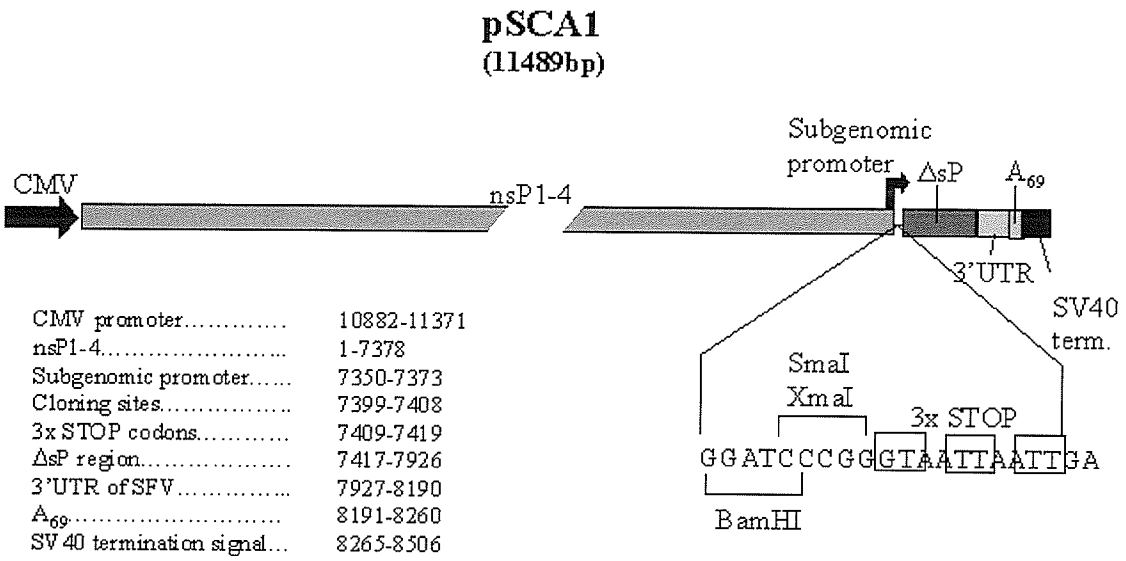
pSMART2a
(12433 bp)



CMV promoter.....	11862-12315
nsP1-4.....	1-7378
Subgenomic promoter.....	7350-7373
Capsid.....	7420-8220
FLAG tag.....	8227-8250
Enterokinase site(cuts 8251)	8235-8250
10xHis tag.....	8251-8280
Thrombin site (cuts 8302)	8290-8307
Cloning sites.....	8308-8352
3x STOP codons.....	8353-8363
ΔsP region.....	8361-8870
3'UTR of SFV.....	8871-9134
A_{69}.....	9135-9203
SV40 termination signal...	9209-9450

Source: DiCiommo *et al*, 2004. http://vsrp.uhnres.utoronto.ca/pSMART2a.pdf

2.



•Drawing is to scale, although full length nsP1-4 region is not shown. Unique cloning sites are indicated.
• ΔsP is the non-functional structural protein ORF containing a large deletion.

Total length of nsP region is 7381 nucleotides. ORF starts at 86, ends at UAA at 7379-7381. ORF codes for 2431aa long polypeptide. Individual nsPs are generated by nsP2-mediated processing.

nsP1: 86-1696 (737aa)
nsP2: 1697-4090 (798aa)
nsP3: 4091-5536 (482aa)
nsP4: 5537-7378 (614aa)

Source: DiCiommo *et al*, 1999. http://vsrp.uhnres.utoronto.ca/SFV_frame5.htmlhttp

3.PCR Reaction for NiV V and NiV W cloning into pSCA and pSMART2a (30 cycles – steps in bold)

Procedure	**Temperature**	**Time**
Denature	94°C	2 minutes
Denature	**94°C**	**15 seconds**
Anneal	**60°C**	**30 seconds**
Extend	**72°C**	**10 minutes**
Final Extend	72°C	10 minutes

4.PCR Master Mix (All components except template are from Platinum Pfx kit by Invitrogen, Cat No. 11708-013)

Reaction component	**Amount added per Reaction**
10x Pfx Buffer	10 µl
10mM dNTP mix	1.5 µl
50mM $MgSO_4$	1 µl
Reverse Primer	1 µl
Forward Primer	1 µl
Pfx Polymerase	1 µl
Template	0.5 µl
dH_2O	34 µl
Total Reaction Mix	50 µl

5. BamHI Restriction digest (All components except DNA are from Roche, Cat No. 10567604001)

Reaction Component	**Amount added per Reaction**
Bam HI (2,500U)	1-2 µl
Buffer	2 µl
DNA (1µg)	5 µl
H_2O	12 µl
Total	20 µl

6. Dephosphorylation (Enzyme and Buffer are supplied by Roche, Cat No. 11097075001)

Reaction Component	**Amount added per Reaction**

Vector (1μg)	10 μl
Buffer	2 μl
Enzyme (1,000U)	1 μl
Total	13 μl

7. Ligation Mix (Enzyme and Buffer are supplied by Roche, Cat No. 10481220001)

Reaction Component	**Amount added per Reaction**
T4 DNA Ligase	2 μl
Buffer (10x)	4 μl
Insert	According to Calculation
Vector	According to Calculation
H_20	Adjust Accordingly
Total	~20 μl

8. DNA Ligation Calculation

$$\frac{\text{ng of Vector} + \text{KB size of insert}}{\text{KB size of Vector}} \times \text{insert:vector ratio} = \text{ng of insert}$$

APPENDIX C

1. Buffers for SDS PAGE and western blot

Resolving Gel (10%)

7.8ml	H_2O
6.5ml	Acrylamide Bis
4.88ml	1.5M Tris
195µl	10% SDS
195 µl	10% APS
13 µl	Temed

Stacking Gel (10%)

7.32ml	H_2O
1.328ml	Acrylamide Bis
1.250ml	1.0M Tris
100µl	10% SDS
120µl	10% APS
10µl	Temed

Anode Buffer (100ml)

5x Tris-CAPS	20ml
MeOH	15ml
H_2O	65ml

Cathode Buffer (100ml)

5x Tris-CAPS	20ml
10% SDS	1ml
H_2O	79ml

Running Buffer (5L)

Tris	30g
Glycine	145g
10% SDS	1%

5x CAPS

Tris Base	36.4g
CAPS	44.26g
H_2O	to 1 Litre

Wash Buffer

1x TBS-T	100ml
Tween	1ml
H_2O	to 1 Litre

10x TBS-T

NaCl	87.6g
Tris	12.1g
Tween-20	0.1%
H_2O	to 1 Litre

Blocking Buffer (100ml)

Skim Milk powder	5g
1x TBS-T	10ml
H_2O	90ml

Lysis Buffer

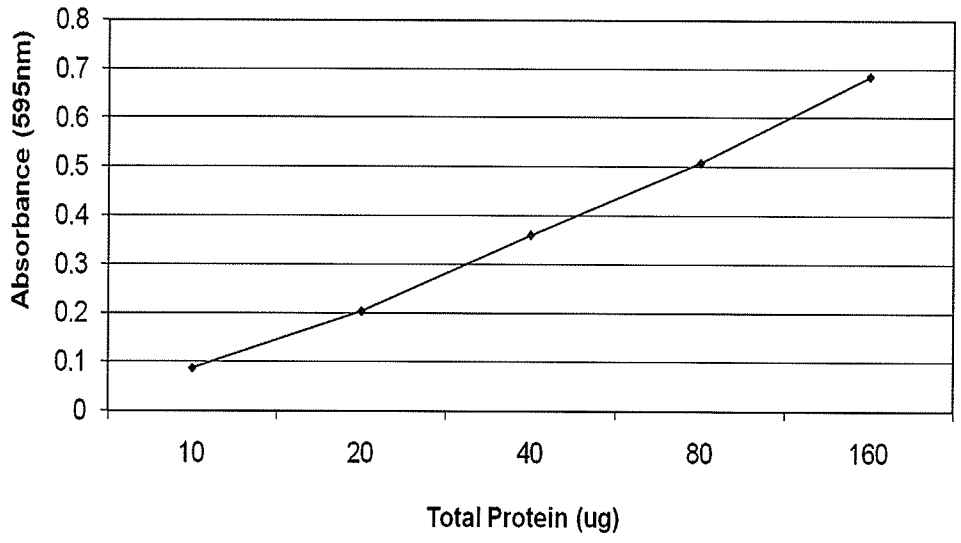
100mM Tris pH 8.0
100mM NaCl
1% Triton X-100

2. Semi dry Transfer

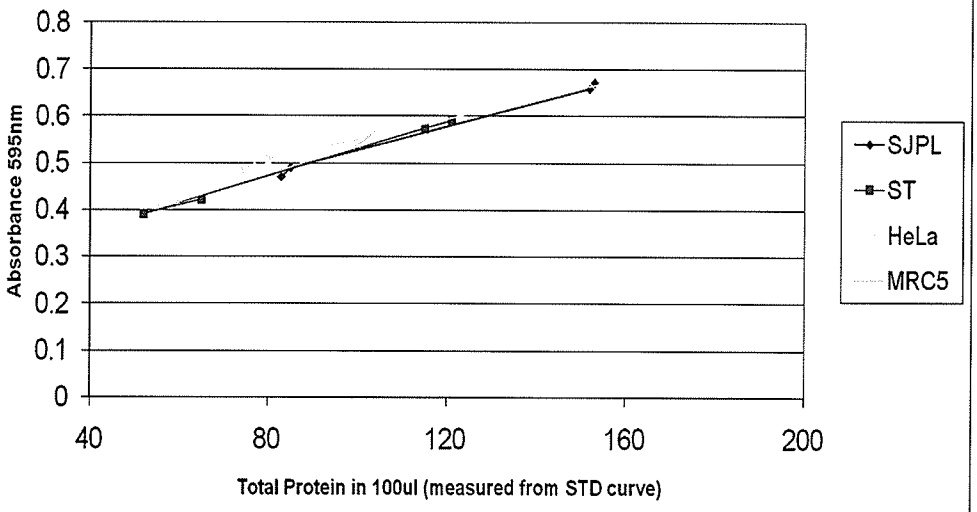
1. Wet PVDF (BioRad, Cat. No. 162-017) membrane in pure methanol for 5 seconds and then into Anode buffer for at least 30 minutes before you begin transfer
2. Remove gel from running equipment and cut off stacking gel
3. Wash gel briefly in water
4. Put gel into Cathode buffer
5. Wet one piece of filter paper in anode buffer and add it to the transfer machine
6. Add the PVDF membrane on top of it and roll out bubbles
7. Add the gel to the membrane so the ladder is on the left side as a guide for transfer efficiency
8. Wet two pieces of filter paper in cathode buffer and add it on top of the gel

9. Run transfer with only two gels in one transblot membrane at 23 volts (no more than 25 volts) consistently for 1 to 1.5 hours
10. After transfer is complete, add the PVDF membranes to blocking buffer (5% skim milk) can leave in blocking buffer for 1 hour at room temperature if western blot is to be carried out the same day or overnight at 4°C

3. **Bovine Serum Albumin (BSA) standard curve of total protein measured at 595nm**



4. **Total Protein in 100ul samples from nuclei, cytoplasm and whole cell lysates of infected SJPL, ST, HeLa and MRC5 cells**



5. Protein Quatification Assay based on the Bradford method using BioRad Protein Assay Dye Reagent Concentrate (Cat. No. 500-0006) provided by the above company and following their provided protocol. NiV infected porcine and human cell extraction of nuclei and cytoplasm and intact whole cell lysate were measured.

Cell Type	Sample	Absorbance (595nm)	Total Protein in 100µl (Measured from STD curve)	Total protein loaded in SDS PAGE gel	Total volume of samples loaded in SDS PAGE gel
SJPL	Whole cell lysate	0.672	153ng	30.6ng	20µl
	Cytoplasm	0.660	152ng	30.4ng	20µl
	Nuclei with membranes attached	0.490	85ng	29.75ng	35µl
	Nuclei with membranes removed	0.471	83ng	29.05ng	35µl
ST	Whole cell lysate	0.591	121ng	24.2ng	20µl
	Cytoplasm	0.575	115ng	23ng	20µl
	Nuclei with membranes attached	0.392	52ng	18.20ng	35µl
	Nuclei with membranes removed	0.422	65ng	22.75ng	35µl
HeLa	Whole cell lysate	0.682	160ng	32ng	20µl
	Cytoplasm	0.508	80ng	16ng	20µl
	Nuclei with membranes attached	0.517	80ng	28ng	35µl
	Nuclei with membranes	0.485	74ng	25.9ng	35µl

	removed				
MRC5	Whole cell lysate	0.563	103ng	20.6ng	20µl
	Cytoplasm	0.538	100ng	20.0ng	20µl
	Nuclei with membranes attached	0.403	55ng	19.25ng	35µl
	Nuclei with membranes removed	0.415	60ng	21ng	35µl