Rapid Development of Optimized Recombinant Adenoviral Vaccines for Biosafety Level 4 Viruses

by

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ABSTRACT

This thesis describes the production of adenovirus-based vaccines containing codon-optimized genes from Nipah virus and Crimean-Congo Hemorrhagic Fever virus. Genes encoding envelope proteins from Crimean-Congo Hemorrhagic Fever Virus and Nipah Virus were codon-optimized for translation in human cells and constructed using a modified method of non-gapped gene synthesis, while the entire M segment encoding the glycoprotein precursor for Crimean-Congo Hemorrhagic Fever Virus was commercially synthesized. Genes were cloned into recombinant human adenovirus serotype 5 and the resulting viral particles were amplified, titred and analyzed for *in vivo* efficacy. Results show that a modified method of non-gapped gene synthesis is an effective and efficient method of producing antigen-encoded DNA and at a fraction of the cost and time required for commercial synthesis. Furthermore, adenovirus-based vaccines induce both cellular and humoral immune responses providing for a highly efficacious vaccine during potential disease outbreaks, where time to completion is of utmost importance. This study has shown that recombinant adenoviral vaccines for Crimean-Congo Hemorrhagic Fever virus and Nipah virus can be produced rapidly and efficiently from virtual DNA sequence to optimized recombinant vaccines in just eight months.

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List of Abbreviations

ABTS: 2,2'Azino-di[3-ethylbenzthiazoline sulfonate(6)]diammonium salt

ADE: Antibody Dependent Enhancement

ALT: Alanine Transferase

AST: Aspartate Transferase

aPTT: Activated Partial Thromboplastin

AUD: Animal User Document

BHK-21: Cell line derived from baby Hamster kidney cells

CAR: Coxsackie Adenovirus Receptor

CCHF(V): Crimean-Congo Hemorrhagic Fever (Virus)

CHO: Cell line derived from Chinese Hamster Ovary Cells

CPE: Cytopathic Effects

DIC: Disseminated Intravascular Coagulation

DMSO: Dimethyl Sulfoxide

ELISA: Enzyme-linked Immunosorbent Assay

Gc: C-terminal glycoprotein

Gn: N-terminal glycoprotein

GTP: Guanosine Triphosphate

GPC: Glycoprotein Precursor

HeV: Hendra Virus

HRP: Horseradish Peroxidase

IFN: Interferon

IRF: Interferon Regulatory Factor

ISG: Interferon Stimulated Gene

IL: Interleukin

ITR: Inverted Terminal Repeats

L: Large segment

LLC-MK2: Cell line derived from Rhesus monkey kidney cells

MCS: Multiple Cloning Site

mRNA: Messenger Ribonucleic Acid (see RNA)

M: Medium segment

NCBI: National Center for Biotechnology Information

NiV: Nipah Virus

ORF: Open Reading Frame

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

RDRP: RNA Dependent RNA Polymerase (see RNA)

RIPA: Radioimmunoprecipitation buffer

PRNT: Plaque Reduction Neutralization Test

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcriptase PCR (see PCR)

S: Small segment

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SLAM: Signalling Lymphocyte Activation Molecule (CD150)

STAT: Signal Transducers and Activators of Transcription

SW13: Cell line derived from human adrenal carcinoma

UAE: United Arab Emirates

UTR: Untranslated Regions

Vero: Cell line derived from African Green Monkey kidney epithelial cells

WWII: World War II

1.0 Introduction

1.1 Adenoviral Vectors as Vaccines

Historically, most vaccines were based on inactivated, live or attenuated pathogens. Advancements in molecular biology and virology now allow for the use of viruses as vectors for antigen expression. Adenoviruses were mainly vectored as gene therapy vehicles, but difficulties with innate and adaptive immune responses induced by adenoviral antigens hampered gene transfer attempts. Despite this, adenoviruses gained increasing popularity as potential vaccine candidates for several reasons. Adenovirus genomes are fairly well characterized, and are relatively easy to render replication-incompetent. Furthermore, adenoviral infections cause relatively mild symptoms for healthy individuals. They can also be grown to high titres in suitable cell lines and have relatively excellent thermostability [1]. Additionally, since adenoviruses have double stranded DNA, this facilitates their use for mammalian systems.

There are currently 51 human serotypes of adenoviruses, divided into six subgroups (A to F; with B being divided into B1 and B2), based on sequence homology and hemagglutination abilities [2]. The 34-43 Kb linear DNA genome is flanked by inverted terminal repeats (ITR) that act as origins of replication. There are 8 transcriptional units, 5 of which are early units, two that are expressed with a delay after viral replication and 1 late unit [2].

Adenoviruses can cause acute symptomatic and persistent asymptomatic infections. Human serotypes, such as AdHu1, AdHu2, and AdHu5 cause mild upper respiratory infections while other serotypes may cause gastrointestinal symptoms. With acute infections, adenoviruses are known to persist in lymphoid cells [3-5]. This persistence, without clinical symptoms, appears to be linked to E3 gene products [6]. E3 gene products are suspected of protecting infected cells from destruction by the immune system and confer a survival advantage to infected cells by inhibiting apoptosis [6] . Due to their prevalence in the human population, adenoviruses generally infect humans during early infancy. Approximately 45-80% of adults carry AdHu5 neutralizing antibodies [7, 8].

Host-virus interactions generally determine adenovirus tropism due to binding of the distal knob domain of the penton fiber attaching to cellular receptors- α_v integrins [9-11]. Most human adenoviruses, such as AdHu5 and AdHu2 initially bind to the coxsackie adenovirus receptor (CAR), which is expressed on various cell types, such as hepatocytes, epithelial cells, endothelial cells, myoblasts, and heart muscle cells [12]. Adenoviruses of the subtype B do not bind CAR but instead bind CD46 (subtype B2), a complement regulatory protein expressed on hematopoietic stem cells and dendritic cells [2]. Secondary receptors are found on the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which bind to the penton fiber base [13].

Adenoviruses are immunogenic due to the fact that they express pathogen associated molecular patterns (PAMPS), which bind to pathogen recognition receptors on host cells [14]. This binding has the effect of activating the innate immune system and initiating the production of proinflammatory cytokines and maturation of dendritic cells into professional APCs [14]. Adenovirus-induced maturation of dendritic cells was shown to be NF-κB-dependent [15]. Systemic administration of high doses of AdHu5 vectors into mice or monkeys was shown to release IL-6, IL-12, and TNF-α with an accumulation of macrophages and dendritic cells in lymphatic tissues [16, 17]. The hexon and fiber protein contain most of the epitopes that are recognized by neutralizing antibodies [18, 19]. Both the early and late antigens of adenoviruses are the targets of the adaptive immune response. Neutralizing antibodies to adenoviral infection are predominantly directed against the surface loops of the viral hexon [20]. Nevertheless, antibodies to the penton base or the fiber can also have a neutralizing effect upon adenoviruses [21].

In a comparison of adenoviral vectors to other subunit vaccines, it has been demonstrated that adenoviral vectors outperform poxvirus vectors, DNA vaccines, and alphavirus vectors with respect to transgene specific antibody titres and CD8+ T-cell responses [22, 23]. In addition, antibody responses can also be elicited by alternate routes of administration, such as mucosal and oral [24-29].

Although most testing of AdHu5 vectors occurs in animals and show promising results, this may be problematic in humans due to the effect of pre-existing immunity. Neutralizing antibodies, even if present at moderate titres, can have a profound effect

upon the uptake of adenoviral vector by the cells [30]. Logically, this can be overcome by increasing the dose of the vaccine, but this approach may be limited by toxicity issues. Furthermore, the levels of neutralizing antibodies across individuals may vary greatly, making it difficult to calculate correct dosages. Studies conducted in AdHu5 pre-exposed nonhuman primates showed that a 1000-fold increase in dose is required to achieve the same transgene expression as in animals that did not have pre-existing immunity [22]. The repeated use of AdHu5 has also been tested, but in most cases, the development of neutralizing antibodies against the vector preclude its use if a significant anamnestic effect is desired [31]. Nevertheless, many different methods are currently being studied to overcome the effects of pre-existing immunity.

Outbreaks of infectious diseases and the continuous threat of bioterrorism have supported a rapid pace for the development of new vaccines. Due to the versatility of adenoviral-based vaccines, it is clear that they are valuable tools for vaccine development; for both improving existing vaccines, and creating new treatments for emerging diseases.

1.2 Crimean –Congo Hemorrhagic Fever Virus

Crimean-Congo hemorrhagic fever (CCHF) is a fatal viral infection endemic in many parts of Asia, Africa, Europe, and the Middle East [32, 33]. The causative agent is a virus, belonging to the family *Bunyaviridae*, genus *Nairovirus*, and causes severe disease in humans with a fatality rate of approximately 3-30% [33]. CCHF virus is a tick-borne virus that is transmitted to humans through infected tick bites, squashed ticks, or by direct

contact with viremic humans or animals [34, 35]. Clinical features of infection include but are not limited to hemorrhage, fever, and myalgia (muscle pain) [33, 34, 36]. There is currently no commercially available vaccine for CCHF, although Ribavirin has been used as a supportive therapy [36].

1.2.1 Historical Background of Crimean- Congo Hemorrhagic Fever

CCHF was first described as a clinical entity in 1944 during an outbreak in which 200 Soviet military personnel were infected in Crimea shortly after World War II (WWII) [32, 33, 36]. In 1967, the virus was isolated from infected patients and was propagated intracerebrally in suckling white mice [32]. This isolate was shown to be virtually identical to the Congo virus, which was isolated in 1956 from a patient in the former Belgian Congo, the present day Democratic Republic of Congo [37, 38]. Due to antigenic similarities between the Eurasian Crimean Hemorrhagic fever strains and African Congo strains, the virus was named Crimean hemorrhagic fever-Congo virus and later renamed the Crimean-Congo Hemorrhagic Fever Virus (CCHFV) [38-43].

1.2.2 Epidemiology

The epidemiology of CCHF reflects the geographic distribution of *Ixodid* ticks, belonging to the genus *Hyalomma* [33, 44-46]. CCHF has been reported in over 30 countries in Africa, southeast Europe, the Middle East, and Asia [32, 34, 47]. Cases of CCHF have been reported in the African nations of the Democratic Republic of Congo, Uganda,

Mauritania, South Africa, Tanzania, Nigeria, and Senegal and in regions of southeastern Europe, including Russia, Bulgaria, Kosovo, Turkey, and Greece [34, 48]. Countries of the Middle East and Asia, such as United Arab Emirates (UAE), Iraq, Iran, Saudi Arabia, Oman, China, Kazakhstan, and Pakistan, have also reported a substantial number of cases of CCHF [33, 34].

Phylogenetic analysis of the M segment, which encodes two glycoproteins, of CCHF isolates has revealed the presence of eight genetically distinct clades [35, 48]. The genetic divergence in the European strains is quite low and are grouped together in one clade with the exception of the Greek isolate, AP92, which forms its own clade [33, 49]. The third clade is comprised of the Asian strains from Kazakhstan, Tajikistan, Uzbekistan and China, which are relatively similar. M segment analysis revealed that the Chinese isolates were clustered into three groups, one of which was homologous to a Nigerian isolate [33, 50, 51]. A fourth clade represents isolates from Iran, Madagascar, and Pakistan [48]. However, some isolates from Iran correspond to a different lineage of CCHFV that resembles strains from Mauritania and Senegal; together these strains comprise a fifth clade, thus, demonstrating the circulation of two genetic variants co-circulating in Iran [52]. In Africa, there are three distinct clades; strains from Senegal, Mauritania, and South Africa are grouped in one clade; strains from Nigeria and Central African Republic are accommodated in a second, and those from Uganda form a third clade [33].

1.2.3 Ecology

Similar to many other tick-borne zoonotic agents, CCHFV undergoes an enzootic tick-vertebrate-tick cycle [36]. CCHFV is transmitted by ticks belonging to the genus *Hyalomma*, particularly *Hyalomma marginatum marginatum,* known as the "Mediterranean hyalomma", which is the main vector in Europe [33]. Recent developments have elucidated many similarities between *Nairovirus* genera and tick phylogenies that indicate the co-evolution of these viruses and their host ticks [53].

As evidenced by viremia and antibody responses, many mammals both wild and domesticated can be infected by CCHFV [36]. Despite this, it appears that birds, with the exception of ostriches, are resistant to infection yet can support large numbers of virus-infected ticks [32, 35, 54, 55]. CCHFV can persist in host ticks through its entire life cycle from larvae to nymph to adult stages, which is known as transstadial transmission [45, 56-59]. It has also been demonstrated that the virus can be transmitted transovarially-from parent to offspring [34, 47, 59]. Larger vertebrates such as sheep, goats and cattle are suspected of amplifying the virus in its life cycle, but many smaller mammals are capable of being infested by ticks that are still in the immature stages of growth [34, 60, 61]. Anti-CCHFV antibodies have been detected in the sera of horses, donkeys, goats, cattle, sheep and pigs throughout Europe, Asia and Africa [33, 34]. Although these mammals are capable of being infected with CCHFV, the symptoms are generally subclinical with the exception of humans and suckling mice [33].

1.2.4 Molecular Biology of CCHFV

The genus *Nairovirus* denotes species that are tick-borne and includes 34 viruses which are segregated into seven serogroups [34]. The CCHF serogroup contains CCHF virus and the closely related Hazara virus [34, 40, 41].

CCHFV is an enveloped virus with a negative sense single-stranded RNA genome (Figure 1.1). The three parts of the genome correspond to the small (S), medium (M), and large (L) segments, which code for the viral nucleocapsid (N), glycoprotein precursor (GPC), and RNA-dependent RNA polymerase (RDRP), respectively [62]. The structure and replication of CCHFV closely resembles those of other *Bunyaviridae* [62].

The 482 amino acid N protein encoded on the S segment associates with the viral RNAs; however, the mechanism of this interaction is poorly understood [34]. In virus-infected mammalian cells, the N protein does not associate with the Golgi Apparatus, but instead localizes to the perinuclear region in the absence of viral glycoproteins and viral RNA [34, 63]. It has been demonstrated that actin filaments are involved in the localization of N protein to the perinuclear regions as depolymerization of actin filaments using Cytochalasin D attenuated localization in CCHFV infected cells and N protein expressing cells [63]. Interestingly, human MxA protein can interact with the N protein, thereby inhibiting CCHFV replication [64]. In addition, inhibition of CCHFV replication in human cells by IFN-α is mediated by MxA GTPase [65].

The entire L segment of CCHF has been sequenced, and data has shown that the L-RNA segment is significantly larger than that found in other *Bunyaviridae*, being almost twice as long, at 12 164 bp [66]. This segment encodes a protein of 3944 amino acids containing an RNA polymerase catalytic domain within a central domain [34].

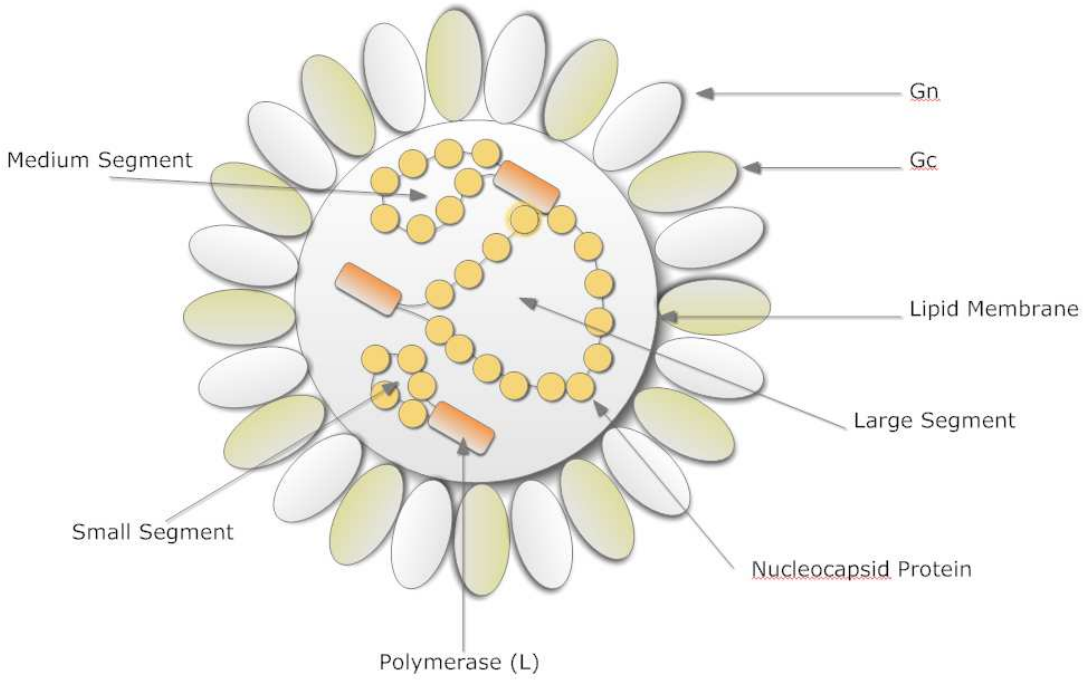

Figure 1.1. Schematic Representation of the CCHF virus. Diagram depicting the lipid bilayer with mature Gn and Gc glycoproteins embedded in the membrane. Virus interior contains the ribonucleocapsids for the S, M, and L segments [33].

Recent research into the M segment of CCHF virus has elucidated the complex coding strategy for the glycoprotein precursor, and the processing events involved in yielding the mature Gn and Gc glycoproteins. The M segment encodes a glycoprotein precursor that is significantly larger than most other *Bunyaviridae*, in which the GPCs are co-translationally processed into mature Gn and Gc proteins [62]. CCHFV GPC is more complex, as further post-translational modifications are needed for the production of the mature glycoproteins. In CCHF infected cells, the mRNA derived from the M segment is translated into a large precursor protein, which is thought to be co-translationally cleaved into 140 kDa PreGn and 85 kDa PreGc proteins [67]. These cleavages are believed to occur at the N-terminus and at the fifth hydrophobic domain by a signalase in the endoplasmic reticulum [67-69]. Both PreGn and PreGc give rise to fully mature envelope glycoproteins, Gn (37 kDa) and Gc (75 kDa) [67-69]. To generate the mature Gn, the PreGn is cleaved at amino acid motif RRLL at Gn's N-terminus by the cellular protease SKI-1, and at an undetermined location within the C-terminus by an unknown protease [69]. Recent research has shown that the potential SKI-1-like protease site ($RKLL_{807}$), when mutated, does not abrogate the production of Gn [69]. Interestingly, this C-terminal site is conserved in all CCHF virus strains as R(R/K)LL and is identical to the N-terminal motif [68]. Mature Gn is presumed to interact with Gc and translocate to the cellular compartment where viral assembly occurs [68]. The membrane-spanning region at the C-terminus of Gn is hypothesized as being a non-structural protein from the M segment (NSm) [70]. The N-terminus region of the Gn, once cleaved by SKI-1, releases the heavily O-glycosylated mucin-like variable region and GP38, a connector protein [68]. In the trans-Golgi network, the $RSKR_{247}$ motif is recognized by furin or furin-like

proprotein convertases, which cleave Gn, releasing GP38 and the mucin protein [68]. Uncleaved versions, referred to as GP85 /GP160 and can be found in the medium of virus- infected cells along with GP38 and the mucin protein (Figure 1.2) [68].

It has been demonstrated that the mature Gn and Gc are localized to the Golgi where assembly and release of the virion occur [34]. The Golgi retention signal resides within the ectodomain of Gn and the N-linked glycosylation is imperative for localization and transport [71]. In addition, it has recently been shown that CCHF virus is capable of reassortment, and that M-segment reassortants can affect the pathogenicity of the virus; a higher fatality rate occurred in patients infected with isolates which had apparently acquired M segments from a group in which predominantly Asian strains are usually found [72].

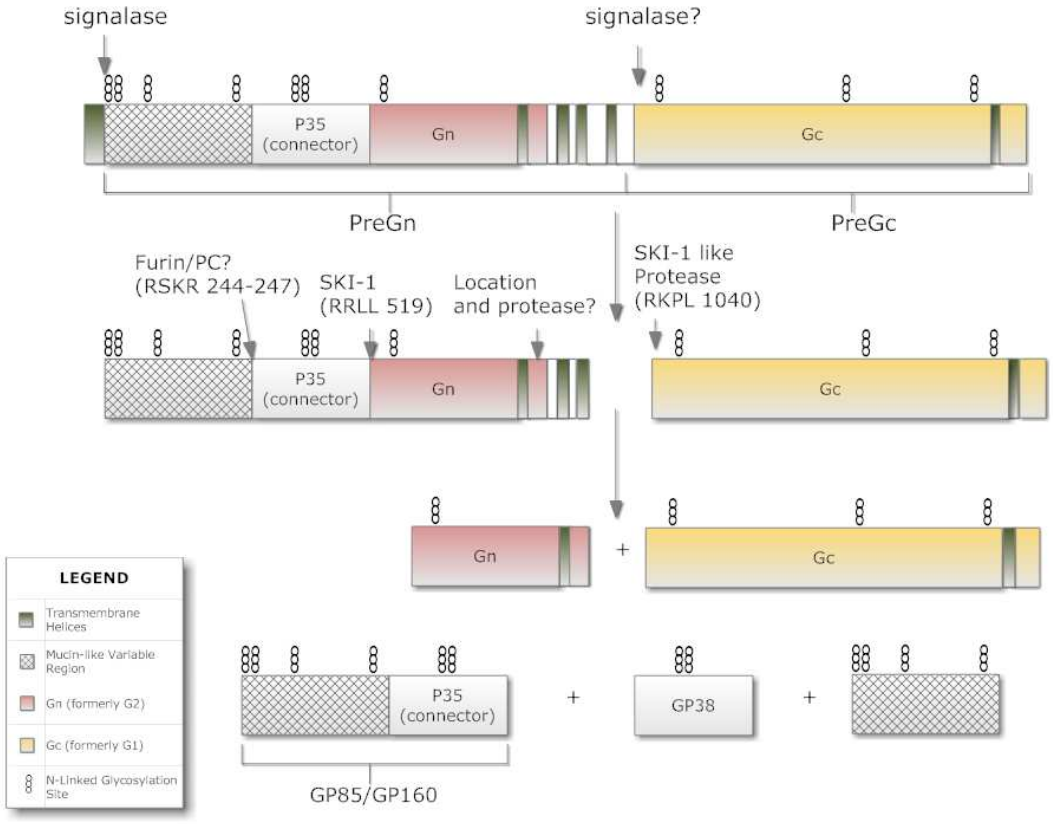

Figure 1.2. CCHF Glycoprotein Processing. Diagram depicting processing of the CCHF glycoprotein of strain IbAr10200 within mammalian cells. The glycoprotein is synthesized as a precursor and is cleaved by cellular proteases. The PreGn is produced by cleavage by a signalase and an unknown protease. The PreGc is produced by cleavage by a signalase and SKI-1-like protease at consensus motif $RKPL_{1040}$. Mature Gn is produced by cleavage by SKI-1 at motif $RRLL_{519}$. This cleavage liberates GP85/GP160, which may be further cleaved by a furin or proprotein convertases at motif $RSKR_{244-247}$. The mucin protein also possesses extensive O-linked glycosylation (not shown) [67-69].

1.2.5 Transmission

CCHF virus can be spread horizontally through infected livestock, as viremia can attain sufficiently high levels to infect previously non-infected ticks. This has been shown to be the case for several animal species such as sheep and cattle [60, 73]. Moreover, infection can occur through aerosols generated from infected excreta [33]. Community-acquired CCHF occurs through contact with blood or other infected tissues of livestock or from the bite of an infected tick. Most cases in humans are generally from workers in the livestock or agricultural industry, and veterinary personnel [33]. Humans with CCHFV can spread the disease through close contact with others, as is the case in the possible horizontal transmission of the virus from mother to daughter [74]. Nosocomial infections do take place as well, as evidenced by a nurse in Albania who had direct skin contact with vomit from an infected patient [75]. In addition, this form of transmission has been well documented in reports from Pakistan, Iraq, UAE, Africa and Iran [76-78]. It has been noted that infections in health care workers have been reported in parallel with outbreaks in the general population (Figure 1.3) [79, 80].

It is apparent that the most important risk factor for CCHFV infection is a tick bite [81]. Other important risk factors include abattoir workers who work with large domesticated mammals; this is included with other high-risk occupations such as butchers, physicians, and agricultural workers [81]. For workers in slaughterhouses, acquisition of the virus generally takes place while slaughtering animals and workers are exposed to infected blood, tissues, and excreta [80, 82].

The consumption of meat is not a significant risk factor, as the virus is inactivated by the corresponding acidification of tissues after slaughter [36]. In addition, if meat is to be cooked, the virus cannot survive elevated temperatures [36].

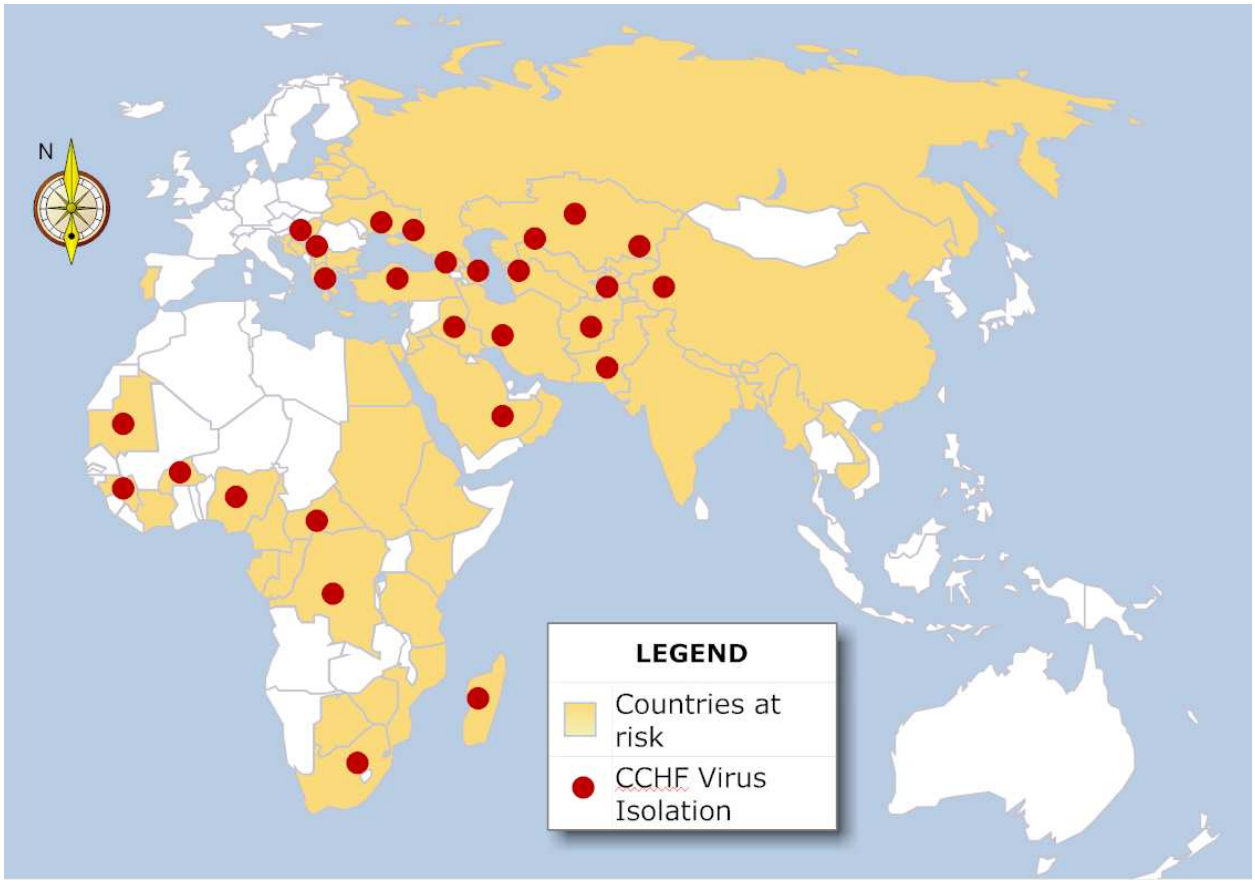

Figure 1.3. CCHFV Distribution Map. The distribution of CCHF virus closely parallels the presence of *Xiodid* ticks in geographical areas [33].

1.2.6 Clinical Manifestations

Humans appear to be the only host of CCHFV in which disease is manifested with the exception of newborn or suckling mice [34, 36]. Although infection in animals is subclinical, infection in humans can result in severe hemorrhagic disease [34, 36]. The course of infection is typically a four-stage event, consisting of incubation, prehemorrhagic, hemorrhagic, and convalescence periods [33, 34, 36]. Incubation periods for CCHFV generally range from about 2- 9 days and may vary based on route of exposure [83]. For instance, in South Africa, for 21 patients, the mean incubation period was 3.2 days after tick bite, 5 days for exposure to infected blood or tissues, and 5.6 days for human blood exposure [44].

The prehemorrhagic period is characterized by the sudden onset of fever, headache, myalgia and dizziness [33, 36, 47, 83]. Typically, fever will persist for approximately 4-5 days [32]. Other symptoms of diarrhea, nausea, vomiting, hyperemia of face, neck and chest, congested sclera, and conjunctivitis are quite common [33, 36]. The prehemorrhagic stage lasts approximately 3 days with a range of 1-7 days [32, 33, 36]. The hemorrhagic period lasts from 2-3 days, and develops quite rapidly despite the lack of relation between fever temperature and onset of hemorrhage [32]. Hemorrhage can range from petechiae to large hematomas of the skin and mucous membrane; it is not uncommon for bleeding to occur at other sites such as gingivum, vagina, nose, GI tract, uterus, urinary tract, and respiratory tract [33, 36]. In addition, hepatomegaly and splenomegaly has been reported in one third of all patients [32].

Convalescence begins in patients about 10-20 days after onset of disease and patients must remain in hospital for 9-10 days [33, 84, 85]. During the convalescent stage, certain symptoms may develop such as labile pulse, tachycardia, xerostomia, poor vision, loss of hearing/memory, loss of hair, and difficulties in breathing have been reported [32, 33]. Hepatorenal insufficiency has been reported in South Africa, but not in Turkey [33, 44, 86]. There have been no reported cases of relapse to date.

Thrombocytopenia has been consistently reported in CCHF cases [33, 83, 84]. Patients present with leucopenia and high levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and creatine phosphokinase [33, 83, 84]. These levels as well as other laboratory tests such as complete blood counts, return to normal levels after 5-9 days in surviving patients [87]. Results from coagulation tests such as prothrombin time and activated partial thromboplastin time are prolonged, and fibrinogen levels may be decreased and fibrinogen degradation products may be increased [33, 83, 84, 88]. It has also been noted that many patients with a fatal outcome often display overt disseminated intravascular coagulation (DIC) with little evidence of an antibody response [33, 83]

1.2.7 Pathogenesis

Due to the lack of a suitable animal model for CCHF, the pathogenesis of the disease is not well described. For most hemorrhagic fever viruses, the ability of the virus to disable the host immune response by affecting cells that regulate the antiviral response leads to a

diseased state in the host [62, 89, 90]. This has a profound effect upon the vascular system and lymphoid organs [89-91].

Infection of the endothelium plays an important role in CCHFV pathogenesis, as the endothelium can be affected into two ways: directly by viral infection and replication, or indirectly by viral factors or virus-induced host factors [88, 90-92]. Endothelial damage contributes to hemostatic failure through the stimulation of platelet aggregation and degranulation and subsequent activation of the coagulation cascade [88, 90-92]. It was found that thrombin activatable fibrinolysis inhibitor (TAFI), a proenzyme that is synthesized in the liver and is responsible for downregulation of fibrinolysis, is downregulated in CCHF patients and may be the cause of bleeding complications [93]. CCHF cases that were fatal displayed a marked dysfunction of the coagulation system from an early stage and disseminated intravascular coagulation was a prominent feature of this process [91, 94]. Furthermore, endothelial damage of the cardiac muscles may play a role in fatality of CCHF patients. In severe cases of CCHF, patients present with impaired cardiac function, such as lower left ventricular ejection fraction, higher systolic pulmonary artery pressure and more frequent pericardial effusion [95]. Whether direct viral invasion of the cardiac muscles or endothelial damage of cardiac structures is the most likely cause remains to be elucidated [95].

In another study, cytokine levels were studied for patients with CCHFV infection. The levels of IL-1, IL-6, and TNF-α were higher among patients who perished as opposed to patients who recovered [96]. In addition the disseminated intravascular coagulation (DIC)

scores correlated positively with the levels of IL-6 and TNF-α, and negatively with IL-10 levels [96]. This suggests that proinflammatory cytokines play an integral role in CCHF pathogenesis and mortality [96, 97].

An analysis of lymphocyte subgroups for CCHF patients (n=77) revealed a significantly higher CD3+CD8+ T-cell count for fatal cases (5/77) versus non-fatal cases [98]. There was no difference in other lymphocyte subgroups, although a positive correlation between viral load and CD3+CD8+ T-cells was also detected [98]. Despite this elevated level of cytotoxic T lymphocytes, a fatal outcome for these patients could not be prevented [98]. In another study, it was found that peripheral blood natural killer cells in severe risk CCHF patients were highly elevated and correlated positively with aspartate transferase (AST), alanine transferase (ALT) and activated partial thromboplastin times (aPTT) [95]. With this finding, it may be possible to use NK cell counts as a prognostic marker in CCHF patients [95].

Laboratory diagnosis of CCHF can be performed by antibody detection using ELISAs, virus isolation, antigen detection and PCR [88, 99-101]. IgG and IgM antibodies may be detected on or after the sixth day of illness [36]. The presence of IgM or a fourfold increase in IgG between the acute and convalescence phases is considered diagnostic for the disease [36]. IgM remains detectable for up to 4 months and IgG levels decrease but remain detectable for up to 4 months [36]. Patients with fatal disease do not develop measurable antibodies and endogenous antibody response was only demonstrated in 2 of 15 fatalities; most patients had low titres of neutralizing antibodies (1:8-1:32 in

fluorescent-focus reduction tests) but some developed titres of 1:256 to 1:512 [101]. For low titre individuals, as well as patients in the first few days of illness, diagnosis is achieved by viral detection in blood or tissue samples [36]. Virus can be isolated from blood or tissue samples during the first 5 days of infection can be grown on cell lines including LLC-MK2, Vero, BHK-21 and SW13 [36, 47]. This method is simple and relatively easy but not as sensitive as the traditional method of intracranial inoculation of suckling mice [100]. The use of cell lines may produce little or no CPE (cytopathic effects), but identification can be confirmed using immunofluorescence with specific monoclonal antibodies [88, 99]. PCR is increasingly popular for detecting the viral genome and has been successfully applied in the diagnosis of viral hemorrhagic fevers, and RT-PCR and antigen detection by ELISA appear to be the most useful in a clinical setting [36].

1.2.8 Treatments

Current treatment for CCHF is mostly supportive, with fluid and electrolyte balance, circulatory volume, and blood pressure monitoring [36]. This may involve the administration of thrombocytes, fresh frozen plasma, and erythrocytes [33]. Ribavirin is the best antiviral agent available for infected patients, although its mechanism of action has not been fully elucidated. Studies conducted *in vitro* have shown that Ribavirin treatment inhibits viral activity in a dose dependent manner [102]. Interestingly, Ribavirin treatment using 50 μg/mL on infected Vero cells inhibited viral activity, while approximately nine times this amount was required to inhibit Rift Valley Fever Virus

infectivity to a comparable level [102]. Studies conducted *in vivo* demonstrate that Ribavirin treatment of CCHFV infected infant mice significantly reduced infant mouse mortality and extended the geometric mean time till death [103]. Control mice showed significantly higher titres in the liver as compared to other organs except the blood [103]. Later, virus was isolated from other organs such as the brain and heart [103]. Ribavirin treatment significantly reduced CCHFV growth in the liver and significantly decreased, but did not prevent viremia [103]. Screening drugs for potential activity against CCHFV revealed that Ribavirin inhibited replication, Ribamidine had antiviral activity that was 4.5- 8 fold lower than Ribavirin and three other drugs 6-azauridine, selenazofurin and tiazofurin had very little effect as antivirals [104].

It has been noted that the interferon-induced MxA protein has antiviral activity against CCHFV [64]. The yield of progeny virus in cells constitutively expressing the MxA protein was reduced up to 1000-fold compared to control cells and the production of viral genomes was blocked [64]. It was determined that the MxA protein co-localizes with the nucleocapsid protein of CCHFV in the perinuclear regions of infected cells [64]. In addition, it was found that IFN-α inhibits the growth of CCHFV in human endothelial cells and hepatoma cells- reducing yields by a factor of 100-1000 [65]. Since the early interferon response in critical in CCHFV infection, it was determined that replicating CCHFV delayed the activation of ISG56 (IFN-stimulated gene 56) until 17-24 hours post-infection [105]. This result was compared to control cells, in which infection with inactivated CCHFV particles was administered to the same cell lines. Further study indicates that IRF-3, an cellular transcription factor, is imperative for the induction of

IFNs during viral infection [105]. Many viruses induce IRF-3 very early during infection; usually from 3-10 hours after infection takes place [105]. It has been shown that CCHFV infection delays the translocation of IRF-3 to the nucleus and biologically active interferon is induced 48 hours after infection [105]. Surprisingly, when IFN-α was administered 6 hours post-infection, no effect on viral titres was observed, indicating a relative insensitivity to interferon once replication has started [105]. These results indicate that host cells must have an established IFN response in order to inhibit CCHFV infection, and may indicate why some patients have fatal cases of CCHFV. These persons, for some reason, may be unable to mount a sufficient IFN response early in infection [105]. The exact mechanism for the evasion of the interferon response by CCHFV is currently unknown and research is underway.

There is currently no vaccine licensed for use for CCHFV. However, in 1974, an immunization program was introduced for medical and military personnel in CCHF endemic areas [106]. This vaccine was prepared from mouse brains inactivated by chloroform, heated to 65°C, and adsorbed on aluminum hydroxide [106]. This vaccine was given to 583 volunteers in Bulgaria and was reported to induce antibody production in 96.6% of the subjects [33]. Despite this, the vaccine is relatively unpopular due to the method of preparation.

A DNA vaccine expressing the entire M-segment of CCHF was constructed and neutralizing antibodies were assessed [107]. This DNA vaccine was able to induce neutralizing antibodies to CCHF in only half of the mice that received the vaccine, and

were vaccinated by gene gun three times at 4 week intervals with 10 µg of DNA coated on gold [107]. Quantitation of the neutralizing antibodies by plaque reduction neutralization tests yielded a $PRNT_{50}$ of 1:40-1:160 [107]. Due to the lack of a suitable animal model, this vaccine was not tested to determine protective immunity.

1.2.9 Future Research Areas

It is clear that the pathogenesis of CCHFV needs to be researched further. Perhaps with the creation of an animal model that closely resembles human disease, more information can be obtained. New small animal models, such as the STAT-1 knockout, would provide valuable information for further studies.

1.3 Nipah Virus

Nipah virus is a highly pathogenic *Paramyxovirus* that has recently emerged from flying foxes to cause serious disease outbreaks in humans and animals in Malaysia, Australia, Singapore and Bangladesh. Nipah virus (NiV) belongs to the genus *Henipavirus* and is designated as a Biosafety Level 4 pathogen (BSL4), which causes severe febrile encephalitis in humans with a mortality rate close to 40% [108, 109]. Since an initial outbreak in Malaysia and Singapore in 1999, NiV has re-emerged in Bangladesh and India [108]. Since the 1990's much effort has been placed on rapidly discovering the reservoir, studying the genome, and formulating potential vaccines and therapies for Nipah virus. NiV has caused particularly high morbidity and mortality on a scale previously unseen with other *Paramyxoviruses*.

1.3.1 Historical Background

Paramyxoviruses have long been implicated in human and animal diseases. The ability of these viruses to cross the host species barrier allows them to have devastating effects upon human and animal life. In 1994, a fatal infection in horses and humans was discovered in Australia [108]. This infection was later identified as being caused by a new member of the family *Paramyxovirus*, termed Hendra virus (HeV) [110]. In 1998 in Malaysia, a closely related virus, Nipah Virus (NiV), was determined to be the cause of many fatal infections of pigs and humans [109, 111-113]. Molecular characterization of the genomes of HeV and NiV in comparison to other paramyxoviruses established that the genes are similarly ordered but are 2 kb larger than the usual 16 kb long genomes of other members of the family [108, 109, 113]. Interestingly, the genome contains the same genes as other paramyxoviruses. This aberration in genome length led to the formation of a new genus, *Henipavirus*, within the *Paramyxovirus* family [109]. The Henipaviruses are unique as they are the only zoonotic paramyxoviruses and are extremely pathogenic [114].

1.3.2 Epidemiology

In 1998, in Malaysia, an epidemic of an unidentified disease infecting pigs and humans was responsible for 258 cases of febrile encephalitis, with 105 of them fatal [108, 109, 113]. The outbreak appeared to have started in the state of Perak, situated north of Kuala Lumpur, among pig farmers [109]. Several months later, a similar outbreak occurred in another pig farming area in the state of Negri Sembilan [109]. These two outbreaks were soon followed by an outbreak in Singapore, in which there were 11 cases and one death [108, 109, 113]. The virus involved was named after one of the afflicted villages, Sungai Nipah. Initial epidemiological evidence that NiV was transmitted from pigs to humans was deduced by the observation that a large number of pigs had been dying before the epidemic in humans, and outbreaks in different areas appeared to be linked to the translocation of pigs from the initial outbreak area in the north [108, 109, 115]. Although the main mode of transmission occurred from pigs to humans, NiV was isolated from the urine, saliva and respiratory secretions from infected patients [115, 116]. The epidemic started in pigs in the northern state of Perak and spread south through the movement and sale of pigs. Disease in pigs was highly contagious and symptoms included acute fever and respiratory problems [108, 113]. Transmission among pigs occurred through direct contact with urine and mucosal secretions, as research has confirmed the rapid spread of NiV from infected to uninfected pigs in close contact [117]. The dominant clinical symptom in humans was encephalitic as opposed to respiratory, and the primary mode of transmission appeared to be through the respiratory tract [108, 113]. The slaughter of nearly 1.2 million pigs following the outbreak in Malaysia and Singapore, it was hoped that an outbreak of this magnitude would not occur again [113].

However, since the initial outbreak in Malaysia, NiV has caused fatal human encephalitis in Bangladesh on several occasions as well as in India [118-123]. These outbreaks, which were smaller than the initial outbreak in Malaysia, have a markedly higher case fatality rate, which ranged from 67-92% [122, 123]. Furthermore, these outbreaks had a few significantly different epidemiological characteristics that differed from the initial outbreak. For instance, in an outbreak in Meherpur, Bangladesh in 2001, close contact with sick patients as well as with sick cows were associated with NiV infection despite the lack of samples available from the cattle [108, 118]. Person-to-person contact was a primary risk factor for an outbreak in Nagoan, Bangladesh in 2003 [124]. Another outbreak in Faridpur, Bangladesh in 2004 demonstrated possible nosocomial transmission, as NiV RNA was isolated on hospital surfaces [125, 126]. A retrospective analysis of an outbreak in West Bengal, India confirmed nosocomial transmission had led to an amplification of the outbreak [122]. Interestingly, consumption of NiV- infected date palm sap was reported as the primary risk factor for an outbreak in Tangail district, Bangladesh [121].

The epidemiological characteristics of the Bangladesh and Indian outbreaks highlight the involvement of person-to-person, food-borne, and nosocomial transmission of NiV. The apparent higher case-fatality rate is indicative of strain specific differences of NiV or the lower supportive care available in those areas relative to the Malaysia and Singapore outbreaks [127].

1.3.3 Ecology

Nipah virus has serological, phylogenetic, and antigenic similarities to HeV. These similarities have allowed for a more specific approach to understanding the wildlife factors surrounding the NiV outbreak in Malaysia [112]. Since HeV had been detected in fruit bats of the *Pteropus* genus, these animals were suspected of harbouring NiV as well [128]. Since then, viral isolation studies have elucidated that fruit bats or flying foxes (*Pteropus* spp.) are the most important natural reservoir host of NiV [129-131]. During initial surveillance studies, NiV neutralizing antibodies were detected in *Pteropus vampyrus* and *Pteropus hypomenalus,* but virus was not isolated [131]. However, when bat urine and swabs of contaminated fruit was collected on Tioman Island, NiV was isolated from *P. hypomenalus* [130]. Pigs appeared to be the amplifying hosts for NiV, and evidence from the Malaysia outbreak did not confirm the transmission of NiV from bats to humans [111]. Despite the fact that NiV can infect across many different species, such as dogs, cats, ferrets, pigs and horses, the relative paucity of neutralizing antibodies in uninfected hosts suggests that these animals are dead-end hosts [108]. The mechanism allowing NiV to jump species barriers is currently unknown, but research has shown that NiV has not undergone any recent significant evolutionary changes [132]. Hence, it appears that the emergence of NiV is likely due to ecological factors [133]. The introduction of NiV into pigs appears to be related to common practice of growing fruit trees in close proximity to pig farms, which allows for the use of pig excreta as fertilizer for the trees [112]. At the index pig farm for the Malaysia outbreak, it was noted that many rambutan and mango trees are proximal to the 30 000-head pig farm, with many trees overhanging the pig enclosures [130]. It is suspected that feeding flying foxes

dropped virus-laden urine and masticated fruit pellets on the pigs below and caused this spill-over event [130].

In addition, large-scale deforestation in Malaysia and Sumatra in conjunction with the large El Niño Southern Oscillation (ENSO) related drought in 1997 might have altered the migration routes and feeding behaviours of *Pteropus* spp. [112]. The primary species of the large fruit bat reservoir for NiV, *Pteropus vampyrus,* travels significantly large distances and has been shown to alter migratory patterns based on seasonal food abundance [112].

Since the elucidation of the primary reservoir host, antibodies to Henipaviruses have been detected in other *Pteropus* species such as *P. lylei* and *P. gignateus*, as well as other non-*Pteropus* species (*Hipposideros larvatus, Scotophiilus kuhlii*) [108]. Characterization of human isolates from Malaysia, Cambodia, India and Bangladesh support evidence that viruses circulating in different areas have coevolved with local animal reservoirs [108]. This is evidenced by the fact that urine collected beneath a *Pteropus lylei* roost Cambodia harboured NiV which was more closely related to NiV strains from India [123]. Experimental infection of *P. poliocephalus* has shown that NiV infected bats present subclinical infection with sporadic viral shedding via urine, seroconversion, and evidence of viral antigen in tissues [134].

1.3.4 Molecular Biology of Nipah Virus

Nipah virus is an enveloped single-stranded, negative-sense RNA virus of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Henipavirus* (Figure 1.4) [108, 109, 112, 113, 118, 127, 129, 132, 133, 135-139].

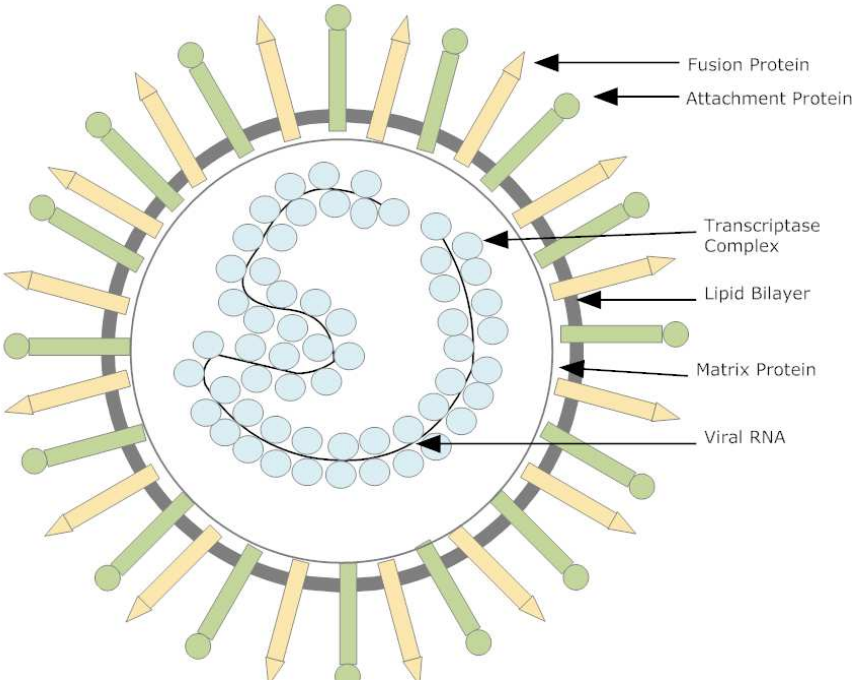

Figure 1.4. Schematic Representation of Nipah virus Structure.

Nipah virus contains a linear ribonucleoprotein (RNP) core consisting of a ssRNA genome of negative polarity to which nucleocapsid proteins (N) are associated to a ratio of 1 N to 6 nucleotides [140]. The RNP contains phospoprotein (P) and the large polymerase (L) protein, which together are required for the creation of mRNA and anti-genome RNA [114]. The RNP core is surrounded by and envelope in which the attachment glycoprotein (G), and fusion protein (F) are anchored [114]. The glycoprotein is a homotetramer and the fusion protein is a homotrimer [114].

Similar to other Paramyxoviruses, the genome of NiV consists of six genes (N, P, M, F, G, L), with a 3' leader and 5' trailer sequence (Figure 1.5) [108, 127, 135, 141]. The unique genus-specific 3' leader and the 5' trailer sequences function as promoters for transcription and genome replication, respectively [114, 141, 142]. Another attribute is the presence of a unique GDNE amino acid sequence in a conserved catalytic region of the transcriptase protein, whereas most other non-segmented negative strand RNA viruses have a GDNQ sequence [141, 143]. A prominent feature of *Henipaviruses* is the abnormally long genome length, with 18, 246 nucleotides for NiV and 18, 234 for HeV [141, 143]. This corresponds to approximately 15% longer than other viruses in the family. This extra length is present in the long untranslated regions, mostly at the 3' end of the six genes, with the L gene as an exception [114, 137, 141, 143]. It has been postulated that these UTRs may serve a purpose in secondary structure, which may enhance translatability and/ or mRNA stability [137]. The genomes of paramyxoviruses have a 3' leader sequence that contains the promoter for transcription of mRNA and a 5' sequence that contains the promoter for RNA genome replication [137]. The first 12 nucleotides of the genomic termini are highly conserved and complementary [137]. A NiV isolate from Rajbari, Bangladesh has demonstrated a markedly different overall nucleotide sequence than isolates from Malaysia, displaying 91.8% homology, warranting the designation of two NiV strains, that of the Malaysian strain (NiV-M) and that of the Bangladesh strain (NiV-B) [127]. The genome of NiV-B is 18, 252 nucleotides in length, 6 nucleotides longer than NiV-M, corresponding to a slightly larger 5' UTR of the F gene [127]. Similar to other members of the *Paramyxovirinae* NiV co-transcriptionally edits P gene mRNA by inserting G residues at a specific editing site

[142, 144]. This results in translation of multiple gene products, the P, V, and W, that share amino termini but have unique carboxyl ends [142, 144]. Expression of these constructs in plasmid DNA has demonstrated the ability of P gene products to inhibit cellular IFN production (V, W), and signaling (P, V, W) [145-151].

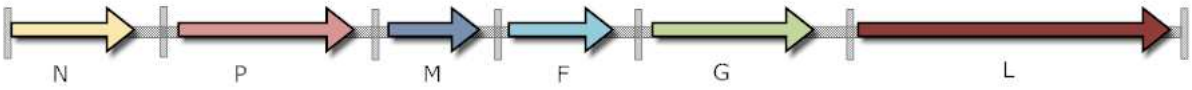

Figure 1.5. Nipah Virus Genome.

Depicted from 3' to 5' orientation due to the negative polarity of the RNA. Note the long untranslated regions for all genes except the L gene. All NiV genes are monocistronic except for the P gene, which undergoes RNA editing [114, 142, 144]. ORFs are indicated by arrows, and start/stop signals by hatched-grey vertical lines.

NiV genes are transcribed by the RNP complex, which associates with the 3' end of the viral genome and subsequently generates discrete mRNAs [114]. The transcription of the viral genes is not equimolar, and there exists a gradient from the N to the L genes, with a significant reduction in transcription from the M-F and G-L junctions [114, 152].

NiV infects mainly neurons and endothelial cells. Experimental infection of animals have shown that guinea pigs and hamsters are also susceptible [138]. Paramyxovirus virulence, host range, and cell tropism is determined largely by the virus attachment (G) and fusion (F) proteins [114]. The G and F proteins function in part by binding to cell surface receptors and fusing the virus and host cell membranes [114].

The attachment proteins of paramyxoviruses are classified as Type II membrane glycoproteins, which have a cytoplasmic tail, transmembrane region, stalk, and globular head [109, 114]. This globular head is organized into a propeller shape, which is consistent for most members of the family [114]. The C-terminal globular head of NiV G protein contains the receptor binding domain, and is also organized in the propeller shape despite the fact that it has low sequence homology with other members of the family [109]. Recent identification of G protein residues has identified a site on the top of the globular head that is implicated in Ephrin B2 binding; this site begins near the shallow depression at the center of the top surface and extends to the rim of the barrel-like structure on the top loops of β-sheet 5 [153]. The topology of this site on the globular head is similar to the SLAM receptor site on the attachment glycoprotein of another paramyxovirus attachment glycoprotein, the measles virus hemagglutinin [153].

Although most paramyxoviruses have attachment proteins that have neuraminidase and hemagglutinin activities, the cognate proteins for the henipaviruses are similar to the attachment proteins of the *Pneumovirus* genus, because they lack both types of activity [132, 137, 142]. The NiV G protein is approximately 602 amino acids in size and is 2 amino acids shorter than the cognate protein in HeV, yet they have 83% sequence homology [137]. Recent research has demonstrated that single amino acid changes in the G protein distinguishes ephrin B2 from ephrin B3 usage, which is predominantly displayed in the brainstem [154]. Moreover, NiV G protein can more efficiently use ephrin B3 as a receptor than can HeV [154]. Localization of the receptor-binding site on the G protein has accelerated the effort in creating therapies, which could potentially block this site, and inhibit NiV attachment.

Receptor binding of the G protein allows the F protein to mediate fusion with the host cell membrane, and permits viral entry [135]. It has been shown that both the F and G proteins are necessary for fusion to occur [110, 155-158]. The coordinated action of the NiV G globular head domain with the stalk domain has been implicated in mediating receptor-induced F triggering [159]. The F protein is a Type I membrane protein which has a single transmembrane domain with an N-terminal lumenal domain with carbohydrate moieties and a C-terminal cytoplasmic domain [114]. The F protein consists of two subunits which are linked by disulphide bonds, and are termed F_1 and F_2 [160]. These subunits are liberated by proteolytic cleavage of a precursor protein (F_0) [114, 161]. Most Paramyxovirus family members, cleave F proteins using furin, a Ca^{2+}-dependent protease, present in the trans-Golgi of host cells [114, 161]. Furin recognizes

the sequence RXXR, which is relatively conserved through most Paramyxovirus F protein species, and cleaves after multiple basic amino acid residues [162, 163]. However, cleavage of henipavirus F proteins is furin-independent, and occurs only after a single basic residue [164, 165]. Further research has shown that F protein cleavage requires a low intracellular pH and occurs within the endosomal compartment [166, 167]. The F protein, unlike other paramyxoviruses, contains an endocytosis signal in the cytoplasmic tail, which is critical for fusion activity and constitutive endocytosis [166]. These results suggest that the endocytosis signal is imperative for henipavirus fusion proteins [166]. Truncation of the cytoplasmic tail of the F protein greatly inhibits cell-cell fusion; in particular, a tribasic KKR motif adjacent to the membrane region is significant in modulating fusion [168]. In addition, the elucidation of Cathepsin L, an endosomal protease, as the enzyme responsible for cleavage of the F proteins has been confirmed [169]. This result has also been confirmed for NiV F proteins [170]. The F protein consists of two heptad repeats (HR) regions, which are α-helical structures that interact during the fusion process [171]. These two heptad regions, HR1 and HR2, have been the subject of potential therapeutic targets, such as heptad-derived peptides [172, 173]. F proteins are N-glycosylated at five sites, and have been implicated in shielding the fusion protein against neutralization; they also appear to confer resistance to heptad repeat inhibition [174].

1.3.5 Transmission

Experimental infection of the reservoir hosts of NiV, has shown that *Pteropus* flying foxes (fruit bats) do not succumb to disease and rarely shed virus in urine [113]. Despite this, they do mount a humoral immune response, although this doesn't occur in all flying foxes [113]. More specifically, neutralizing antibodies were detected in four fruit bat species and one insectivourous species in Malaysia [131]. Antibodies to NiV have also been detected in Cambodian bats [131, 175]. Transmission to humans generally occurs through an intermediary host such as pigs (Figure 1.6). Despite this, foodborne transmission of NiV has occurred through the consumption of raw date palm sap in the Tangail District of Bangladesh [121]. Fruit bat species such as *Pterpous giganteus* are a nuisance to date palm sap collectors because the bats drink from clay pots used to collect the sap at night [121]. Illness correlated significantly with consumption of the date palm sap (64% among case patients versus 18% among controls) [121]. Furthermore, NiV has been isolated from partially eaten fruit [130]. Human-to-human cases have also been reported with a significantly higher proportion of those displaying respiratory difficulty as being transmitters of the virus (12% vs. 0%, p=0.03) [118]. Despite the small number of infected patients that transmit NiV, more than half of identified cases result from person-to-person transmission [118]. In the outbreak in Faridpur, Bangladesh, person-to-person transmission was implicated and the possibility of nosocomial infection was suspected due to the detection of NiV RNA on many hospital surfaces [125, 126, 176]. Analysis of an outbreak in Siliguri, India in 2001 confirmed nosocomial transmission of NiV and led to an amplification of the outbreak [123].

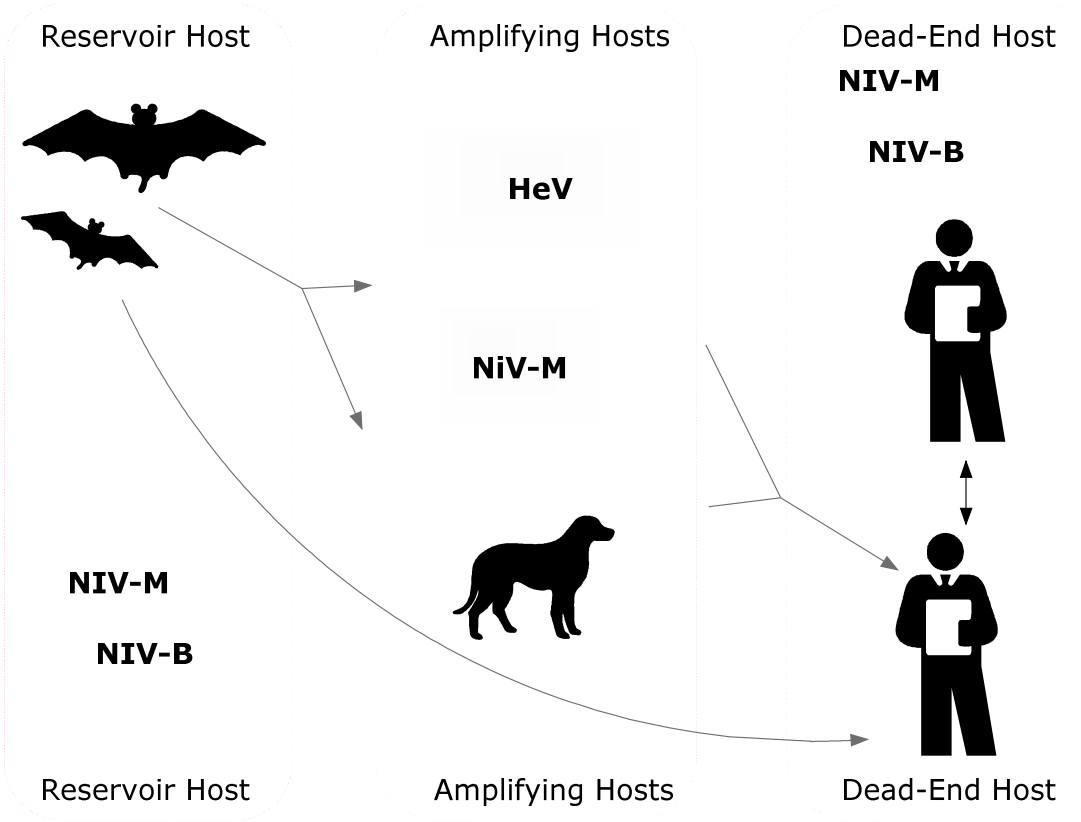

Figure 1.6. Cycle of Henipavirus Transmission.

HeV can infect horses, as a spillover host and thereby infect the human population. For NiV, the Malaysian strain infects amplifying hosts such as pigs and dogs, thereby transmitting the virus to humans, a dead-end host. The situation in Bangladesh, is indicated by direct transfer of NiV-B from bats to humans, as well as from human-to-human.

1.3.7 Clinical Manifestations

NiV infection causes rapid acute encephalitis with high mortality rates [108]. The exact incubation period in humans is not known, but ranges from a few days to 4 months, but generally two weeks or less in the majority of patients [108, 135]. Disease in patients is characterized by fever (97%), headache (65%), dizziness (36%), vomiting (27%) as evidenced by the outbreaks in Malaysia and Singapore [177]. Three out of 11 patients in Singapore experienced atypical pneumonia with fever and chest infiltrates [178]. Case fatality rates were 40% for Malaysia and 75% in Bangladesh and likely reflect differences in supportive care [135].

From information gathered during the Malaysian outbreak, it was found that 7.5% of patients who recovered from NiV infection had developed a relapse in encephalitis, and 3.7% of those with asymptomatic/non-encephalitic infection had late-onset encephalitis [179]. Patients who had late-onset encephalitis displayed a reduced mortality rate (18%) as compared to acute encephalitis [179]. Patients from the Bangladesh outbreak who survived illness were examined for long-term neurologic and functional outcomes via MRI [180, 181]. Among these 22 patients, those who suffered acute encephalitis developed neurological sequelae such as encephalopathy, cranial nerve palsies and dystonia [179].

In addition to encephalitis, a vasculitis associated with infection of endothelial cells was the main pathologic feature of NiV infection [115]. Infection appeared predominantly in the central nervous system, characterized by endothelial cell damage, mural necrosis, and

infiltration by polymorphonuclear leukocytes and mononuclear cells [182]. Evidence of endothelial infection and vasculitis was also observed for other organs such as the lung, heart, spleen and kidney [182].

1.3.7 Pathogenesis

As mentioned previously, NiV P gene products have the ability to inhibit IFN production and signaling [145, 148-151]. The P gene produces four proteins: P, V, W and C [114]. Although the target of the C protein is not known, the P, V, and W proteins all act upon IFN signaling or the JAK/STAT pathway by acting directly upon STAT-1 [114]. Most paramyxoviruses affect IFN signaling through their V proteins by inducing proteosomal degradation of IFN-responsive transcription factors, STAT-1 or STAT-2 [145]. NiV V protein is somewhat different as it does not induce STAT degradation, but inhibits IFN by forming high molecular weight complexes with both STAT-1 and STAT-2, causing it to accumulate in the cytoplasm [145]. Consistent with the formation of complexes, it has been shown that STAT protein tyrosine phosphorylation is attenuated in cells expressing the NiV V protein [145]. Further research has shown that a NiV V segment between amino acids 100-160 and STAT-1 residues 509 to 712 [151]. Furthermore, interaction with STAT-2 requires a larger portion of the V protein, encompassing amino acids 100-300 [151]. Interestingly, STAT-1 and V protein interactions are a prerequisite for STAT-2 binding [151]. Through this mechanism, the nuclear translocation of STAT-1/STAT-2 is prevented, causing cellular unresponsiveness to both IFN-α and IFN-γ [145]. In a

study conducted involving V protein mutants, it was found that a single amino acid mutation results in the inability of the protein to bind STAT-1 and STAT-2 [183].

Since the P, V and W proteins are all encoded by the same viral gene, they share an identical 407 amino acid N-terminal region but have distinct C-terminal regions [149]. It has been shown that the P protein also has anti-IFN functions, confirming that the N-terminal region contains the binding regions to STAT-1/STAT-2 [149]. In addition, subcellular localization studies have shown that the V and P proteins are mainly cytoplasmic and the W protein localizes to the nucleus- but in all cases, STAT-1 colocalizes with the corresponding NiV protein [149]. Through this, NiV has the ability to sequester STAT-1 in the cytoplasm and the nucleus of host cells [149]. In addition, the V and W proteins have functions in blocking activation of the IFN-β promoter and the IRF3-responsive ISG-54 promoter [148]. Only the W protein shows inhibition of promoter activation in response to dsRNA stimulation of TLR3, an effect dependent upon nuclear localization of the W protein [148]. A nuclear localization signal was discovered on the C-terminus domain that allows the W protein to target the JAK/STAT pathway in addition to the TLR3 pathways [148]. These mechanisms allow NiV to effectively attenuate host responses to viral infection and contribute to the overall virulence and pathogenicity of the virus.

1.3.9 Treatments

Analysis of serum samples obtained from patients during outbreaks has indicated that NiV generates a humoral immune response in humans [135]. Serum IgM was present early after infection and nearly all patients were seropositive by the third day of infection [135]. IgG was detectable within the first 10 days of infection (10-29% of patients), and 100% of patients by day 17-18 [135].

There are currently no available vaccines or post-exposure therapeutics specifically for NiV infection [113]. Nevertheless, an open-label trial using Ribavirin in 140 patients, showed that the use of Ribavirin reduced mortality by 36% ($p=0.011$), compared to controls [184]. Furthermore, there appeared to be no associated side effects from treatment [184]. Despite this, studies performed on golden hamsters, a model for NiV infection, showed delayed death using Ribavirin, and no protection with cholorquine; either alone, or in combination with Ribavirin [185].

Although neutralizing antibodies elicited by a vaccine can be effective at preventing disease, purified neutralizing antibodies administered passively to patients with acute infections can be equally effective. This has been demonstrated through the use of monoclonal antibodies (mAB) directed against the NiV F and G proteins. Evidently, as little as 1.2 μg of anti-G mAb was required to provide complete protection against challenge in a hamster model of infection [186]. However, 1.8 μg of anti-F mAbs were required for protection [186]. As a more useful measure of protection, post-exposure administration of the mAbs was determined and it was found that anti-G mAbs protected

more than 50% of the animals at 24 hours, but offered no protection if administered later [186]. Interestingly, administration of anti-F mAbs 1 hour after infection completely protected the animals and 50% survived after a 96-hour administration [186]. These results indicate a promising treatment for people exposed to NiV infections.

Vaccination with recombinant vaccinia viruses encoding the F and G proteins have been shown to be protective in golden hamsters, these recombinant viral vaccines are not a suitable human vaccine candidate due to the inherent risks of vaccinia virus vaccination [157, 187]. Nevertheless, poxvirus vaccines have been developed as licensed veterinary vaccines, and the safety concerns have led to the development of attenuated poxviruses such as modified vaccinia virus Ankara (MVA), which is the vaccinia virus strain most suitable for clinical investigation [188, 189]. Virus-like particle (VLP) based strategies, where non-infectious viral particles composed of a limited number of structural proteins, can be produced in cell lines and may provide suitable methods of vaccination. A trivalent recombinant MVA virus encoding the M, F, and G of NiV is currently underway and poses an advancement for veterinary vaccines [188].

1.4. Hypothesis and Statements of Objectives

Adenoviral-based vaccines are excellent vectors in terms of vaccine immunogenicity. We hypothesize that adenoviral-based vaccines for Crimean-Congo Hemorrhagic Fever virus and Nipah virus can be generated that are capable of stimulating significant immune responses within 6 months.

This would represent a relatively short time frame compared to conventional methods requiring gene isolation and consequently access to the infectious agent.

Objective 1

Design and synthesis of optimized expression cassettes encoding glycoproteins for Crimean-Congo Hemorrhagic Fever virus and Nipah virus and clone into recombinant adenovirus.

Objective 2

Evaluate expression of recombinant adenoviral vaccines in vitro.

Objective 3

Evaluate immune responses in vivo using STAT-1 mice for CCHF AdX and guinea pigs for NiG AdX and NiF AdX.

In cases of outbreaks, as a corollary, the approximate time to completion was determined from initial gene synthesis to *in vivo* testing. In-house gene synthesis for glycoproteins of large insert size, such as CCHF, was attempted but also ordered commercially for a comparison in time frames.

Glycoprotein genes from each respective virus were chosen as vaccine candidates due to their accessibility to the immune system, and potential for induction of neutralizing antibodies.

2.0 Materials and Methods

2.1 Optimization of Glycoprotein Sequences

Glycoprotein gene sequences were obtained from Genbank (National Centre for Biotechnology Information, NCBI) and accession numbers are listed in braces: CCHF M segment-Strain IbAr10200 (NC_005300.2), Nipah Virus G (NP_112027.1), and Nipah Virus F (NP_112026.1). Because the genes encoding glycoproteins are encoded on the M segment and extensive post-translational modification events are required, the entire M segment was used for CCHF virus. Gene Designer software from DNA2.0 (Menlo Park, California) was used to optimize the codon DNA sequences using a codon-bias table for *Homo sapiens* to a threshold of 21%. The resulting DNA sequence was appended with a Kozak consensus sequence (GCCGCCACC), and appropriate restriction enzyme sites (Table 2.1). The region of the optimized sequence showing the Kozak consensus sequence and restriction sites are shown in Figure 2.1. The sequences were back-translate using the most optimal DNA codon; to yield DNA sequences, this was performed using Gene Designer.

Table 2.1. Restriction sites added to DNA sequences at 5' and 3' ends, respectively.

Gene	Restriction Sites
CCHF M segment	SacI, SphI
Nipah Virus G protein	SacI, NheI
Nipah Virus F protein	NheI, DraI

The entire CCHF M optimized sequence was ordered from Geneart (Regensburg, Germany). Nipah Virus glycoproteins were synthesized. The resulting DNA sequences were split into oligonucleotides using web-based software termed Gene2Oligo (http://berry.engin.umich.edu/gene2oligo/); Gene2Oligo is a program that divides long sequences into a set of contiguous oligonucleotides that correspond to both DNA strands. Design mode options consist of creating oligonucleotides using various parameters, such as oligonucleotide length priority and software optimized Tm, oligonucleotide Tm priority, or hybridization unit sizes. All other parameters were left in default values. For construction of Nipah virus F, the design priority was set to 20 bp hybridization units resulting in oligonucleotides of uniform 40 bp length without considering Tm values of individual oligonucleotides. The Nipah virus G protein oligonucleotides, and all subsequent genes were created using oligonucleotide Tm priority according to default parameters specified by Gene2Oligo. The output generated from Gene2Oligo was compiled and oligonucleotides were ordered from Integrated DNA Technologies (Coralville, Iowa). The oligonucleotides were ordered in wet plate format at 150 μM for each oligonucleotide. (See Appendix B for the list of oligonucleotides).

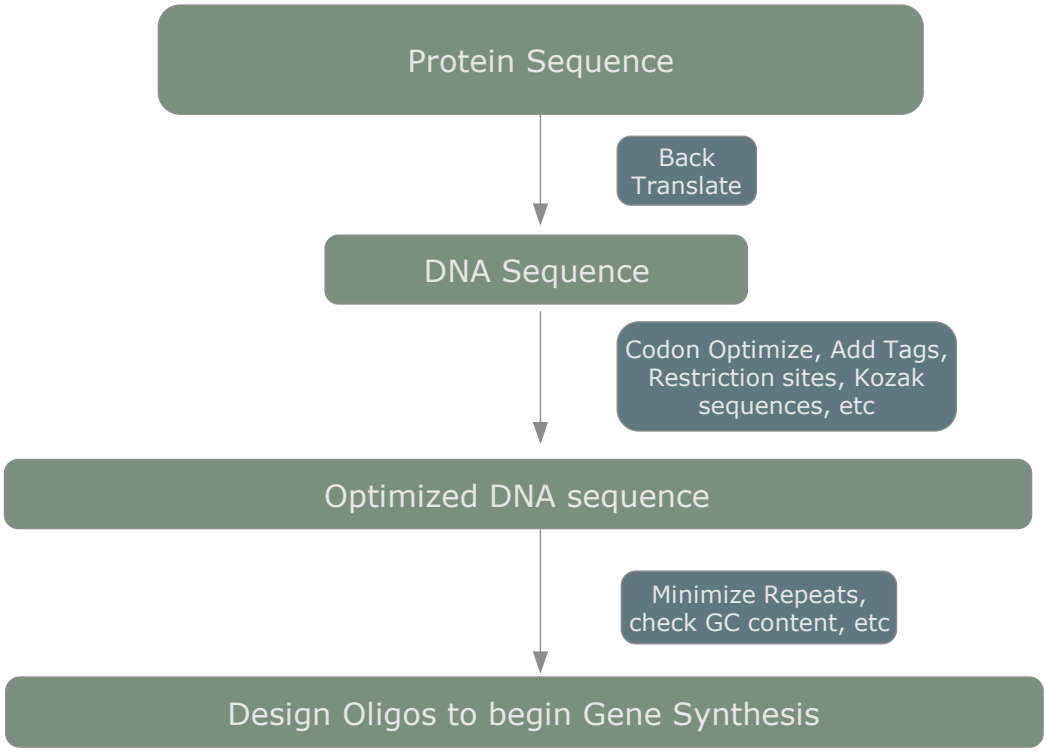

Figure 2.1. Oligonucleotide Design Procedure.

Flow diagram indicating procedure for obtaining an optimized DNA sequence from the glycoprotein amino acid sequences. Back translation allows for the selection of optimal codons for expression in *Homo sapiens*.

2.2 Non-gapped Gene Synthesis PCR

Gene synthesis was performed using three successive rounds of PCR using iProof High Fidelity DNA Polymerase (BioRad, Hercules, CA). The primers obtained at 150 μM were pooled into one tube and 2 μL of this solution was used in the first PCR step (see Table 2.2). This method of gene synthesis was performed for the Nipah F/G glycoproteins and all subsequent gene synthesis methods were carried out using a modified 4-step PCR, to reflect oligonucleotide design parameters. Since oligonucleotides were designed for Tm specificity, a cycle-variable PCR protocol was performed.

Nipah F was synthesized successfully using the unmodified gene synthesis protocol but Nipah G failed to assemble correctly. Consequently Nipah G oligonucleotides were reordered and the modified method of gene synthesis was performed successfully (see Table 2.3).

Table 2.2 Gene Synthesis Protocol. Non-gapped PCR assembly for Nipah virus F protein.

Step 1	**Amount (µL)**		**Cycling Parameters**				
Primer Mix	2		Step	Temp (°C)	Time (sec)		
dNTPs (10 mM)	2		Initial Denaturation	98	30		Cycled 50X
MgCl2	0.2		Denaturation	98	10		
HF Buffer (5x)	4		Annealing	40	30		
iProof (2U/µL)	0.5		Extension	72	30		
Water	13		Hold	4	∞		
Total	**20**						
Step 2			Step	Temp (°C)	Time (sec)		
Step 1 Reaction mix	6.5		Initial Denaturation	98	30		Cycled 35X
dNTP	0.5		Denaturation	98	10		
MgCl2	0.2		Annealing	40	30		
Buffer (5x)	4		Extension	72	30		
iProof (2U/µL)	0.5		Hold	4	∞		
water	8.5						
Total	**20**						
Step 3			Step	Temp (°C)	Time (sec)		
Step 2 Reaction mix	6.5		Initial Denaturation	98	30		
dNTP	2		Denaturation	98	10		Cycled 35X
MgCl2	1		Annealing	40	30		
Buffer (5x)	20		Extension	72	30		
iProof (2U/µL)	1.5		Hold	4	∞		
water	68						
Forward Primer (10mM)	1						
Reverse Primer (10mM)	1						

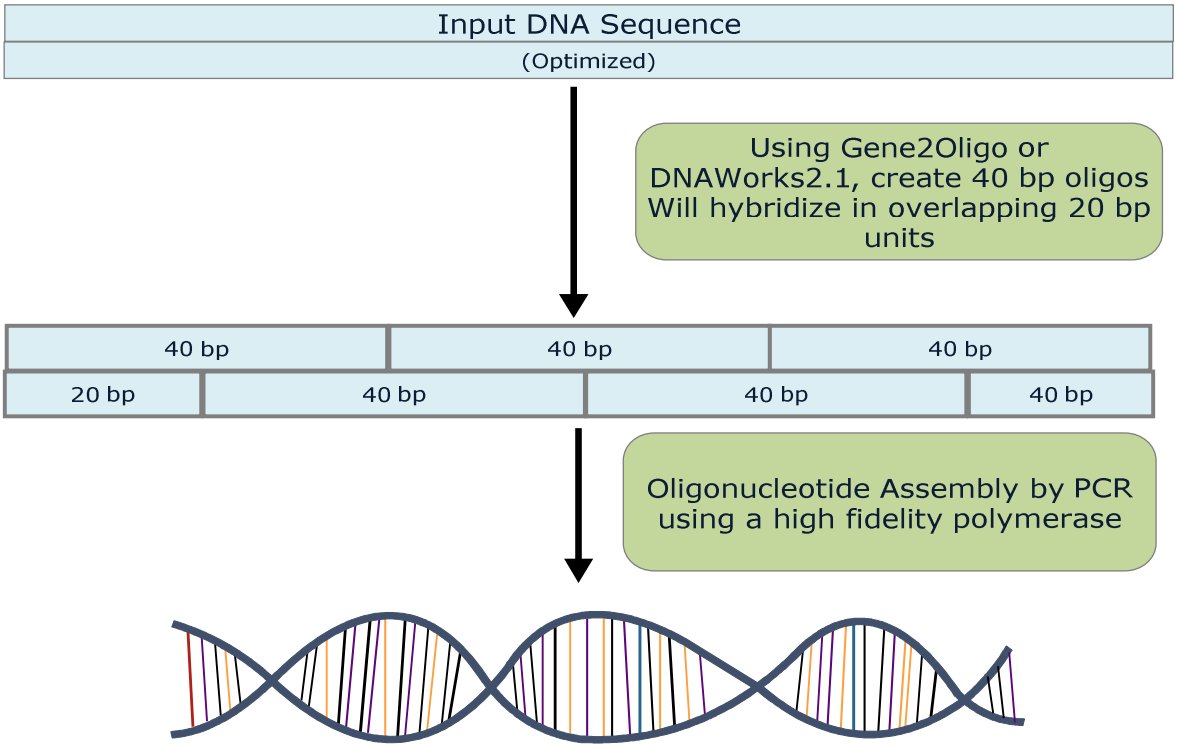

Figure 2.2. Oligonucleotide Assembly by PCR.

Oligonucleotides are divided into 20 bp hybridization units without gaps. The oligonucleotides, in the absence of mispriming, will align and create a contiguous DNA sequence during the PCR process.

Table 2.3. Modified Gene Synthesis PCR Protocol. Note the varying cycling parameters for each step.

Step 1	**Amount (µL)**			**Temperature (°C)**	**Time (sec)**	**Cycles**
Primer Mix	5		1	98	60	
dNTP	1		2	98	10	
$MgCl_2$	0.5		3	55	30	
Buffer (5x)	10		4	72	15	Go to 2 for 10 cycles
iProof	0.5		5	98	10	
Water	32.5		6	60	30	
Total	50		7	72	30	Go to 5 for 40 cycles
			8	4	∞	
Step 2	Amount			Temperature	Time	Cycles
Step 1 Reaction Mixture	10		1	98	1 min	
dNTP	1		2	98	10	
$MgCl_2$	0.5		3	60	30	
Buffer GC (5x)	10		4	72	40	Go to 2 for 35 cycles
iProof	0.5		5	72	10 min	
Water	27.5		6	4	∞	
Total	50					
Step 3	Amount			Temperature	Time	Cycles
Step 2 Reaction Mixture	2		1	98	1 min	
Forward Primer	1		2	98	10	
Reverse Primer	1		3	60	30	
dNTP	1		4	72	45	Go to 2 for 35 cycles
$MgCl_2$	0.5		5	72	10 min	
Buffer GC (5x)	10		6	4	∞	
iProof	0.5					
Water	34					
Total	50					
Step 4	Amount			Temperature	Time	Cycles
Step 3 Reaction Mixture	3		1	98	1 min	
Forward Primer	1		2	98	10	
Reverse Primer	1		3	60	30	
dNTP	1		4	72	45	Go to 2 for 35 cycles
Buffer GC	10		5	72	10 min	
iProof	0.5		6	4	∞	
Water	33.5					
Total	50					

2.3 Cloning Procedures

The entire 50-µL reaction mixture obtained from step 4 of the gene synthesis protocol was loaded onto a 1% agarose gel in TAE buffer (Life Technologies, Carlsbad, California). For Nipah virus G and F, gels were run at 100 V for approximately 1 hour. 2 Log Ladder (Life Technologies) was used as a marker. Following the run, the agarose gel was imaged and the corresponding band was gel extracted using a Gel Extraction Kit from Qiagen Corporation (Germantown, Maryland) using manufacturer's recommended protocol. Since iProof® DNA Polymerase was used in the gene synthesis steps, dATPs (Life Technologies) were added to the resulting PCR fragments for use with pCR 2.1 Topo TA® Cloning Kits (Life Technologies). The gel extracted PCR product was incubated with TAQ DNA polymerase (Life Technologies) and dATPs at 72˚C according to the manufacturer's recommended procedure. This reaction was allowed to proceed for 30 minutes.

The Topo TA® Cloning Kit (Life Technologies) was used to clone PCR products according to manufacturer's recommended protocol using the pCR2.1 vector. The reaction mix was transformed into Top 10® chemically competent cells (Life Technologies) according to manufacturer's recommended protocol. The transformed bacteria were inoculated onto LB supplemented with 100 µg/mL of ampicillin and X-Gal for screening using blue-white selection. Plates were incubated at 37˚C overnight. White and slightly blue colonies were selected to inoculate 5 mL of LB Lenox (1.5% NaCl) Broth and placed in an incubator/shaker at 37˚C at 250 rpm. All plasmid purification was

performed using DNA Miniprep Kits (Qiagen) according to manufacturer's recommended protocol. Samples were eluted with 50 µL of deionized water. Restriction digests were performed using enzymes purchased from New England Biolabs (NEB, Pickering, Ontario) according to manufacturer's recommended protocols. Nipah F Topo was screened with EcoRI and suitable clones were sent for sequencing using primers ordered from IDT DNA Technologies (Appendix B). Nipah G Topo was screened with SacI and NheI (NEB). The resulting clones were sequenced by the National Microbiology Laboratory DNA core facilities (Winnipeg, Manitoba). All primers were diluted in deionized water to a final concentration of 1 µM and all DNA samples were diluted to 150 uM. After confirmation by sequencing, Nipah F Topo was digested with NheI and DraI, and the resulting fragment was cloned into pShuttle2. Nipah G Topo was digested with SacI and NheI and cloned into pCAGα [190]. This vector contains a chicken β-actin promoter and was derived from an 829 basepair deletion between the Eco47III/XbaI sites [190, 191]. CCHF M was cloned directly into pCAGαpShuttle2, using SacI/SphI. DNA ligations were performed using T4 DNA ligase (NEB) using the manufacturer's recommended protocol. The cloning strategies employed are described in Figures 2.3-2.6.

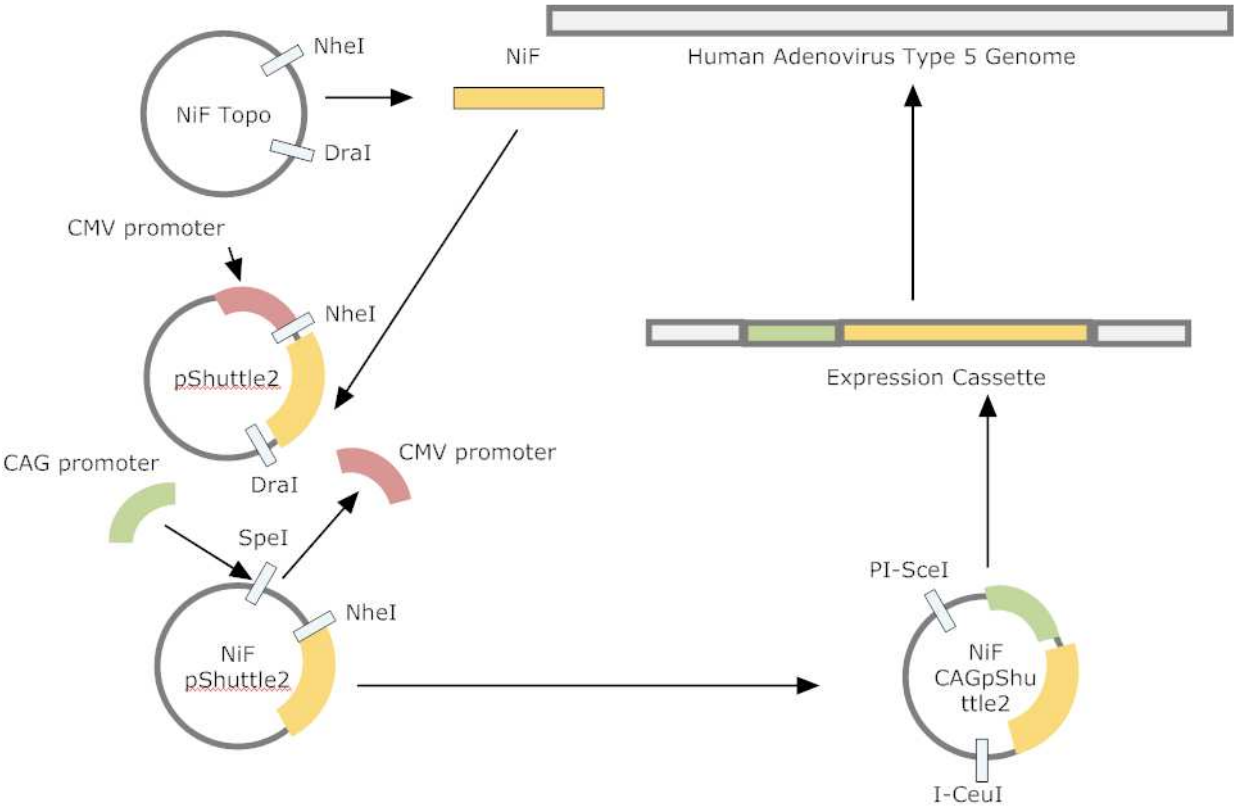

Figure 2.3. Nipah F Cloning Strategy.

Once Nipah F glycoprotein was digested out of pCR2.1 Topo, it was subcloned into pShuttle2, bearing the CMV promoter. The CMV promoter was then excised and the CAGα promoter was inserted for better expression of the viral glycoprotein. The resulting plasmid, NiFCAGpShuttle2, was digested with the rare-cutting LAGLIDADG-type homing endonucleases, PI-SceI and I-CeuI to liberate the expression cassette for subsequent cloning into linearized Human Adenovirus Type 5 (AdHu5) termed pAdenoX® (Clonetech).

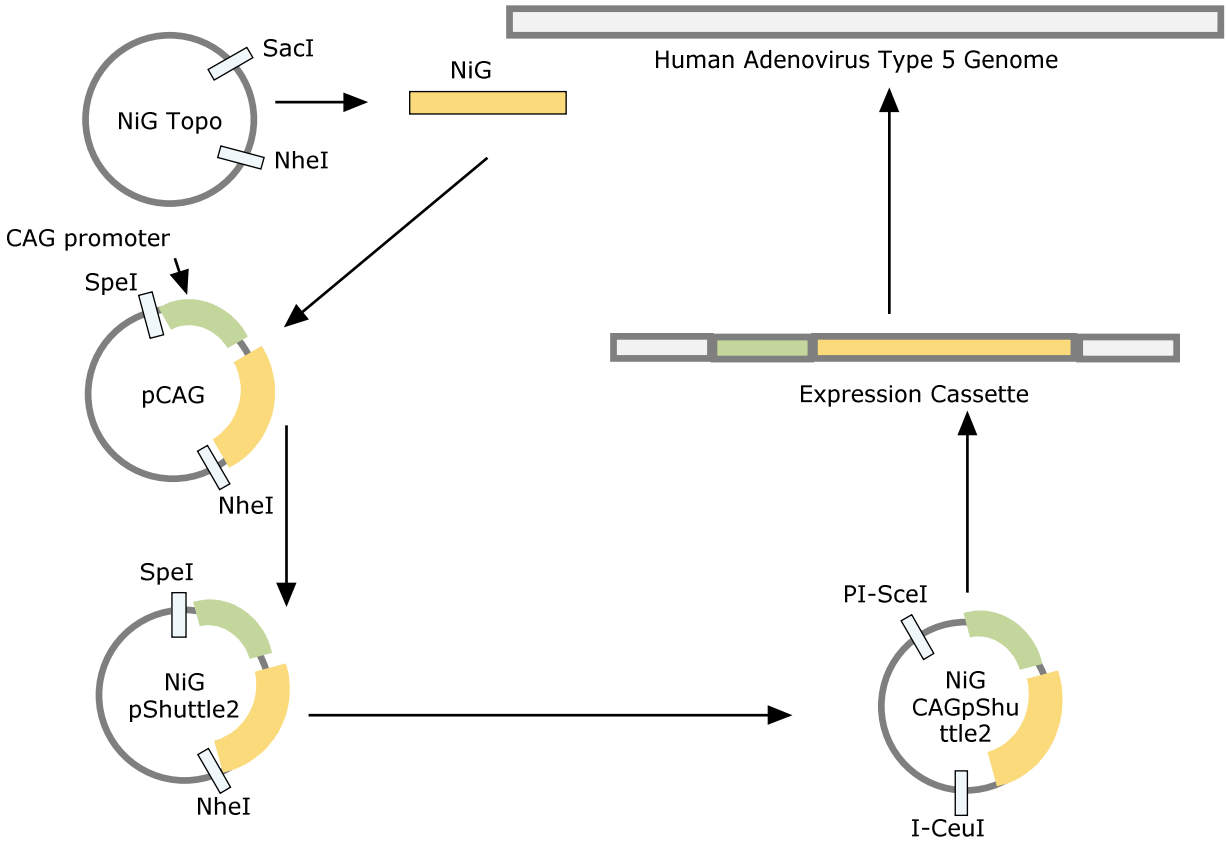

Figure 2.4. Nipah G Cloning Strategy.

Nipah G was cloned directly in pCAGα, and excised using SpeI and NheI, which removed the promoter and gene for subsequent ligation into pShuttle2. The shuttle construct, termed NiGCAGpShuttle2, was digested with rare-cutting LAGLIDADG-type homing endonucleases, PI-SceI and I-CeuI, and the entire expression cassette was ligated into the Human Adenovirus Type 5 genome (AdHu5) termed pAdenoX® (Clonetech).

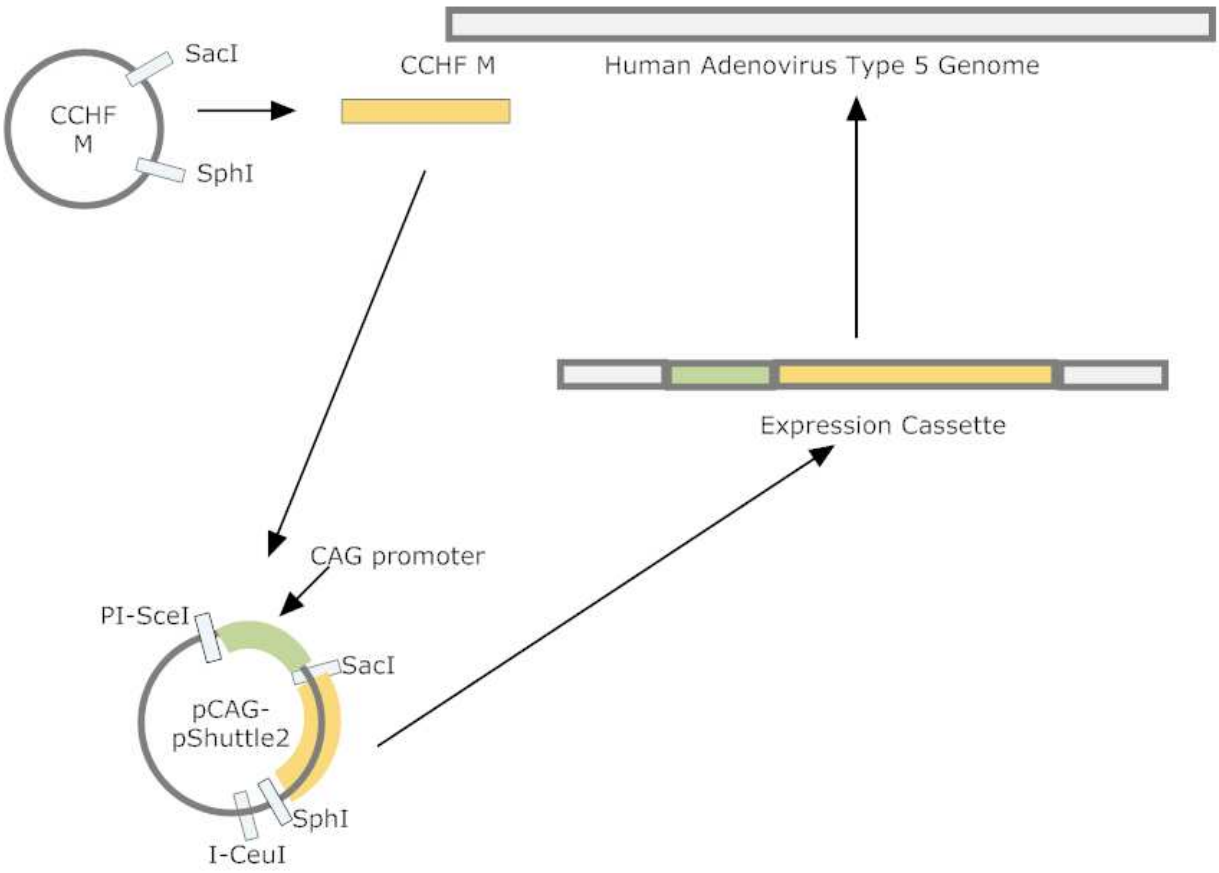

Figure 2.5. CCHF M Segment Cloning Strategy.

CCHF M was synthesized by GeneArt, and was created with SacI and SphI sites on the 5' and 3' ends, respectively. This was cloned directly into pCAGpShuttle2, a shuttle vector displaying the CAG promoter instead of the CMV promoter. The resulting CCHFMpCAGpShuttle2 vector was digested with PI-SceI and I-CeuI to liberate the expression cassette, which was subsequently ligated into the Human Adenovirus Type 5 genome (AdHu5).

Ligations of Nipah F, Nipah G and CCHF M into pAdenoX®, (Clonetech), a human adenovirus type 5, were performed using in-gel ligations. This consisted of separating the digested band on a 0.7% low melt agarose gel (Life Technologies), excising the band of interest, and freezing the gel band at –80°C and then melting at 70°C. Three ratios of vector to insert were used (3:7, 2:8, 1:9), along with a no insert control. T4 DNA ligase (NEB) was prepared according to the manufacturer's recommended protocol and added to the melted DNA once it had cooled to below 37°C. The ligations were placed at 16°C overnight, and heated to 37°C the next morning. To prepare for the transformation, 100 µL of SOC medium was added, and the solution was heated to 72°C for 2 minutes. The solution was vortexed, centrifuged, and incubated at 72°C twice, and added to Top 10 chemically competent cells (Life Technologies), and placed on ice for a total of 1 hour with intermittent mixing. Cells were heat-shocked for 1 minute at 42°C and 700 µL of SOC medium was added. The competent cells were then placed into a 37°C incubator/shaker and allowed to shake for 90 minutes at 225 rpm. Competent cells were inoculated onto LB agar supplemented with 100 µg/mL of kanamycin and incubated at 37°C overnight. Purification was performed using DNA Midi Kits (Qiagen), and eluted with deionized water. This vector was linearized using PacI, purified and used for transfection of HEK 293A cells (Life Technologies) to recover recombinant adenovirus.

2.4 Cell Culture and DNA Transfections

All cell culture was performed using Dulbecco's Modified Eagles Medium (DMEM, Gibco, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS, Wisent,

St.Bruno, Quebec), 1% penicillin/streptomycin (Gibco), 1% L-glutamine (Gibco), and 1% sodium pyruvate (Gibco). Human embryonic kidney (HEK 293A) cells were generously provided by L. Espira, Institute of Cardiovascular Sciences, St. Boniface Research Center, Winnipeg, Manitoba.

DNA transfections of HEK 293A cells were performed using Calcium Phosphate Transfection Kits from Clonetech (Mountain View, California) in a 6-well plate format according to manufacturer's recommended protocol, using 4 μg of DNA per well. After transfection, plates were centrifuged at 1850 rpm for 20 minutes and incubated under an atmospheric headspace of 5% CO_2 for 16 hours. Following incubation, cell monolayers were washed, and passaged into T-175 flasks until CPE was noted. For testing the expression of viral glycoproteins, Nipah F, Nipah G, and CCHF M, all in pCAGα, were transfected into 15 cm tissue culture plates using 54 μg of DNA using calcium phosphate precipitation as described above.

2.5 Protein Expression

Western blots were performed using the standard methodology previously described [192, 193]. Briefly, HEK 293A cells were cultured in 6-well plates to approximately 80% confluence and were infected with recombinant adenovirus at a M.O.I of 5 (based on total infectious particle per mL as determined by immunocytochemistry against the adenovirus hexon protein using pAdenoX® rapid titration kit). At 48 hours post-infection, infectious cell supernatants were discarded and 50 μL of 2X radioimmunoprecipitation (RIPA)

buffer was added per cm^2 of surface area. Cell lysates were collected, normalized, and analyzed under reducing conditions with 5x sodium dodecyl sulfate (SDS) gel loading buffer on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred by electroblotting to a PVDF membrane (Bio-Rad, Hercules, California) and visualized with appropriate immune sera as the primary antibody, followed by a horseradish peroxidase conjugated secondary antibody. Immunodetection was performed using Amersham ECL detection system (Amersham, Piscataway, NJ) according to manufacturer's recommended protocol. Western blots using the LI-COR (Lincoln, Nebraska) Odyssey® imaging system were performed according to manufacturer's recommended protocol.

Enzyme-linked Immunosorbent Assays (ELISA) were performed using standard methods for infected cell lysates. Briefly, adenoviral constructs were used to infect HEK 293A cells and were tested for expression after 48 hours. The cells were subjected to 3 freeze-thaw cycles and were resuspended in borate saline. The cells were then centrifuged at 2500 rpm for 10 minutes and resuspended in borate saline. The cell lysates were then subjected to light intermittent sonication for 10 minutes and subsequently centrifuged for 10 minutes at 10,000 x g. The supernatant was used to coat 96 well ELISA plates overnight. Following incubation, the ELISA plates were blocked with 5% skim milk for 2 hours at 37°C, washed twice and the sera, diluted in phosphate buffered saline (PBS), was then used in a double dilution fashion and incubated at 37°C for 1 hour. The plate was then washed, and secondary horseradish peroxidase (HRP) conjugated antibody (1:200 in PBS) was added and allowed to incubate for 1 hour at 37°C. For a peroxidase

substrate, 2,2'Azino-di[3-ethylbenzthiazoline sulfonate(6)]diammonium salt (ABTS), (Roche, Mannheim, Germany) was used according to manufacturer's recommended protocol. Plates were read using an ELISA plate reader (Molecular Devices, Sunnyvale, California), at 405 nm.

2.6 Cellular Immune Responses

Enzyme-linked immunosorbent spots (ELISPOT) assays were performed using the ELISPOT Mouse Set (BD Biosciences, Mississauga, Ontario) according to the manufacturer's instructions. PVDF membrane 96-well plates were coated overnight at 4°C using purified anti-murine IFN-γ antibody. Afterwards, each plate was blocked for 3 hours using RPMI 1640 supplemented with 10% FBS, and 1% penicillin/streptomycin. Splenocytes were harvested from mice 10 days post-vaccination and ground against a fine mesh filter in L-15 medium (Gibco/ Life Technologies) and mononuclear cells were isolated by filtration and resusupended in L-15 medium. Peptides (Mimotopes, Clayton, Victoria, Australia) consisting of 15 amino acids with 10 amino acid overlaps were resuspended in dimethyl sulfoxide (DMSO) and pooled into either 10 or 20 peptides per pool. Each pool was diluted in RPMI 1640 and added to the microtiter plate wells at a final concentration of 2.5 μg/mL of each peptide per well. Splenocytes were resuspended in RPMI 1640 (supplemented with 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-glutamine, 5 x 10^{-3} β-mercaptoethanol, 1% sodium pyruvate, 1% HEPES) and added at 5 x 10^5 cells per well. The plates were then incubated overnight at 37°C in an atmospheric headspace of 5% CO_2. The following day, samples were

washed and incubated with biotinylated anti-mouse IFN-γ for approximately 2 hours at room temperature. After the addition of streptavidin conjugated-HRP IFN-γ positive cells were visualized through use of the BD 3- amino-9-ethyl carbazole (AEC) Substrate Reagent Set (BD Biosciences). Spots were counted using an ELISPOT Plate Reader (AID ELISPOT Reader, Cell Technology, Colombia, Maryland).

2.7 Adenovirus Amplification and Titration

Adenoviral particles were purified based on previously published protocols [190, 193, 194]. Briefly, 293A cells were harvested 48 hours post infection and centrifuged at 3,000 rpm for 20 minutes. The supernatant was discarded and the cell pellet was resuspended in 1X Tris-Cl (pH 8). The pellets were then subjected to three freeze-thaw cycles and centrifuged at 4,000 rpm for 20 minutes. The supernatant was loaded onto a cesium chloride gradient (see Appendix B) and centrifuged for 4 hours at 20,000 rpm. The resulting band was extracted, diluted in an equal volume of Tris-Cl, loaded onto a cesium chloride gradient, and spun at 20,000 rpm overnight. The following day, the adenoviral particles were recovered and subjected to buffer exchange using Slide-A-Lyzer® (Pierce, Rockford, Illinois) cartridges in Tris-Cl (pH 8). Total particle counts were determined using A_{260} spectrophotometer readings as previously described [193]. Adenovirus particles were titred using AdenoX® Rapid Titre Kit (Clonetech) according to manufacturer's recommended protocol.

2.8 Immunization and Challenge

For CCHF AdX, immunizations were performed on STAT-1 mice using 1 $x10^{10}$ total particles diluted to 100 μL in PBS. Injections were administered intra-muscularly, and mice were housed in filtered cages. At day 28, STAT-1 mice were relocated to BSL4 and allowed to acclimatize for several days. At day 32, mice were subjected to a challenge with 100 PFU of CCHF strain IbAr10200 (25 x LD_{50}) administered intra-peritoneally.

3.0 Results

3.1 Gene Synthesis and Cloning

The glycoprotein sequences for Nipah viruses F and G and for CCHFV were obtained from the National Center for Biotechnology Information (NCBI) and used to create a codon-optimized DNA sequence for translation in human cell lines. The software used for this purpose was Gene Designer, freely available from DNA2.0 (Menlo Park, California). Gene Designer allows for the use of codons according to a codon bias table integrated into the software. For Nipah F and G and the CCHFV M sequences, the codon bias table for *Homo sapiens* was used. A threshold parameter, which allows for the elimination of codons that are not prevalent in the organism of choice, was set to 21%. Once these parameters were established, the program allowed for the manual removal of large repeats and for the addition of restriction enzyme sites and Kozak sequences without altering the optimization threshold. The resulting DNA sequence is an optimized version of the original glycoprotein gene. The optimized DNA sequences were inputted into Gene2Oligo and the corresponding 20 bp hybridization unit oligonucleotides were created in a non-gapped fashion (Appendix B). These oligonucleotides were pooled and a 3-step PCR protocol was performed.

The DNA sequence corresponding to the Nipah G glycoprotein yielded an indistinct band from the 3 step PCR product (Figure 3.1), but Nipah F yielded a band at the correct length of 1.6 Kb, indicated by the arrow in Figure 3.1, and was excised from the gel. Nipah G oligonucleotides were redesigned using melting temperature priority. Nipah G

assembled correctly but due to the faint banding pattern, was not imaged. The Nipah F glycoprotein gene was found to have a deletion and was corrected using PCR. The two segments were then assembled together using PCR.

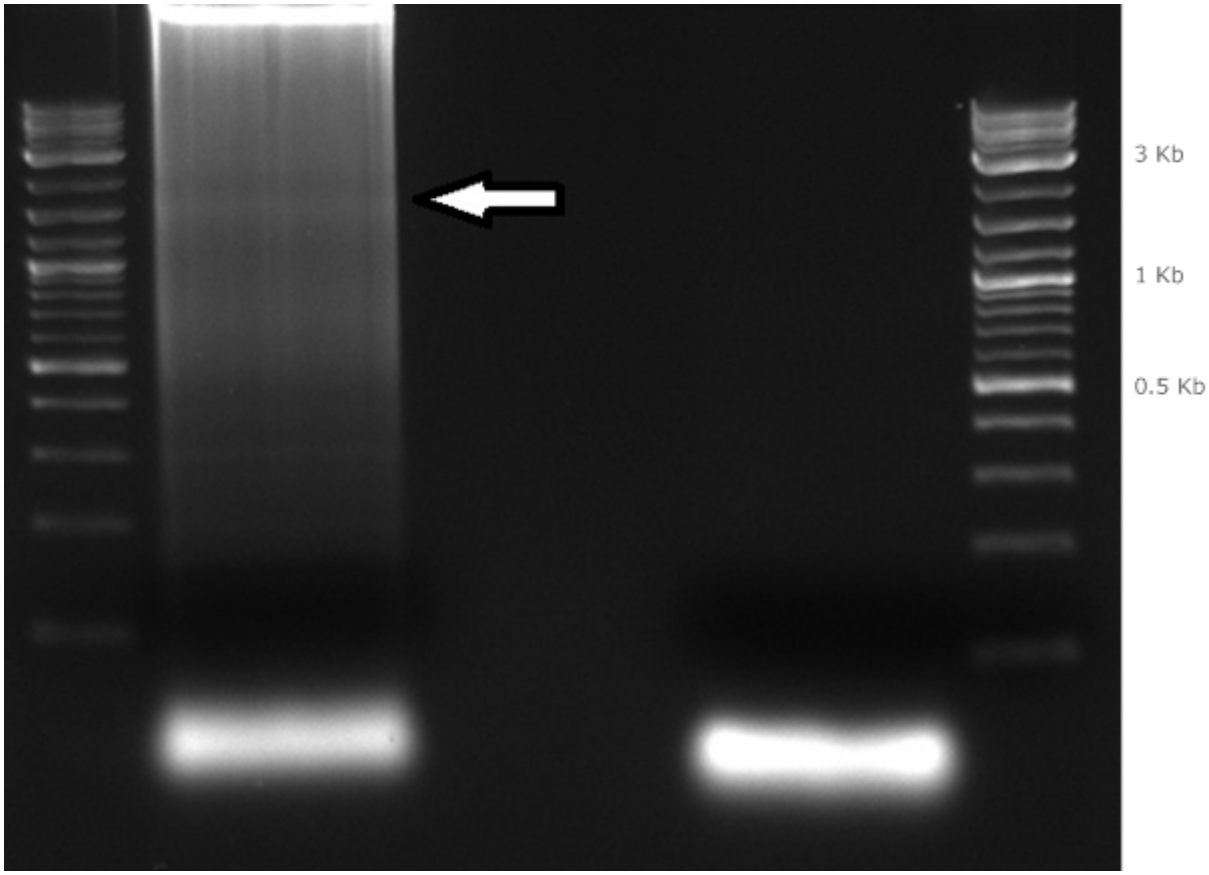

Figure 3.1. Nipah G and Nipah F Gene Synthesis.

The first lane to the right of the ladder (on the left-hand side of the gel) corresponds to the gene synthesis product for Nipah F and adjacent lane corresponds to the product obtained for Nipah G. The arrow corresponds to an amplicon of 1.6 Kb. 2-log ladder (NEB) was used as a DNA ladder.

Due to the large size of CCHF M Segment (5.1 Kb), the DNA sequence was split into five portions around unique restriction sites (Figure 3.3). The five pieces assembled correctly during PCR, verified by agarose gel electrophoresis, and were excised. Following verification of restriction digests and sequence confirmation, inserts were subcloned.

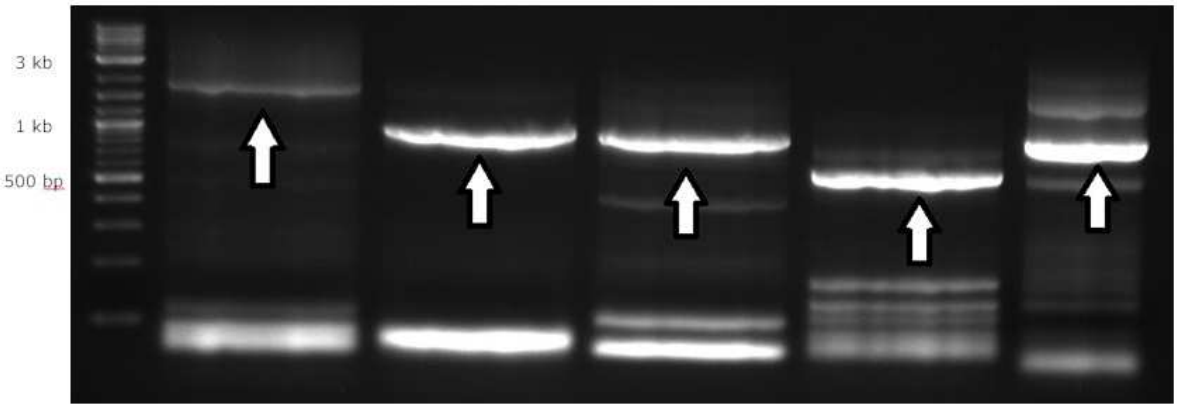

Figure 3.2. CCHF M Segment Gene Synthesis.

The entire codon-optimized sequence was split into 5 individual segments with unique restriction sites. Arrows indicate bands that were excised at the correct lengths.

3.2 Adenoviral Particle Rescue Times

Adenoviral DNA containing the transgene under control of the CAGα promoter was transfected into HEK 293A cells for CCHF AdX, NiF AdX and NiG AdX. DNA was linearized with PacI (NEB), gel purified, and transfected using calcium phosphate precipitation. Particle rescue times (Table 3.1) were calculated from the date of transfection until the formation of CPE was visualized. Nipah F AdX was approximated due to a blind passage, as CPE was not visible after 28 days. Despite the large size of the CCHF AdX insert, the recombinant adenovirus was recovered within 25 days. Furthermore, this large insert size had little effect upon the infectivity of the viral particles.

Table 3.1. Adenoviral rescue times. Time in days until the formation of cytopathic effects observed on transfected cells.

Adenoviral particle rescue times	
Virus	Time to rescue (days)
CCHFV AdX	25
Nipah G AdX	20
Nipah F AdX	~ 28

3.2 Virus Titration

Once CPE was visualized, the cells were harvested and concentrated by centrifugation, and the pellet was subjected to three freeze-thaw cycles to liberate the virus. This crude lysate was then used to infect progressively larger cell volumes until a sufficient viral stock was achieved to infect 50 large 15 cm tissue culture plates. Once 50 plates were harvested 48 hours post infection, the virus was purified and titrated (Table 3.2). Total particles were determined using A_{260} readings, which utilizes a spectrophotometer to determine the concentration of viral particles in the sample. In this assay, 0.1% SDS in adenovirus excipient (see Media Formulations) was used to lyse the viral particles and liberate the viral capsid into component proteins and DNA. The viral concentration was measured using an absorbance of 1.00 AU (1-cm pathlength) at 260 nm which corresponds to 1.1×10^{12} viral particles per mL.

Table 3.2. **Viral Titration of Adenoviral Constructs**. Particles were titrated using AdenoX® Rapid Titre Kit, and total particles were determined using A_{260} readings.

Sample	Total Particles	Infectious Particles	Total to Infectious Particle Ratio
CCHF AdX	4.47×10^{12}	7.3×10^{10}	1:63
NiF AdX	3.0×10^{8}	3.0×10^{6}	1:100
NiG AdX	1.27×10^{12}	1.75×10^{10}	1:73

3.3 Transgene expression

To determine expression of the glycoproteins in the adenovirus constructs, HEK 293A cells were infected with CCHF AdX and western blots were performed using the cell lysate. Immune serum from non-human primates (Figure 3.3) was used as well as CCHF anti-Gc mouse ascietes fluid (Figure 3.4). A further expression test using infected cell lysate was performed in ELISA format (Figure 3.5) using pooled murine sera from mice that were vaccinated three times with wild-type virus. Due to the lack of suitable reagents for testing expression of NiV F and G proteins, DNA constructs of NiF CAGα and NiG CAGα, were transfected into HEK 293A cells and the formation of syncitia was observed (Figure 3.6).

Cellular immune responses were determined using STAT-1 mice for CCHF AdX. Mice were vaccinated using 10^{10} total particles and harvested for splenocytes 10 days later (Figure 3.7). The immunodominant epitopes were identified and mapped according to their location on the CCHF M segment (Figure 3.8). In addition, STAT-1 mice were immunized and subjected to challenge with CCHF strain IbAr10200 (Figure 3.9). Once the adenovirus-based vaccine was purified and expression confirmed, times to completion were determined according to procedural times (Table 3.3).

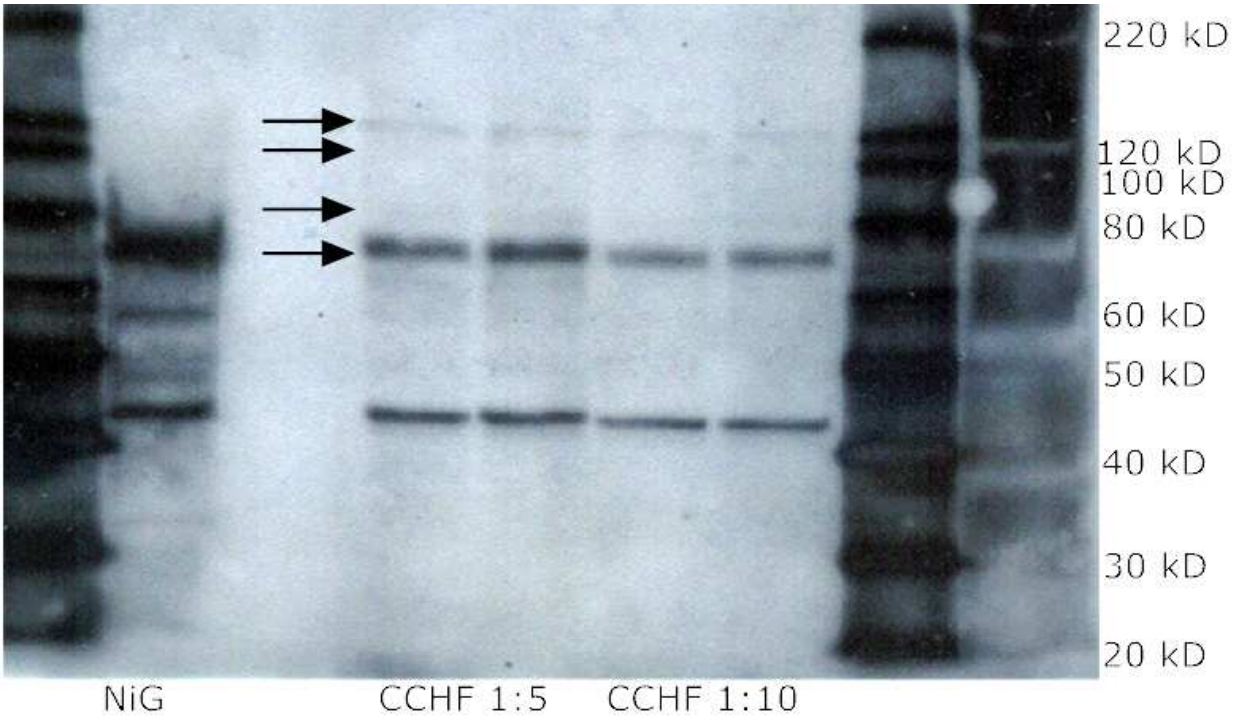

Figure 3.3. Western blot of CCHF AdX Infected Cells.

Primary antibody was immune sera from non-human primates (NHP). Serum was diluted 500-fold and anti-human HRP conjugated secondary, 2000-fold. The second lane, adjacent to Magic Mark™ (Life Technologies) protein ladder contains NiG AdX infected cell lysates as a negative control. Lysates were diluted 1:5, and 1:10, as shown in the following lanes, to enable visualization of the glycoprotein bands. The last lane contains SeeBlue® prestained ladder (Life Technologies).

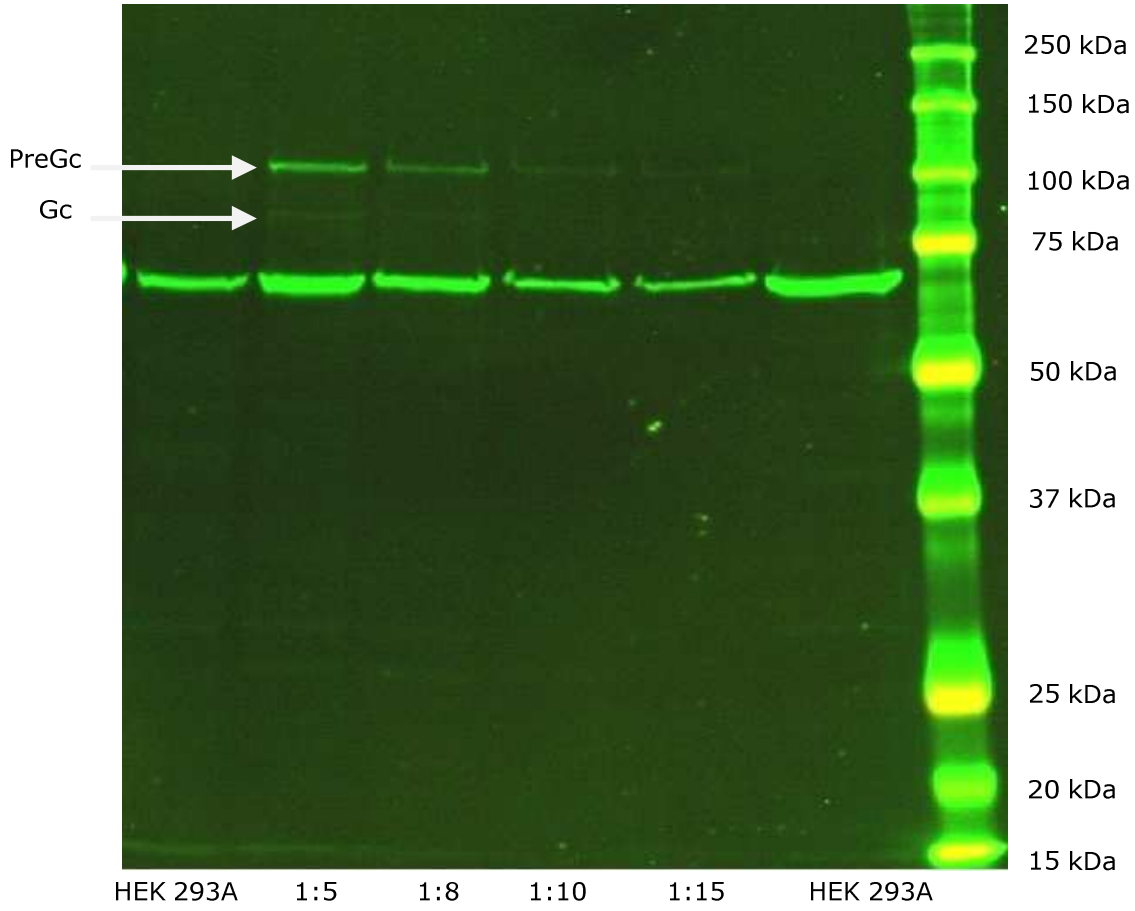

Figure 3.4. Fluorescent Western blot of CCHF AdX Infected Cells.

As a primary antibody, used CCHF anti- Gc mouse ascietes fluid (MAF). Anti-Gc MAF was diluted 1000-fold, and goat anti-mouse IRDye® secondary was diluted 15 000-fold. The first and sixth lanes correspond to uninfected HEK 293A cells, and lanes two to five correspond to CCHF AdX infected HEK 293A cells in increasing dilutions. Odyssey® Two Color Protein Molecular Weight Marker (LI-COR) denotes the last lane. Arrows indicate glycoprotein bands.

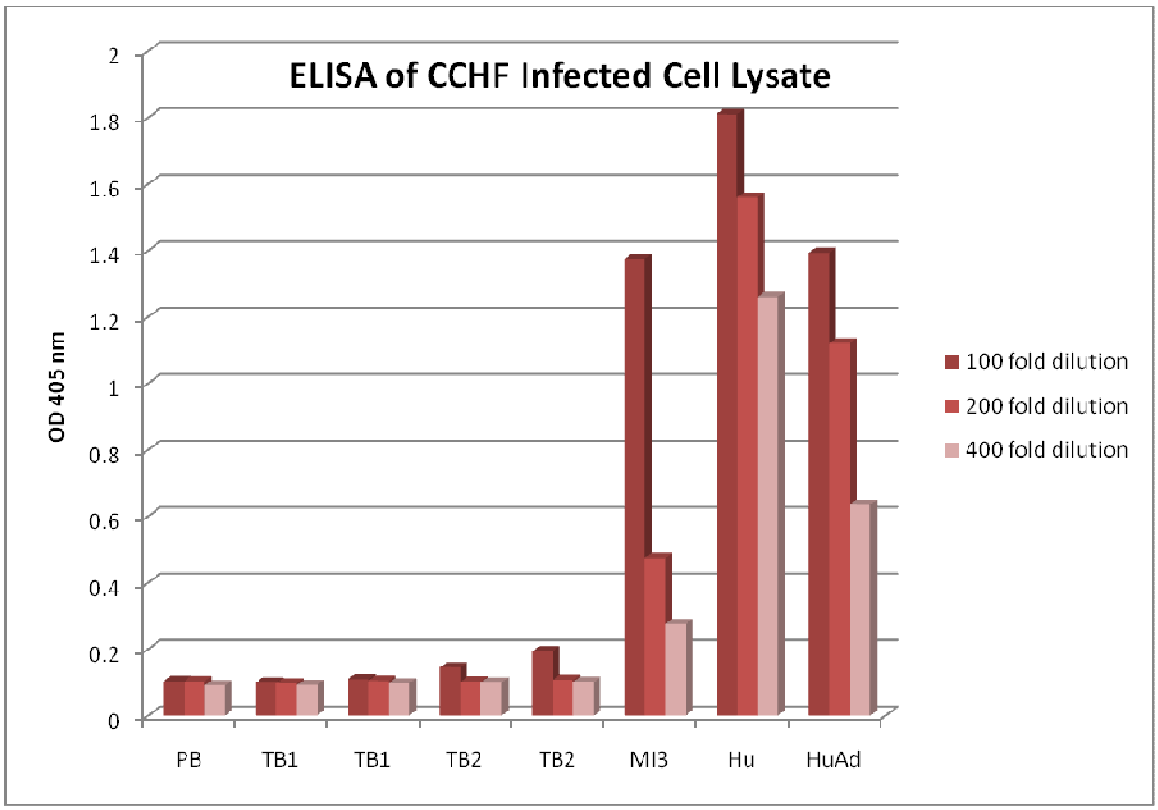

Figure 3.5. ELISA of CCHF AdX infected HEK 293A cells.

Infected cell lysate was tested against various sera to determine expression of the viral glycoproteins. Prebleed (PB) corresponds to naïve BALB/c mouse sera with BALB/c mice vaccinated with 100 µg of CCHF CAGα DNA (TB1). A second and third vaccination was administered at 3 week intervals and corresponds to test bleed 1, and test bleed 2, respectively. Positive mouse serum (MI3) was obtained from mice vaccinated three times with wild-type CCHF virus. Human serum was also tested and is indicated as Hu. To determine reactivity towards AdHu5, the human serum was tested using Ebola Zaire AdX lysate and is indicated by HuAd. Legend on the right indicates sera dilutions performed for the ELISA.

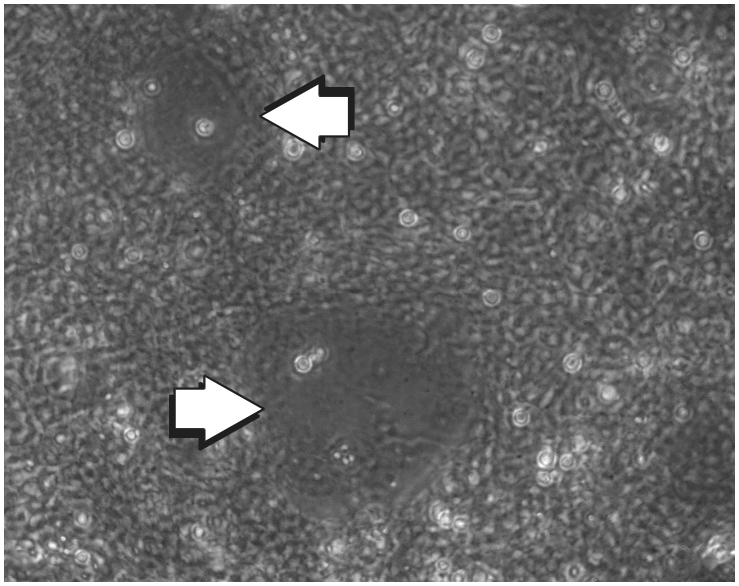

Figure 3.6. Syncitia Formation of Nipah F and G.

Image of syncitia formation induced by expression of Nipah F and Nipah G glycoproteins. HEK293A cells were transfected with NiFpCAGα and NiGpCAGα and the cell monolayer was imaged approximately 48 hours post transfection. Arrows indicate syncitia.

3.4 Cellular Immune Responses

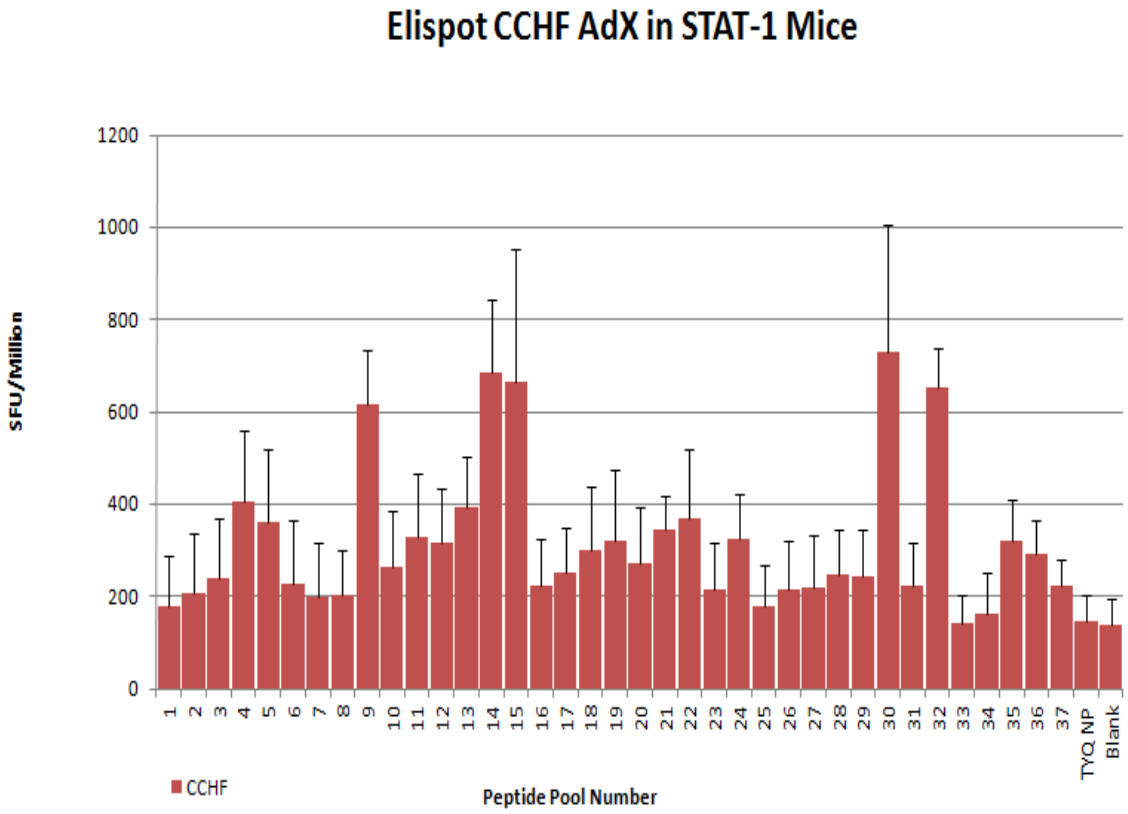

Figure 3.7. CCHF ELISPOT Assay.

ELISPOT assay of 6 STAT-1 mice vaccinated with 1 x10^{10} total particles of CCHF AdX intramuscularly. ELISPOTS were performed 10 days post-vaccination. Peptide TYQNP is an immunogenic, non- relevant peptide from hemagglutinin (H5N1).

PEPTIDE	SCORE	AMINO ACID
VMLAVCKL	11	948-955
NRALFIRS	11	966-973
TTFVLCIL	10	977-984
YSPVFEYL	23	1144-1151
GCTSSTCL	11	1174-1181

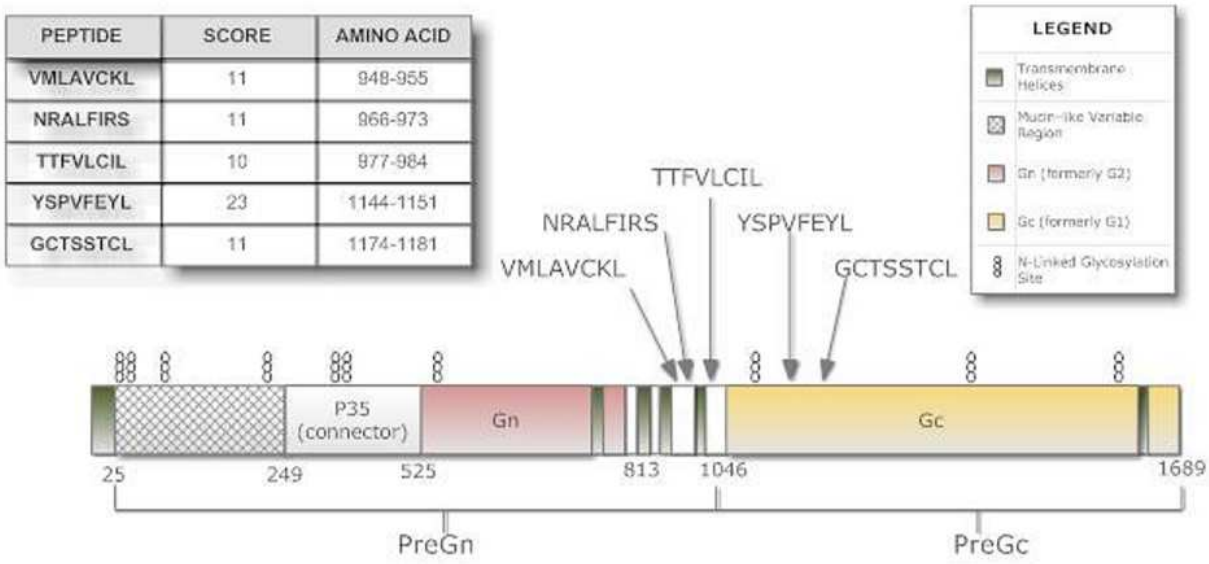

Figure 3.8. Immunodominant epitopes of CCHF ELISPOT assay.

Peptides were analyzed using web-based software SYFPEITHI (www.syfpeithi.de), to generate relative scores. Peptide locations are indicated by arrows on the glycoprotein precursor.

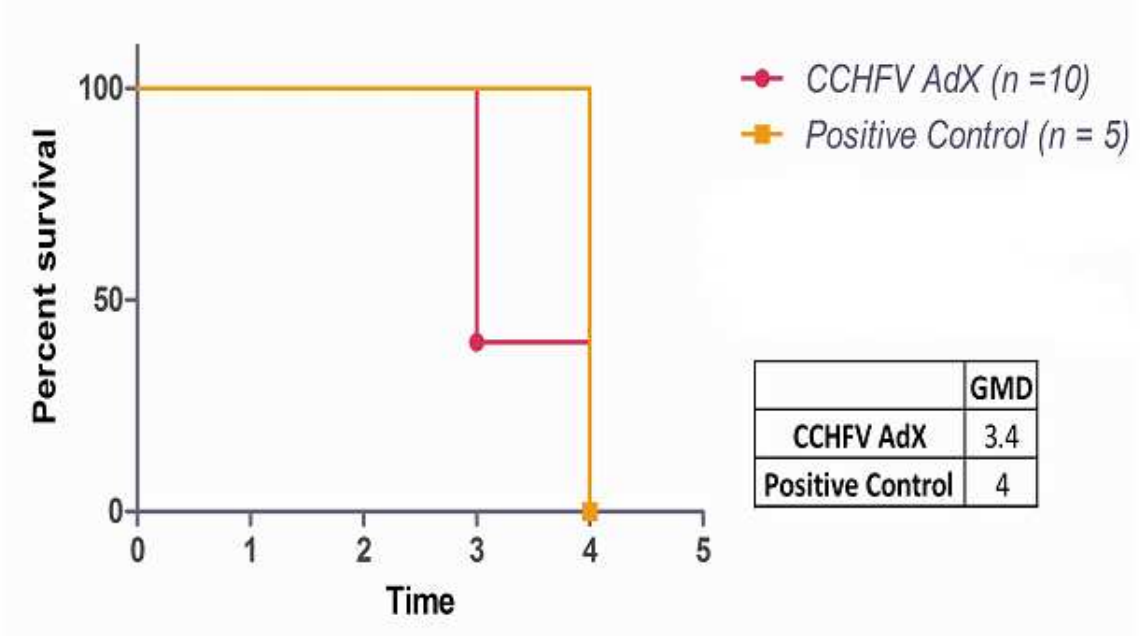

Figure 3.9. Challenge of STAT-1 Mice.

Survival curve of mice challenged intra-peritoneally with 100 PFU (LD_{50} x 25) of CCHF strain IbAr10200. Mice were challenged 32 days post immunization with 1 x 10^{10} total particles of CCHF AdX. Time is depicted in days.

3.5 Time to Completion of Adenoviral-based Vaccines

Table 3.3 Time Required for Completion of Adenoviral- based Vaccines. Times are based on a single person performing all procedures and do not account for trouble-shooting the significant deletions and/or mutations detected in samples during the gene synthesis process, or from delays experienced during ordering oligonucleotides and/or reagents.

Procedural step	**Time**
Antigen selection and planning	2 days
Codon-optimization and oligonucleotide splitting	1 day
Ordering oligonucleotides	1 week[1]
Gene synthesis	2 days
Cloning and subcloning	4-6 weeks
Sequencing	3 days
Adenoviral particle rescue	3-5 weeks
Viral amplification and purification	2 weeks
Expression testing	1 week
Animal ordering	1 week
In vivo testing	6 weeks
Immunology-cellular/humoral responses	2 weeks
TOTAL	28 weeks

[1]Additional time for oligonucleotide ordering may be required.

4.0 Discussion

4.1 Gene Synthesis and Cloning

The flexibility and ease of utility available in synthetic genes has led to its increasing popularity in research. Gene synthesis is a powerful technique; it is widely used for a number of purposes, such as facilitation of site-directed mutagenesis, structural analysis, and generation of proteins [195-197]. In addition, it allows for the generation of a DNA sequence that is codon-optimized for expression in a particular species or cell line, with individual tags and restriction sites. In this study, glycoprotein genes were constructed for optimal expression in *Homo sapiens*. Codon-optimization is an important factor in establishing gene expression; however, it is generally less significant than promoter strength, locus, and type of vector [195].

Methodology for gene synthesis involves the inputting of the desired amino acid sequence, back translating to a DNA sequence, and then modifying the sequence to meet specified needs without altering the protein sequence. This involves the insertion of silent restriction sites and allows for the removal of restriction sites for cloning into the multiple cloning site (MCS) of the chosen vector. Furthermore, this design step also allows for the adjustment of GC content and manual removal of long repeats which could potentially hinder the oligonucleotide assembly process. Once the final sequence is obtained, which is codon-optimized and contains necessary restriction sites and tags, it is divided into individual hybridizing oligonucleotides that are assembled by polymerase chain reactions or ligase chain reactions.

The methods involved in creating a codon-optimized synthetic gene *in silico* can be a tedious and error prone process. To date there are a few publicly available web-based software programs that can aid in the process. In this study, Gene Designer, available from DNA2.0, was used to back-translate and codon-optimize the glycoprotein genes used. For the creation of oligonucleotides, Gene2Oligo was used to generate 20bp hybridizing units [198]. PCR assembly of synthetic genes is relatively straight-forward and generally occurs in a three-step process, with individual oligonucleotides hybridizing in overlapping units of predefined length [199].

The construction of the glycoprotein for Nipah F was performed in a three-step process that assembled the entire gene but failed to produce a clearly defined band when visualized on agarose gel electrophoresis. This was most likely due to the fact that individual oligonucleotides were designed simply by limiting the size of the DNA sequence to 20 bp hybridization units with a total length of 40bp; this led to difficulties in establishing T_m parameters during the PCR process. This procedure was modified for Nipah G by setting the parameters for oligonucleotide design to T_m priority. In this way, oligonucleotides were designed based, not on length, but by melting temperature. The end result was that during the three step process, a clearly defined T_m could be used that would accommodate all the oligonucleotide melting temperatures.

It was noted, however, that genes that are significantly longer in length are more difficult to synthesize, as the final DNA band on agarose gel electrophoresis was significantly

fainter for larger size inserts. This was observed during the synthesis of Nipah G glycoprotein, which is 1.6 kb in length; this difficulty was corrected by introducing a fourth step to the PCR process using terminal forward and reverse gene synthesis primers and by altering the cycling parameters. This procedure was tested during the construction of Rift Valley Fever Virus M Segment (see Supplementary Data Figure S.2), which was synthesized in two fragments of 1.3 and 1.8 kb, and utilized PCR cycling at two different melting temperatures (55°C and 60°C).

With this additional step, the frequency of mutations in the final PCR product was reduced in comparison with the 3- step procedure. The web-based software used in this study, Gene2Oligo, does not contain any parameter for misprime analysis; a utility useful for determining if an oligonucleotide has sufficient sequence homology to other areas of the sequence and may bind elsewhere. Other web-based software such as DNAWorks, not only designs oligonucleotides and reverse translates, but provides the user with a misprime analysis [200].

Another frequently overlooked variable in gene synthesis is the potential for cryptic splice sites, which are sequences that resemble authentic splice sites and may result in aberrant splicing. This would lead to sections of the mRNA that could possibly be spliced out, producing a truncated or deviant protein. Although many splice site junction sequences are known [201], the software used in this study did not allow for scanning sequences for the presence of possible cryptic splice sites.

Current gene synthesis software also lacks parameters for determining stable secondary structures. For smaller gene inserts such as Nipah F and Nipah G, this was not problematic, but is reported to occur with larger DNA segments with Rift Valley Fever Virus (RVFV) M segment (see Supplementary Data), which was assembled from two smaller inserts, the final gene segment was difficult to sequence, amplify and digest with certain enzymes. Sequencing yielded partial results with sections of the M segment missing or incomplete, although restriction digests yielded correct sequence length. Due to these difficulties, RVFV M segment was put on hold to concentrate on creation of Nipah F AdX, Nipah G AdX and CCHF AdX. It is plausible that stable secondary structures, formed from tracts of self-complementary DNA sequences including di-nucleotide tandem repeats, may have contributed to these difficulties. CCHF M was divided into 5 smaller segments to facilitate ease of synthesis. The resulting fragments synthesized correctly, and ligation was underway, but commercially synthesized CCHF M arrived before the final assembly occurred. Consequently, the commercially synthesized CCHF M segment was used.

In this study, it was noted that the improved method of gene synthesis involving a 4-step PCR procedure and codon-optimization utilizing Gene Designer, was a superior procedure for the creation of synthetic genes at a fraction of the cost of commercially synthesized genes. These improvements increased the efficiency of obtaining synthetic genes in house, without compromising extended time frames that may be prevalent when ordering from external sources. It is clear from this work that for urgent situations such as

outbreaks, the modified 4-step PCR system, in conjunction with versatile *in silico* design, is most advantageous for creating antigens for vaccine development.

4.2 Adenoviral Particle Rescue Times and Titration

Rescue times for adenoviral particles varied considerably for constructs used in this study as compared to the recommended time for pAdenoX® constructs. According to the manufacturer's protocol, adenoviral particles are to be harvested after 1 week post-transfection. CCHF AdX, NiG AdX and NiF AdX all required more than 20 days. This was possibly due to the high levels of expression by the CAGα promoter. The shorter rescue times obtained through the use of CAGαGFP AdX, and CMVGFP AdX DNA constructs that were used as transfection controls illustrate this effect. In addition, at 5 days post-transfection of CAGαGFP AdX, there were considerably more cells (data not shown) that showed signs of necrosis than the CMV GFP counterpart. Overall, this suggests that high levels of transgene expression may have contributed to lengthy rescue times.

NiF AdX required the longest time for particle rescue, and after 28 days, CPE was not clearly visible. The cells were passaged and particles were recovered shortly thereafter. As with other paramyxoviruses, the F protein from NiV bears similarities with the hemagglutinin of influenza, which may have toxic effects upon overexpression in cells. As Cathepsin L is required for cleavage of the F protein [169], which occurs in the acidic endosomal compartment, it is plausible that overexpression of the F proteins could disrupt endosomal compartments and result in acidification of the cytosolic compartment.

NiG AdX rescue times were the shortest with HEK 293A cells showing CPE at only 20 days. Despite the extensive size of the CCHF M segment at 5.2 kb, rescue time was 25 days, which was shorter than rescue times for NiF AdX.

NiG AdX was titrated to 1.75 x 10^{10} total particles with a total particle to infectious particle ratio of 1:73. NiF AdX was titrated to 3.0 x 10^{8} total particles with a total particle to infectious ratio of 1:100. CCHF AdX had the highest titre with 4.47 x 10^{12} total particles and a total particle to infectious particle ratio of 1:63. Upon several viral amplifications, NiF AdX produced lower titres than NiG AdX and CCHF AdX consistently in HEK 293A cells. Similar results were produced with other HEK subtypes. Once again, it appears that toxicity issues experienced with the NiV F protein may have interfered with sufficient viral particle amplification.

4.3 Expression Testing

CCHF AdX expression was tested using serum from CCHF-infected non-human primates. Western blot analysis yielded several proteins of approximately 130 kDa, 100 kDa, 75 kDa and 80 kDa. A previous study reported sizes of the glycoprotein precursors corresponding to 140 kDa for PreGn and 85kDa for PreGc using hyperimmune MAF against Gn in Chinese Hamster Ovary cells (CHO) [69]; the differences observed in this work may be due to glycosylation patterns developed with HEK 293A cells. The authors also found fully processed glycoproteins corresponding to Gn and Gc to be 37 and 75

kDa, respectively [69]. The 37 kDa protein was not visible, yet the 80 kDa protein was detected, although control cell lysate infected with NiG AdX demonstrated a band at the same molecular weight. The relatively faint banding patterns observed in the western blot analysis may be due to the relatively low level of antibodies against the glycoproteins present in the serum tested. There are currently no monoclonal antibodies available for the CCHF Gn and Gc glycoproteins. A western blot assay using the Odyssey™ (LI-COR) system using mouse ascietes fluid against Gc clearly depicts two bands at approximately 85 kDa and 105 kDa. The 85 kDa protein is consistent with results obtained by Vincent et al [69], and the 105 kDa protein is indicative of the PreGc.

To confirm expression of CCHF glycoproteins, an ELISA using human sera and mouse sera was performed on CCHF AdX infected cell lysates. Pooled sera from six mice per group which received 100 μg of pCAGαCCHF DNA at three week intervals were also used to detect the CCHF glycoproteins. Results confirm the expression of CCHF AdX in HEK 293A cells, as pooled serum from mice immunized with wild-type CCHF virus, showed a strong response. The human serum also indicated a significant response to the CCHF glycoproteins, although the serum was also positive for AdHu5, as determined by its reactivity to Ebola Zaire AdX infected cell lysate.

For Nipah F and G proteins, cells were transfected with DNA plasmids, pCAGαNiF and pCAGαNiG, respectively. Cells were examined for the formation of syncitia, which would be indicative of the expression of the fusion and attachment protein on HEK 293A cell membranes. Cells transfected with both plasmids clearly showed areas of syncitia

formation, whereas cells transfected with either plasmid alone showed no syncitia formation (data not shown). The formation of syncitia is particularly useful, as it depicts proper processing of the F protein and indicates that both glycoproteins are expressed, as fusion can only occur with both proteins.

4.4 Immune Responses

CCHF AdX was further characterized by performing ELISPOT assays to determine cellular immune responses. STAT-1 mice were immunized with 10^{10} total particles and were assessed for cellular responses 10 days later. The ELISPOT assay identified five particular peptides, which elicited the strongest response on the assay according to web-based software referred to as Syfpeithi (www.syfpeithi.de). Syfpeithi is a database of MHC ligands and peptide motifs, which can predict immunodominant epitopes according to specific mouse genomes. Mapping of these peptides to the glycoprotein precursor identified that three of the peptides were present on the NSm protein and the remaining two near the amino terminus of the Gc protein. This study for the first time has identified immunodominant epitopes of the M segment of CCHF. Data obtained from the ELISPOT assay was inputted in Syfpeithi as 15-mers and was predicted to yield the most immunodominant 8-mers. These sequences have been identified as VMLAVCKL, NRALFIRS, TTFVLCIL, YSPVFEYL, GCTSSTCL, with NRALFIRS being the most immunodominant. ELISPOTS were not conducted on NiF AdX or NiG AdX.

4.5 STAT-1 Mouse Challenge

A new STAT-1 mouse model for studying CCHF pathogenesis was developed and mice were immunized with 1 x 10^{10} CCHF AdX particles. These mice were then challenged intra-peritoneally with 100 PFU of CCHF strain IbAr10200. The survival curves indicate that six out of ten mice died one day before the control animals, with four mice dying on the same day as the control mice. In this instance, it appears that immunization with CCHF AdX did not provide protection against CCHFV infection. The immune responses induced by adenoviruses, particularly T-cell responses, can be improved by heterologous prime-boost regimens, which may aid in providing protection [31, 202-205].

4.6 Time to Completion

For CCHF AdX, NiF AdX and NiG AdX, times to completion were determined based upon times required for selected procedures for one person. During times of outbreaks when more personnel participate to mitigate the impact, the time required for completion can be significantly reduced. The key factor in creation of optimized vaccines is clearly the time required for gene synthesis. If gene synthesis proceeds well, the number of mutations, deletions, and substitutions are kept to a minimum, and cloning can begin relatively quickly. Most delays occur in trying to ameliorate any defects in the glycoprotein genes. In this study, the modified gene synthesis protocol has been an advancement allowing the creation genes of less than 2 kb in under a week, which includes the time taken for oligonucleotides to arrive. These genes were synthesized

without errors in the DNA sequence. In house gene synthesis was quicker and more cost-effective than commercially synthesized genes.

For this study, a construct referred to as pCAGαpShuttle2, was created. This construct contains the pShuttle2 backbone, for expression cassette delivery into pAdenoX® but contains the high-expression CAGα promoter. This development improved the time required for completion of cloning, as it eliminated one subcloning step. Through the use of this construct, genes can be synthesized using restriction sites present in the MCS of pCAGpShuttle2, and directly cloned into the AdHu5 genome.

The most challenging aspect of the procedure in developing adenovirus-based vaccines is the difficulties encountered when trying to rescue (recover virus expressing the transgene). Rescue times alone account for approximately 30% of the time required for adenoviral production until *in vivo* testing, and 15% of the entire procedure. This extended time frame may be related to the high expression rates of the CAGα promoter in conjunction with codon-optimization and transfection efficiencies. In this study, all of the constructs were rescued. Preliminary work conducted in this study to assess transfection efficiencies of calcium phosphate precipitation using pCAGαGFP showed that centrifugation greatly enhanced transfection rates. Centrifugation of transfected cells immediately after transfection at 1850 rpm for 20 minutes had the greatest increase in fluorescence of cells. Work conducted by Boussif et al, showed that centrifugation of 3T3 cells using polyethylenimine (PEI) and DNA complexes at 280 x g for 5 minutes, increased transfection levels 40 to 50-fold [206]. Nevertheless, this study found that

using calcium phosphate and a more aggressive centrifugation treatment yielded excellent results even with the use of large DNA constructs, enabling a more efficient rescue process.

Since all the adenovirus-based vaccines used in this study were created to protect against biosafety level 4 (BSL4) agents, the quality of reagents, such as sera, made expression testing more difficult. With the increasing number of monoclonal antibodies available, expression testing of constructs is becoming easier. CCHF AdX expression was confirmed using Western Blots and ELISA procedures that clearly illustrated the expression of viral glycoproteins. Due to the complex nature of the Nipah virus glycoprotein and fusion protein, it has been postulated that conformational epitopes are predominant. Consequently, procedures in removing immune sera from BSL4 laboratories may have had an impact upon expression studies due to gamma irradiation and subsequent degradation of samples. Research is currently underway to develop a suitable method to test expression of NiG AdX and NiF AdX.

Viral amplification and purification is a relatively quick and easy process. Adenoviruses can be recovered at fairly high titres in HEK 293 cells, and are easy to purify using cesium chloride gradients. All adenovirus constructs were isolated at high titres with the exception of the NiF AdX construct, which may have had low titres due to toxicity issues associated with high expression levels.

As most of the immunology of the vaccines in this study required *in vivo* testing, the process of animal ordering can be a lengthy. For instance, ordering STAT-1 can take from 12-16 weeks and can greatly increase time to completion if not ordered in advance. Mice strains such as BALB/c only require a few days to order, and can arrive relatively rapidly.

Challenges to determine protection generally require 28 days and this time frame is a requirement for sufficient immune responses to occur. However, there is some flexibility to when ELISPOT assays are performed, as they can be done concurrently with challenges.

This study has validated our hypothesis that recombinant adenoviral based vaccines can be created in under six months. Furthermore, Objective 1 was completed despite the fairly large insert size of CCHF M segment, at over 5 kb in length and the infectious titre ratios obtained for CCHFAdX, NiF AdX and NiG AdX were all sufficient for vaccine purposes. Objective 2 was validated for CCHF AdX using three different methods that showed expression. NiG AdX and NiF AdX was expression tested through syncitia formation and confirmatory methods are currently underway. Objective 3, the determination of immune responses was completed for CCHF AdX and ELISPOT results clearly define immunodominant epitopes. Neutralizing antibody responses not performed in BSL4 due to the lack of a validated procedure. A challenge was completed through the use of STAT-1 mice as an animal model.

In summary, many of the procedures involved in producing adenovirus-based vaccines can be shortened greatly as underlined in this study. It is feasible to complete an adenoviral- based vaccine in less than 6 months, if adequate reagents and animal models exist.

5.0 Future Directions

The products of this study have yielded three adenoviral vaccines, CCHF AdX, NiG Adx and NiF AdX. These vaccines, allow for further testing and insights into vaccine- induced immunity and glycoprotein characteristics.

For NiF AdX and NiG AdX further studies need to be conducted with respect to cellular immune responses and neutralizing antibodies. These studies can be completed *in vivo* using guinea pigs or golden hamsters as lethal animal models.

The glycoprotein genes for RVFV M segment should be divided into two individual genes, as this approach would foster a more direct immune response without the formation of any non-structural proteins, such as the NSm. Furthermore, cloning two separate Gn and Gc proteins would ameliorate any difficulties encountered with the formation of stable secondary structures. A disadvantage of this approach entails the development of two separate adenovirus constructs, unless an adenovirus vector capable of expressing two genes is developed, such as that developed by Holman et al., 2009 [207].

CCHF AdX was tested *in vivo* using STAT-1 mice. Although STAT-1 mice are useful for studying CCHF pathogenesis, as it somewhat resembles human disease, it may not be adequate for testing viral vaccines. As adenoviral vaccines would benefit from a fully

functional IFN response. Therefore, a STAT-1 knockout model may not be the best choice for CCHF AdX, as the adaptive immune response is hampered.

In CCHF AdX, the use of the entire M segment produces many extraneous proteins in addition to the Gn and Gc glycoprotein. These proteins are GP38, GP85/GP160, NSm, and the mucin-like variable region. Since the cleavage sites of the cellular proteases are known, it would be ideal to construct optimized recombinant adenoviruses against each individual glycoprotein based on the perceived cleavage sites. This approach would facilitate the use of each construct separately or together to determine relative protective capabilities, and eliminate the need for complex processing events by host cells.

Taken together, the adenoviral constructs of CCHF AdX, NiG AdX and NiF AdX, with further study, not only show promise for gaining insights into immune responses relevant to protect against CCHF and Nipah infection. In conclusion, this study has shown that adenoviral- vectored vaccines for BSL4 viruses can be quickly and easily produced during times of disease outbreaks.

References

1. Evans, R.K., et al., *Development of stable liquid formulations for adenovirus-based vaccines.* J Pharm Sci, 2004. **93**(10): p. 2458-75.
2. Tatsis, N. and H.C. Ertl, *Adenoviruses as vaccine vectors.* Mol Ther, 2004. **10**(4): p. 616-29.
3. Horvath, J., L. Palkonyay, and J. Weber, *Group C adenovirus DNA sequences in human lymphoid cells.* J Virol, 1986. **59**(1): p. 189-92.
4. Green, M., et al., *Analysis of human tonsil and cancer DNAs and RNAs for DNA sequences of group C (serotypes 1, 2, 5, and 6) human adenoviruses.* Proc Natl Acad Sci U S A, 1979. **76**(12): p. 6606-10.
5. Garnett, C.T., et al., *Latent species C adenoviruses in human tonsil tissues.* J Virol, 2009. **83**(6): p. 2417-28.
6. McNees, A.L., C.T. Garnett, and L.R. Gooding, *The adenovirus E3 RID complex protects some cultured human T and B lymphocytes from Fas-induced apoptosis.* J Virol, 2002. **76**(19): p. 9716-23.
7. Farina, S.F., et al., *Replication-defective vector based on a chimpanzee adenovirus.* J Virol, 2001. **75**(23): p. 11603-13.
8. Vogels, R., et al., *Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity.* J Virol, 2003. **77**(15): p. 8263-71.
9. Wickham, T.J., et al., *Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment.* Cell, 1993. **73**(2): p. 309-19.

10. Wickham, T.J., M.E. Carrion, and I. Kovesdi, *Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs.* Gene Ther, 1995. **2**(10): p. 750-6.

11. Wickham, T.J., et al., *Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization.* J Cell Biol, 1994. **127**(1): p. 257-64.

12. Bergelson, J.M., et al., *Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5.* Science, 1997. **275**(5304): p. 1320-3.

13. Kojaoghlanian, T., P. Flomenberg, and M.S. Horwitz, *The impact of adenovirus infection on the immunocompromised host.* Rev Med Virol, 2003. **13**(3): p. 155-71.

14. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

15. Morelli, A.E., et al., *Recombinant adenovirus induces maturation of dendritic cells via an NF-kappaB-dependent pathway.* J Virol, 2000. **74**(20): p. 9617-28.

16. Schnell, M.A., et al., *Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors.* Mol Ther, 2001. **3**(5 Pt 1): p. 708-22.

17. Zhang, Y., et al., *Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages.* Mol Ther, 2001. **3**(5 Pt 1): p. 697-707.

18. Horowitz, M., *Adenoviruses*, in *Fields Virology*, D.e.a. Knipe, Editor. 2001, Lippincott-Williams & Wilkins: Philadelphia. p. 2301-2326.

19. Sumida, S.M., et al., *Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein.* J Immunol, 2005. **174**(11): p. 7179-85.

20. Wohlfart, C., *Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms.* J Virol, 1988. **62**(7): p. 2321-8.

21. Hong, S.S., et al., *Identification of adenovirus (ad) penton base neutralizing epitopes by use of sera from patients who had received conditionally replicative ad (addl1520) for treatment of liver tumors.* J Virol, 2003. **77**(19): p. 10366-75.

22. Casimiro, D.R., et al., *Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene.* J Virol, 2003. **77**(11): p. 6305-13.

23. Xiang, Z.Q., et al., *A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier.* Virology, 1996. **219**(1): p. 220-7.

24. Baer, G.M., R.C. Brooks, and C.M. Foggin, *Oral vaccination of dogs fed canine adenovirus in baits.* Am J Vet Res, 1989. **50**(6): p. 836-7.

25. Zhou, D., et al., *A chimpanzee-origin adenovirus vector expressing the rabies virus glycoprotein as an oral vaccine against inhalation infection with rabies virus.* Mol Ther, 2006. **14**(5): p. 662-72.

26. Kumari, T.V., et al., *Prevalence of antibodies against herpes simplex and adenovirus in oral and cervical cancer patients--a preliminary report.* Indian J Med Res, 1982. **75**: p. 590-2.

27. Charlton, K.M., et al., *Oral rabies vaccination of skunks and foxes with a recombinant human adenovirus vaccine.* Arch Virol, 1992. **123**(1-2): p. 169-79.

28. Hammond, J.M., et al., *Oral and sub-cutaneous vaccination of commercial pigs with a recombinant porcine adenovirus expressing the classical swine fever virus gp55 gene.* Arch Virol, 2001. **146**(9): p. 1787-93.

29. Sharpe, S., et al., *Single oral immunization with replication deficient recombinant adenovirus elicits long-lived transgene-specific cellular and humoral immune responses.* Virology, 2002. **293**(2): p. 210-6.

30. Fitzgerald, J.C., et al., *A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag.* J Immunol, 2003. **170**(3): p. 1416-22.

31. Casimiro, D.R., et al., *Vaccine-induced immunity in baboons by using DNA and replication-incompetent adenovirus type 5 vectors expressing a human immunodeficiency virus type 1 gag gene.* J Virol, 2003. **77**(13): p. 7663-8.

32. Hoogstraal, H., *The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa.* J Med Entomol, 1979. **15**(4): p. 307-417.

33. Ergonul, O., *Crimean-Congo haemorrhagic fever.* Lancet Infect Dis, 2006. **6**(4): p. 203-14.

34. Morikawa, S., M. Saijo, and I. Kurane, *Recent progress in molecular biology of Crimean-Congo hemorrhagic fever.* Comp Immunol Microbiol Infect Dis, 2007. **30**(5-6): p. 375-89.

35. Shepherd, A.J., et al., *Field and laboratory investigation of Crimean-Congo haemorrhagic fever virus (Nairovirus, family Bunyaviridae) infection in birds.* Trans R Soc Trop Med Hyg, 1987. **81**(6): p. 1004-7.

36. Mardani, M. and M. Keshtkar-Jahromi, *Crimean-Congo hemorrhagic fever.* Arch Iran Med, 2007. **10**(2): p. 204-14.

37. Simpson, D.I., et al., *Congo virus: a hitherto undescribed virus occurring in Africa. I. Human isolations--clinical notes.* East Afr Med J, 1967. **44**(2): p. 86-92.

38. Woodall, J.P., M.C. Williams, and D.I. Simpson, *Congo virus: a hitherto undescribed virus occurring in Africa. II. Identification studies.* East Afr Med J, 1967. **44**(2): p. 93-8.

39. Begum, F., C.L. Wisseman, Jr., and J. Casals, *Tick-borne viruses of West Pakistan. 3. Dera Ghazi Khan virus, a new agent isolated from Hyalomma dromedarii ticks in the D. G. Khan District of West Pakistan.* Am J Epidemiol, 1970. **92**(3): p. 195-6.

40. Begum, F., C.L. Wisseman, Jr., and J. Casals, *Tick-borne viruses of West Pakistan. IV. Viruses similar to or identical with, Crimean hemorrhagic fever (Congo-Semunya), Wad Medani and Pak Argas 461 isolated from ticks of the Changa Manga Forest, Lahore District, and of Hunza, Gilgit Agency, W. Pakistan.* Am J Epidemiol, 1970. **92**(3): p. 197-202.

41. Begum, F., C.L. Wisseman, Jr., and J. Casals, *Tick-borne viruses of West Pakistan. II. Hazara virus, a new agent isolated from Ixodes redikorzevi ticks from the Kaghan Valley, W. Pakistan.* Am J Epidemiol, 1970. **92**(3): p. 192-4.

42. Begum, F., C.L. Wisseman, Jr., and R. Traub, *Tick-borne viruses of West Pakistan. I. Isolation and general characteristics.* Am J Epidemiol, 1970. **92**(3): p. 180-91.

43. Casals, J., *Antigenic similarity between the virus causing Crimean hemorrhagic fever and Congo virus.* Proc Soc Exp Biol Med, 1969. **131**(1): p. 233-6.

44. Swanepoel, R., et al., *Epidemiologic and clinical features of Crimean-Congo hemorrhagic fever in southern Africa.* Am J Trop Med Hyg, 1987. **36**(1): p. 120-32.

45. Shepherd, A.J., et al., *Experimental studies on the replication and transmission of Crimean-Congo hemorrhagic fever virus in some African tick species.* Am J Trop Med Hyg, 1989. **40**(3): p. 326-31.

46. Logan, T.M., et al., *Replication of Crimean-Congo hemorrhagic fever virus in four species of ixodid ticks (Acari) infected experimentally.* J Med Entomol, 1990. **27**(4): p. 537-42.

47. Whitehouse, C.A., *Crimean-Congo hemorrhagic fever.* Antiviral Res, 2004. **64**(3): p. 145-60.

48. Anagnostou, V. and A. Papa, *Evolution of Crimean-Congo Hemorrhagic Fever virus.* Infect Genet Evol, 2009. **9**(5): p. 948-54.

49. Papa, A., et al., *A case of Crimean-Congo haemorrhagic fever in Greece, June 2008.* Euro Surveill, 2008. **13**(33).

50. Papa, A., et al., *Genetic characterization of the M RNA segment of Crimean Congo hemorrhagic fever virus strains, China.* Emerg Infect Dis, 2002. **8**(1): p. 50-3.

51. Ma, B., C. Hang, and A. Papa, *[Sequencing and comparative analysis of the complete glycoprotein gene of three Crimean-Congo hemorrhagic fever virus*

Chinese isolates]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 2001. **15**(2): p. 105-11.

52. Chinikar, S., et al., *Genetic analysis of Crimean-congo hemorrhagic fever virus in Iran.* J Med Virol, 2004. **73**(3): p. 404-11.

53. Honig, J.E., J.C. Osborne, and S.T. Nichol, *The high genetic variation of viruses of the genus Nairovirus reflects the diversity of their predominant tick hosts.* Virology, 2004. **318**(1): p. 10-6.

54. Zeller, H.G., J.P. Cornet, and J.L. Camicas, *Experimental transmission of Crimean-Congo hemorrhagic fever virus by west African wild ground-feeding birds to Hyalomma marginatum rufipes ticks.* Am J Trop Med Hyg, 1994. **50**(6): p. 676-81.

55. Swanepoel, R., et al., *Experimental infection of ostriches with Crimean-Congo haemorrhagic fever virus.* Epidemiol Infect, 1998. **121**(2): p. 427-32.

56. Dohm, D.J., et al., *Transmission of Crimean-Congo hemorrhagic fever virus by Hyalomma impeltatum (Acari:Ixodidae) after experimental infection.* J Med Entomol, 1996. **33**(5): p. 848-51.

57. Gordon, S.W., K.J. Linthicum, and J.R. Moulton, *Transmission of Crimean-Congo hemorrhagic fever virus in two species of Hyalomma ticks from infected adults to cofeeding immature forms.* Am J Trop Med Hyg, 1993. **48**(4): p. 576-80.

58. Logan, T.M., et al., *Experimental transmission of Crimean-Congo hemorrhagic fever virus by Hyalomma truncatum Koch.* Am J Trop Med Hyg, 1989. **40**(2): p. 207-12.

59. Gonzalez, J.P., et al., *Sexual and transovarian transmission of Crimean-Congo haemorrhagic fever virus in Hyalomma truncatum ticks.* Res Virol, 1992. **143**(1): p. 23-8.

60. Wilson, M.L., et al., *Transmission of Crimean-Congo haemorrhagic fever virus from experimentally infected sheep to Hyalomma truncatum ticks.* Res Virol, 1991. **142**(5): p. 395-404.

61. Shepherd, A.J., et al., *Antibody to Crimean-Congo hemorrhagic fever virus in wild mammals from southern Africa.* Am J Trop Med Hyg, 1987. **36**(1): p. 133-42.

62. Schmaljohn, C.S., *Bunyaviridae: the viruses and their replication*. 4th Edition ed. Fields Virology, ed. D. Howley. 2001, Philadelphia, PA: Lippincott-Raven.

63. Andersson, I., et al., *Role of actin filaments in targeting of Crimean Congo hemorrhagic fever virus nucleocapsid protein to perinuclear regions of mammalian cells.* J Med Virol, 2004. **72**(1): p. 83-93.

64. Andersson, I., et al., *Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus.* J Virol, 2004. **78**(8): p. 4323-9.

65. Andersson, I., et al., *Type I interferon inhibits Crimean-Congo hemorrhagic fever virus in human target cells.* J Med Virol, 2006. **78**(2): p. 216-22.

66. Kinsella, E., et al., *Sequence determination of the Crimean-Congo hemorrhagic fever virus L segment.* Virology, 2004. **321**(1): p. 23-8.

67. Sanchez, A.J., M.J. Vincent, and S.T. Nichol, *Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus.* J Virol, 2002. **76**(14): p. 7263-75.

68. Sanchez, A.J., et al., *Crimean-congo hemorrhagic fever virus glycoprotein precursor is cleaved by Furin-like and SKI-1 proteases to generate a novel 38-kilodalton glycoprotein.* J Virol, 2006. **80**(1): p. 514-25.

69. Vincent, M.J., et al., *Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1.* J Virol, 2003. **77**(16): p. 8640-9.

70. Altamura, L.A., et al., *Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN that leads to generation of an NSM protein.* J Virol, 2007. **81**(12): p. 6632-42.

71. Erickson, B.R., et al., *N-linked glycosylation of Gn (but not Gc) is important for Crimean Congo hemorrhagic fever virus glycoprotein localization and transport.* Virology, 2007. **361**(2): p. 348-55.

72. Burt, F.J., et al., *Genetic relationship in southern African Crimean-Congo haemorrhagic fever virus isolates: evidence for occurrence of reassortment.* Epidemiol Infect, 2009. **137**(9): p. 1302-8.

73. Shepherd, A.J., et al., *Viraemic transmission of Crimean-Congo haemorrhagic fever virus to ticks.* Epidemiol Infect, 1991. **106**(2): p. 373-82.

74. Saijo, M., et al., *Possible horizontal transmission of crimean-congo hemorrhagic Fever virus from a mother to her child.* Jpn J Infect Dis, 2004. **57**(2): p. 55-7.

75. Harxhi, A., et al., *Crimean-Congo hemorrhagic fever: a case of nosocomial transmission.* Infection, 2005. **33**(4): p. 295-6.

76. Aradaib, I.E., et al., *Nosocomial outbreak of Crimean-Congo hemorrhagic fever, Sudan.* Emerg Infect Dis. **16**(5): p. 837-9.

77. Mardani, M., et al., *Crimean-Congo hemorrhagic fever virus as a nosocomial pathogen in Iran.* Am J Trop Med Hyg, 2009. **81**(4): p. 675-8.

78. Gurbuz, Y., et al., *A case of nosocomial transmission of Crimean-Congo hemorrhagic fever from patient to patient.* Int J Infect Dis, 2009. **13**(3): p. e105-7.

79. Suleiman, M.N., et al., *Congo/Crimean haemorrhagic fever in Dubai. An outbreak at the Rashid Hospital.* Lancet, 1980. **2**(8201): p. 939-41.

80. el-Azazy, O.M. and E.M. Scrimgeour, *Crimean-Congo haemorrhagic fever virus infection in the western province of Saudi Arabia.* Trans R Soc Trop Med Hyg, 1997. **91**(3): p. 275-8.

81. Saluzzo, J.F., et al., *Haemorrhagic fever caused by Crimean Congo haemorrhagic fever virus in Mauritania.* Trans R Soc Trop Med Hyg, 1985. **79**(2): p. 268.

82. Hassanein, K.M., O.M. el-Azazy, and H.M. Yousef, *Detection of Crimean-Congo haemorrhagic fever virus antibodies in humans and imported livestock in Saudi Arabia.* Trans R Soc Trop Med Hyg, 1997. **91**(5): p. 536-7.

83. Swanepoel, R., et al., *The clinical pathology of Crimean-Congo hemorrhagic fever.* Rev Infect Dis, 1989. **11 Suppl 4**: p. S794-800.

84. Schwarz, T.F., H. Nsanze, and A.M. Ameen, *Clinical features of Crimean-Congo haemorrhagic fever in the United Arab Emirates.* Infection, 1997. **25**(6): p. 364-7.

85. Ergonul, O., et al., *Characteristics of patients with Crimean-Congo hemorrhagic fever in a recent outbreak in Turkey and impact of oral ribavirin therapy.* Clin Infect Dis, 2004. **39**(2): p. 284-7.

86. Karti, S.S., et al., *Crimean-Congo hemorrhagic fever in Turkey.* Emerg Infect Dis, 2004. **10**(8): p. 1379-84.

87. Ergonul, O., et al., *Analysis of risk-factors among patients with Crimean-Congo haemorrhagic fever virus infection: severity criteria revisited.* Clin Microbiol Infect, 2006. **12**(6): p. 551-4.

88. Burt, F.J., et al., *Serodiagnosis of Crimean-Congo haemorrhagic fever.* Epidemiol Infect, 1994. **113**(3): p. 551-62.

89. Feldmann, H., et al., *Ebola virus: from discovery to vaccine.* Nat Rev Immunol, 2003. **3**(8): p. 677-85.

90. Geisbert, T.W. and P.B. Jahrling, *Exotic emerging viral diseases: progress and challenges.* Nat Med, 2004. **10**(12 Suppl): p. S110-21.

91. Schnittler, H.J. and H. Feldmann, *Viral hemorrhagic fever--a vascular disease?* Thromb Haemost, 2003. **89**(6): p. 967-72.

92. Schnittler, H.J., et al., *Replication of Marburg virus in human endothelial cells. A possible mechanism for the development of viral hemorrhagic disease.* J Clin Invest, 1993. **91**(4): p. 1301-9.

93. Sonmez, M., et al., *Plasma activity of thrombin activatable fibrinolysis inhibitor in Crimean-Congo hemorrhagic fever.* J Infect, 2007. **55**(2): p. 184-7.

94. Burt, F.J., et al., *Immunohistochemical and in situ localization of Crimean-Congo hemorrhagic fever (CCHF) virus in human tissues and implications for CCHF pathogenesis.* Arch Pathol Lab Med, 1997. **121**(8): p. 839-46.

95. Yilmaz, M., et al., *Peripheral blood natural killer cells in Crimean-Congo hemorrhagic fever.* J Clin Virol, 2008. **42**(4): p. 415-7.

96. Ergonul, O., et al., *Evaluation of serum levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-alpha in patients with Crimean-Congo hemorrhagic fever.* J Infect Dis, 2006. **193**(7): p. 941-4.

97. Papa, A., et al., *Cytokine levels in Crimean-Congo hemorrhagic fever.* J Clin Virol, 2006. **36**(4): p. 272-6.

98. Akinci, E., et al., *Analysis of lymphocyte subgroups in Crimean-Congo hemorrhagic fever.* Int J Infect Dis, 2009. **13**(5): p. 560-3.

99. Blackburn, N.K., et al., *Preparation and use of monoclonal antibodies for identifying Crimean-Congo hemorrhagic fever virus.* Am J Trop Med Hyg, 1987. **37**(2): p. 392-7.

100. Shepherd, A.J., et al., *Comparison of methods for isolation and titration of Crimean-Congo hemorrhagic fever virus.* J Clin Microbiol, 1986. **24**(4): p. 654-6.

101. Shepherd, A.J., R. Swanepoel, and P.A. Leman, *Antibody response in Crimean-Congo hemorrhagic fever.* Rev Infect Dis, 1989. **11 Suppl 4**: p. S801-6.

102. Watts, D.M., et al., *Inhibition of Crimean-Congo hemorrhagic fever viral infectivity yields in vitro by ribavirin.* Am J Trop Med Hyg, 1989. **41**(5): p. 581-5.

103. Tignor, G.H. and C.A. Hanham, *Ribavirin efficacy in an in vivo model of Crimean-Congo hemorrhagic fever virus (CCHF) infection.* Antiviral Res, 1993. **22**(4): p. 309-25.

104. Paragas, J., et al., *A simple assay for determining antiviral activity against Crimean-Congo hemorrhagic fever virus.* Antiviral Res, 2004. **62**(1): p. 21-5.

105. Andersson, I., et al., *Crimean-Congo hemorrhagic fever virus delays activation of the innate immune response.* J Med Virol, 2008. **80**(8): p. 1397-404.

106. Papa, A., et al., *Genetic detection and isolation of crimean-congo hemorrhagic fever virus, Kosovo, Yugoslavia.* Emerg Infect Dis, 2002. **8**(8): p. 852-4.

107. Spik, K., et al., *Immunogenicity of combination DNA vaccines for Rift Valley fever virus, tick-borne encephalitis virus, Hantaan virus, and Crimean Congo hemorrhagic fever virus.* Vaccine, 2006. **24**(21): p. 4657-66.

108. Lo, M.K. and P.A. Rota, *The emergence of Nipah virus, a highly pathogenic paramyxovirus.* J Clin Virol, 2008. **43**(4): p. 396-400.

109. Wild, T.F., *Henipaviruses: a new family of emerging Paramyxoviruses.* Pathol Biol (Paris), 2009. **57**(2): p. 188-96.

110. Tamin, A., et al., *Functional properties of the fusion and attachment glycoproteins of Nipah virus.* Virology, 2002. **296**(1): p. 190-200.

111. Chua, K.B., *Nipah virus outbreak in Malaysia.* J Clin Virol, 2003. **26**(3): p. 265-75.

112. Olival, K.J. and P. Daszak, *The ecology of emerging neurotropic viruses.* J Neurovirol, 2005. **11**(5): p. 441-6.

113. Halpin, K. and B.A. Mungall, *Recent progress in henipavirus research.* Comp Immunol Microbiol Infect Dis, 2007. **30**(5-6): p. 287-307.

114. Eaton, B.T., et al., *Hendra and Nipah viruses: different and dangerous.* Nat Rev Microbiol, 2006. **4**(1): p. 23-35.

115. Parashar, U.D., et al., *Case-control study of risk factors for human infection with a new zoonotic paramyxovirus, Nipah virus, during a 1998-1999 outbreak of severe encephalitis in Malaysia.* J Infect Dis, 2000. **181**(5): p. 1755-9.

116. Chua, K.B., et al., *The presence of Nipah virus in respiratory secretions and urine of patients during an outbreak of Nipah virus encephalitis in Malaysia.* J Infect, 2001. **42**(1): p. 40-3.

117. Middleton, D.J., et al., *Experimental Nipah virus infection in pigs and cats.* J Comp Pathol, 2002. **126**(2-3): p. 124-36.

118. Luby, S.P., et al., *Recurrent zoonotic transmission of Nipah virus into humans, Bangladesh, 2001-2007.* Emerg Infect Dis, 2009. **15**(8): p. 1229-35.

119. Hsu, V.P., et al., *Nipah virus encephalitis reemergence, Bangladesh.* Emerg Infect Dis, 2004. **10**(12): p. 2082-7.

120. *Nipah virus outbreak(s) in Bangladesh, January-April 2004.* Wkly Epidemiol Rec, 2004. **79**(17): p. 168-71.

121. Luby, S.P., et al., *Foodborne transmission of Nipah virus, Bangladesh.* Emerg Infect Dis, 2006. **12**(12): p. 1888-94.

122. Harit, A.K., et al., *Nipah/Hendra virus outbreak in Siliguri, West Bengal, India in 2001.* Indian J Med Res, 2006. **123**(4): p. 553-60.

123. Chadha, M.S., et al., *Nipah virus-associated encephalitis outbreak, Siliguri, India.* Emerg Infect Dis, 2006. **12**(2): p. 235-40.

124. Hossain, M.J., et al., *Clinical presentation of nipah virus infection in Bangladesh.* Clin Infect Dis, 2008. **46**(7): p. 977-84.

125. Gurley, E.S., et al., *Person-to-person transmission of Nipah virus in a Bangladeshi community.* Emerg Infect Dis, 2007. **13**(7): p. 1031-7.

126. Gurley, E.S., et al., *Risk of nosocomial transmission of Nipah virus in a Bangladesh hospital.* Infect Control Hosp Epidemiol, 2007. **28**(6): p. 740-2.

127. Harcourt, B.H., et al., *Genetic characterization of Nipah virus, Bangladesh, 2004.* Emerg Infect Dis, 2005. **11**(10): p. 1594-7.

128. Halpin, K., et al., *Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus.* J Gen Virol, 2000. **81**(Pt 8): p. 1927-32.

129. Halpin, K., et al., *Newly discovered viruses of flying foxes.* Vet Microbiol, 1999. **68**(1-2): p. 83-7.

130. Chua, K.B., et al., *Isolation of Nipah virus from Malaysian Island flying-foxes.* Microbes Infect, 2002. **4**(2): p. 145-51.

131. Yob, J.M., et al., *Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia.* Emerg Infect Dis, 2001. **7**(3): p. 439-41.

132. Field, H., et al., *The natural history of Hendra and Nipah viruses.* Microbes Infect, 2001. **3**(4): p. 307-14.

133. Stephens, D.S., et al., *Emerging and reemerging infectious diseases: a multidisciplinary perspective.* Am J Med Sci, 1998. **315**(2): p. 64-75.

134. Middleton, D.J., et al., *Experimental Nipah virus infection in pteropid bats (Pteropus poliocephalus).* J Comp Pathol, 2007. **136**(4): p. 266-72.

135. Bellini, W.J., et al., *Nipah virus: an emergent paramyxovirus causing severe encephalitis in humans.* J Neurovirol, 2005. **11**(5): p. 481-7.

136. Halpin, K., et al., *Emerging viruses: coming in on a wrinkled wing and a prayer.* Clin Infect Dis, 2007. **44**(5): p. 711-7.

137. Wang, L., et al., *Molecular biology of Hendra and Nipah viruses.* Microbes Infect, 2001. **3**(4): p. 279-87.

138. Weingartl, H.M., Y. Berhane, and M. Czub, *Animal models of henipavirus infection: a review.* Vet J, 2009. **181**(3): p. 211-20.

139. Yoneda, M., et al., *Establishment of a Nipah virus rescue system.* Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16508-13.

140. Lamb, R.K., D., *Paramyxoviridae: the viruses and their replication*. 4th ed. Fields Virology, ed. D.e.a. Knipe. 2001, Washington, DC: Lipincott, Williams and Williams. 1305-1340.

141. Wang, L.F., et al., *The exceptionally large genome of Hendra virus: support for creation of a new genus within the family Paramyxoviridae.* J Virol, 2000. **74**(21): p. 9972-9.

142. Harcourt, B.H., et al., *Molecular characterization of Nipah virus, a newly emergent paramyxovirus.* Virology, 2000. **271**(2): p. 334-49.

143. Harcourt, B.H., et al., *Molecular characterization of the polymerase gene and genomic termini of Nipah virus.* Virology, 2001. **287**(1): p. 192-201.

144. Lo, M.K., et al., *Determination of the henipavirus phosphoprotein gene mRNA editing frequencies and detection of the C, V and W proteins of Nipah virus in virus-infected cells.* J Gen Virol, 2009. **90**(Pt 2): p. 398-404.

145. Rodriguez, J.J., J.P. Parisien, and C.M. Horvath, *Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation.* J Virol, 2002. **76**(22): p. 11476-83.

146. Ciancanelli, M.J., et al., *Nipah virus sequesters inactive STAT1 in the nucleus via a P gene-encoded mechanism.* J Virol, 2009. **83**(16): p. 7828-41.

147. Kulkarni, S., et al., *Nipah virus edits its P gene at high frequency to express the V and W proteins.* J Virol, 2009. **83**(8): p. 3982-7.

148. Shaw, M.L., et al., *Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways.* J Virol, 2005. **79**(10): p. 6078-88.

149. Shaw, M.L., et al., *Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively.* J Virol, 2004. **78**(11): p. 5633-41.

150. Rodriguez, J.J. and C.M. Horvath, *Host evasion by emerging paramyxoviruses: Hendra virus and Nipah virus v proteins inhibit interferon signaling.* Viral Immunol, 2004. **17**(2): p. 210-9.

151. Rodriguez, J.J., C.D. Cruz, and C.M. Horvath, *Identification of the nuclear export signal and STAT-binding domains of the Nipah virus V protein reveals mechanisms underlying interferon evasion.* J Virol, 2004. **78**(10): p. 5358-67.

152. Wright, P.J., G. Crameri, and B.T. Eaton, *RNA synthesis during infection by Hendra virus: an examination by quantitative real-time PCR of RNA accumulation, the effect of ribavirin and the attenuation of transcription.* Arch Virol, 2005. **150**(3): p. 521-32.

153. Guillaume, V., et al., *Evidence of a potential receptor-binding site on the Nipah virus G protein (NiV-G): identification of globular head residues with a role in fusion promotion and their localization on an NiV-G structural model.* J Virol, 2006. **80**(15): p. 7546-54.

154. Negrete, O.A., et al., *Single amino acid changes in the Nipah and Hendra virus attachment glycoproteins distinguish ephrinB2 from ephrinB3 usage.* J Virol, 2007. **81**(19): p. 10804-14.

155. Bossart, K.N., et al., *Functional expression and membrane fusion tropism of the envelope glycoproteins of Hendra virus.* Virology, 2001. **290**(1): p. 121-35.

156. Bossart, K.N., et al., *Membrane fusion tropism and heterotypic functional activities of the Nipah virus and Hendra virus envelope glycoproteins.* J Virol, 2002. **76**(22): p. 11186-98.

157. Bossart, K.N. and C.C. Broder, *Viral glycoprotein-mediated cell fusion assays using vaccinia virus vectors.* Methods Mol Biol, 2004. **269**: p. 309-32.

158. Bossart, K.N., et al., *Receptor binding, fusion inhibition, and induction of cross-reactive neutralizing antibodies by a soluble G glycoprotein of Hendra virus.* J Virol, 2005. **79**(11): p. 6690-702.

159. Aguilar, H.C., et al., *A novel receptor-induced activation site in the Nipah virus attachment glycoprotein (G) involved in triggering the fusion glycoprotein (F).* J Biol Chem, 2009. **284**(3): p. 1628-35.

160. Diederich, S., et al., *The nipah virus fusion protein is cleaved within the endosomal compartment.* J Biol Chem, 2005. **280**(33): p. 29899-903.

161. Scheid, A. and P.W. Choppin, *Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus.* Virology, 1974. **57**(2): p. 475-90.

162. Molloy, S.S., et al., *Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen.* J Biol Chem, 1992. **267**(23): p. 16396-402.

163. Vey, M., et al., *Maturation of the trans-Golgi network protease furin: compartmentalization of propeptide removal, substrate cleavage, and COOH-terminal truncation.* J Cell Biol, 1994. **127**(6 Pt 2): p. 1829-42.

164. Michalski, W.P., et al., *The cleavage activation and sites of glycosylation in the fusion protein of Hendra virus.* Virus Res, 2000. **69**(2): p. 83-93.

165. Moll, M., et al., *Ubiquitous activation of the Nipah virus fusion protein does not require a basic amino acid at the cleavage site.* J Virol, 2004. **78**(18): p. 9705-12.

166. Vogt, C., et al., *Endocytosis of the Nipah virus glycoproteins.* J Virol, 2005. **79**(6): p. 3865-72.

167. Pager, C.T., M.A. Wurth, and R.E. Dutch, *Subcellular localization and calcium and pH requirements for proteolytic processing of the Hendra virus fusion protein.* J Virol, 2004. **78**(17): p. 9154-63.

168. Aguilar, H.C., et al., *Polybasic KKR motif in the cytoplasmic tail of Nipah virus fusion protein modulates membrane fusion by inside-out signaling.* J Virol, 2007. **81**(9): p. 4520-32.

169. Pager, C.T. and R.E. Dutch, *Cathepsin L is involved in proteolytic processing of the Hendra virus fusion protein.* J Virol, 2005. **79**(20): p. 12714-20.

170. Pager, C.T., et al., *A mature and fusogenic form of the Nipah virus fusion protein requires proteolytic processing by cathepsin L.* Virology, 2006. **346**(2): p. 251-7.

171. Lou, Z., et al., *Crystal structures of Nipah and Hendra virus fusion core proteins.* Febs J, 2006. **273**(19): p. 4538-47.

172. Xu, Y., et al., *Crystallization and preliminary crystallographic analysis of the fusion core from two new zoonotic paramyxoviruses, Nipah virus and Hendra virus.* Acta Crystallogr D Biol Crystallogr, 2004. **60**(Pt 6): p. 1161-4.

173. Bossart, K.N., et al., *Inhibition of Henipavirus fusion and infection by heptad-derived peptides of the Nipah virus fusion glycoprotein.* Virol J, 2005. **2**: p. 57.

174. Aguilar, H.C., et al., *N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry.* J Virol, 2006. **80**(10): p. 4878-89.

175. Reynes, J.M., et al., *Nipah virus in Lyle's flying foxes, Cambodia.* Emerg Infect Dis, 2005. **11**(7): p. 1042-7.

176. Tan, C.T. and K.S. Tan, *Nosocomial transmissibility of Nipah virus.* J Infect Dis, 2001. **184**(10): p. 1367.

177. Goh, K.J., et al., *Clinical features of Nipah virus encephalitis among pig farmers in Malaysia.* N Engl J Med, 2000. **342**(17): p. 1229-35.

178. Paton, N.I., et al., *Outbreak of Nipah-virus infection among abattoir workers in Singapore.* Lancet, 1999. **354**(9186): p. 1253-6.

179. Tan, C.T., et al., *Relapsed and late-onset Nipah encephalitis.* Ann Neurol, 2002. **51**(6): p. 703-8.

180. Sarji, S.A., et al., *MR imaging features of Nipah encephalitis.* AJR Am J Roentgenol, 2000. **175**(2): p. 437-42.

181. Sejvar, J.J., et al., *Long-term neurological and functional outcome in Nipah virus infection.* Ann Neurol, 2007. **62**(3): p. 235-42.

182. Maisner, A., J. Neufeld, and H. Weingartl, *Organ- and endotheliotropism of Nipah virus infections in vivo and in vitro.* Thromb Haemost, 2009. **102**(6): p. 1014-23.

183. Hagmaier, K., et al., *A single amino acid substitution in the V protein of Nipah virus alters its ability to block interferon signalling in cells from different species.* J Gen Virol, 2006. **87**(Pt 12): p. 3649-53.

184. Chong, H.T., et al., *Treatment of acute Nipah encephalitis with ribavirin.* Ann Neurol, 2001. **49**(6): p. 810-3.

185. Freiberg, A.N., et al., *Combined chloroquine and ribavirin treatment does not prevent death in a hamster model of Nipah and Hendra virus infection.* J Gen Virol. **91**(Pt 3): p. 765-72.

186. Guillaume, V., et al., *Antibody prophylaxis and therapy against Nipah virus infection in hamsters.* J Virol, 2006. **80**(4): p. 1972-8.

187. Guillaume, V., et al., *Nipah virus: vaccination and passive protection studies in a hamster model.* J Virol, 2004. **78**(2): p. 834-40.

188. Bossart, K.N. and C.C. Broder, *Developments towards effective treatments for Nipah and Hendra virus infection.* Expert Rev Anti Infect Ther, 2006. **4**(1): p. 43-55.

189. Sutter, G. and C. Staib, *Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery.* Curr Drug Targets Infect Disord, 2003. **3**(3): p. 263-71.

190. Patel, A., et al., *Mucosal delivery of adenovirus-based vaccine protects against Ebola virus infection in mice.* J Infect Dis, 2007. **196 Suppl 2**: p. S413-20.

191. Patel, A., et al., *Evaluation of conserved and variable influenza antigens for immunization against different isolates of H5N1 viruses.* Vaccine, 2009. **27**(23): p. 3083-9.

192. Burnette, W.N., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.* Anal Biochem, 1981. **112**(2): p. 195-203.

193. Richardson, J.S., et al., *Enhanced protection against Ebola virus mediated by an improved adenovirus-based vaccine.* PLoS One, 2009. **4**(4): p. e5308.

194. Croyle, M.A., et al., *Nasal delivery of an adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves the immune response in mice.* PLoS One, 2008. **3**(10): p. e3548.

195. Richardson, S.M., et al., *GeneDesign: rapid, automated design of multikilobase synthetic genes.* Genome Res, 2006. **16**(4): p. 550-6.

196. Neves, F.O., et al., *Overexpression of a synthetic gene encoding human alpha interferon in Escherichia coli.* Protein Expr Purif, 2004. **35**(2): p. 353-9.

197. Patterson, S.S., et al., *Codon optimization of bacterial luciferase (lux) for expression in mammalian cells.* J Ind Microbiol Biotechnol, 2005. **32**(3): p. 115-23.

198. Rouillard, J.M., et al., *Gene2Oligo: oligonucleotide design for in vitro gene synthesis.* Nucleic Acids Res, 2004. **32**(Web Server issue): p. W176-80.

199. Stemmer, W.P., et al., *Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides.* Gene, 1995. **164**(1): p. 49-53.

200. Hoover, D.M. and J. Lubkowski, *DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis.* Nucleic Acids Res, 2002. **30**(10): p. e43.

201. Mount, S.M., *A catalogue of splice junction sequences.* Nucleic Acids Res, 1982. **10**(2): p. 459-72.

202. Casimiro, D.R., et al., *Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag.* J Virol, 2005. **79**(24): p. 15547-55.

203. Goonetilleke, N., et al., *Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes.* J Virol, 2006. **80**(10): p. 4717-28.

204. Harari, A., et al., *An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses.* J Exp Med, 2008. **205**(1): p. 63-77.

205. Lemckert, A.A., et al., *Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity.* J Virol, 2005. **79**(15): p. 9694-701.

206. Boussif, O., M.A. Zanta, and J.P. Behr, *Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold.* Gene Ther, 1996. **3**(12): p. 1074-80.

207. Holman, D.H., et al., *A complex adenovirus-vectored vaccine against Rift Valley fever virus protects mice against lethal infection in the presence of preexisting vector immunity.* Clin Vaccine Immunol, 2009. **16**(11): p. 1624-32.

Appendix A Media Formulations

10X Gel Loading Buffer

20% Glycerol
0.1 M disodium EDTA, pH=8
1% SDS
0.25% Bromophenol Blue
0.25% Xylene Cyanol

TAE Buffer

93.05 g EDTA disodium salt
500 mL of deionized water, pH adjusted to 8.0 using NaOH

Cesium Chloride Gradients

Light cesium chloride

22.39g of cesium chloride
77.61 mL of 10mMTris-Cl pH8.0. Filter sterilize.

Heavy cesium chloride

42.23g of cesium chloride
57.77ml of 10mM Tris-Cl pH 8.0. Filter sterilize.

Radioimmunoprecipitation (RIPA) Buffer

10 mL of TritonX-100
10 g lauryl sulfate
5 mL of 10% SDS
30 mL of 5M NaCl
20 mL of 1M Tris, pH 7.7
20 mL of 0.5M EDTA pH 8.0

5x SDS Gel Loading Buffer

50 mM Tris-HCl pH 6.8
100 mM dithiothreitol
2% (w/v) electrophoresis grade SDS
0.1% bromophenol blue
10% (v/v) glycerol

Adenovirus Excipient

20 mM Tris
25 mM NaCl
2.5% Glycerol (w/v) pH 8.0

Appendix B Gene Sequences and Oligonucleotides

Nipah F Optimized Oligonucleotides

R0	TTATCCAGGATCACGACCAT
F0	ATGGTCGTGATCCTGGATAAACGGTGCTACTGTAATCTGC
R20	AATCATCAGAATCAGGATCAGCAGATTACAGTAGCACCGT
F40	TGATCCTGATTCTGATGATTAGCGAGTGTAGCGTGGGGAT
R60	ACAGCTTCTCGTAATGCAGAATCCCCACGCTACACTCGCT
F80	TCTGCATTACGAGAAGCTGTCCAAAATTGGCCTGGTGAAG
R100	TTGTATTTCCGTGTCACCCCCTTCACCAGGCCAATTTTGG
F120	GGGGTGACACGGAAATACAAGATCAAATCCAATCCACTGA
R140	CTTAATGACGATGTCTTTAGTCAGTGGATTGGATTTGATC
F160	CTAAAGACATCGTCATTAAGATGATTCCAAATGTGTCCAA
R180	TCCCTGTGCATTGGCTCATATTGGACACATTTGGAATCAT
F200	TATGAGCCAATGCACAGGGAGCGTCATGGAAAATTATAAA
R220	AGGATTCCGTTCAGCCGGGTTTTATAATTTTCCATGACGC
F240	ACCCGGCTGAACGGAATCCTGACTCCTATTAAAGGAGCAC
R260	GTTGTTCTTGTAAATCTCCAGTGCTCCTTTAATAGGAGTC
F280	TGGAGATTTACAAGAACAACACACATGACCTGGTGGGCGA
R300	TCACCCCAGCCAGCCGCACATCGCCCACCAGGTCATGTGT
F320	TGTGCGGCTGGCTGGGGTGATCATGGCTGGAGTGGCTATC
R340	TGGGCGGCAGTAGCAATTCCGATAGCCACTCCAGCCATGA
F360	GGAATTGCTACTGCCGCCCAGATTACAGCTGGCGTGGCCC
R380	GTTCTTCATAGCCTCGTACAGGGCCACGCCAGCTGTAATC
F400	TGTACGAGGCTATGAAGAACGCTGATAACATTAACAAGCT
R420	TGGACTCGATGCTGCTCTTCAGCTTGTTAATGTTATCAGC
F440	GAAGAGCAGCATCGAGTCCACAAACGAGGCAGTGGTGAAG
R460	TTTTCGGCTGTCTCCTGCAGCTTCACCACTGCCTCGTTTG
F480	CTGCAGGAGACAGCCGAAAAGACCGTGTACGTCCTGACCG
R500	GTTAATGTAATCCTGCAGAGCGGTCAGGACGTACACGGTC
F520	CTCTGCAGGATTACATTAACACTAACCTGGTGCCCACTAT
R540	GCTTGCAGGAAATCTTATCGATAGTGGGCACCAGGTTAGT
F560	CGATAAGATTTCCTGCAAGCAAACTGAGCTGAGCCTGGAT
R580	AGGTACTTGCTCAGGGCCAGATCCAGGCTCAGCTCAGTTT
F600	CTGGCCCTGAGCAAGTACCTGTCCGATCTGCTGTTCGTCT
R620	GTCCTGCAGATTTGGCCCAAAGACGAACAGCAGATCGGAC
F640	TTGGGCCAAATCTGCAGGACCCCGTGAGCAACTCCATGAC
R660	CTTGGGAAATGGCCTGAATTGTCATGGAGTTGCTCACGGG
F680	AATTCAGGCCATTTCCCAAGCTTTCGGAGGCAACTACGAA
R700	CCCAGTGTCCGCAGCAGGGTTTCGTAGTTGCCTCCGAAAG
F720	ACCCTGCTGCGGACACTGGGCTATGCTACAGAGGATTTCG
R740	ATCGCTTTCCAGCAGATCGTCGAAATCCTCTGTAGCATAG
F760	ACGATCTGCTGGAAAGCGATAGCATCACTGGACAGATCAT
R780	AGCTGGACAGATCCACGTAGATGATCTGTCCAGTGATGCT
F800	CTACGTGGATCTGTCCAGCTACTATATCATCGTGCGGGTC

R820	TCAGTCAGAATTGGGAAATAGACCCGCACGATGATATAGT
F840	TATTTCCCAATTCTGACTGAAATTCAACAGGCCTACATCC
R860	GGACACGGGCAGCAGCTCCTGGATGTAGGCCTGTTGAATT
F880	AGGAGCTGCTGCCCGTGTCCTTCAACAATGATAATAGCGA
R900	TGGGCACGATGCTGATCCACTCGCTATTATCATTGTTGAA
F920	GTGGATCAGCATCGTGCCCAACTTCATTCTGGTCCGGAAC
R940	TCAATGTTGCTGATCAGGGTGTTCCGGACCAGAATGAAGT
F960	ACCCTGATCAGCAACATTGAAATTGGATTTTGCCTGATCA
R980	ACAGATCACGGACCGCTTAGTGATCAGGCAAAATCCAATT
F1000	CTAAGCGGTCCGTGATCTGTAATCAGGATTACGCTACCCC
R1020	CCCGCATGTTATTGGTGGTAGGGGTAGCGTAATCCTGATT
F1040	TACCACCAATAACATGCGGGAATGCCTGACCGGGAGCACC
R1060	AGCTCCCGTGGACACTTTTCGGTGCTCCCGGTCAGGCATT
F1080	GAAAAGTGTCCACGGGAGCTGGTGGTGAGCAGCCACGTCC
R1100	ATTGCTCAGTGCAAACCGAGGGACGTGGCTGCTCACCACC
F1120	CTCGGTTTGCACTGAGCAATGGCGTCCTGTTTGCTAACTG
R1140	ACTGGCAAGTCACGGAGATACAGTTAGCAAACAGGACGCC
F1160	TATCTCCGTGACTTGCCAGTGCCAGACCACAGGCCGGGCA
R1180	TGTTCGCCGCTCTGGGAGATTGCCCGGCCTGTGGTCTGGC
F1200	ATCTCCCAGAGCGGCGAACAGACCCTGCTGATGATCGATA
R1220	TGCGGTTGGGCAGGTAGTGTTATCGATCATCAGCAGGGTC
F1240	ACACTACCTGCCCAACCGCAGTGCTGGGCAATGTGATCAT
R1260	CCAGATACTTGCCCAGGGAGATGATCACATTGCCCAGCAC
F1280	CTCCCTGGGCAAGTATCTGGGCTCCGTGAACTACAACAGC
R1300	GGCCCAATGGCAATGCCTTCGCTGTTGTAGTTCACGGAGC
F1320	GAAGGCATTGCCATTGGGCCCCCTGTGTTCACCGACAAAG
R1340	GATCTGGGAGCTGATGTCGACTTTGTCGGTGAACACAGGG
F1360	TCGACATCAGCTCCCAGATCTCCTCCATGAACCAGTCCCT
R1380	TATAGTCTTTGGATTGCTGCAGGGACTGGTTCATGGAGGA
F1400	GCAGCAATCCAAAGACTATATTAAGGAGGCCCAGCGGCTG
R1420	GATGGGTTCACTGTGTCCAGCAGCCGCTGGGCCTCCTTAA
F1440	CTGGACACAGTGAACCCATCCCTGATCTCCATGCTGAGCA
R1460	CAGCACGTACAGAATAATCATGCTCAGCATGGAGATCAGG
F1480	TGATTATTCTGTACGTGCTGTCCATCGCCAGCCTGTGTAT
R1500	TGATAAATGTAATCAGGCCAATACACAGGCTGGCGATGGA
F1520	TGGCCTGATTACATTTATCAGCTTTATCATCGTGGAGAAG
R1540	CGGGAGTAGGTGTTCCGCTTCTTCTCCACGATGATAAAGC
F1560	AAGCGGAACACCTACTCCCGGCTGGAGGACCGGCGGGTCC
R1580	GTCCCCGCTGCTGGTAGGCCGGACCCGCCGGTCCTCCAGC
F1600	GGCCTACCAGCAGCGGGGACCTGTACTATATCGGCACATGA
R1620	TTCATGTGCCGATATAGTACAG

Nipah F Primers

NiF Forward	AACCTGTGACGCTAGCGCCGCCACCATGGTCGTGATCCTGGATAAA
NiF Reverse	GTCACAGGTTTTTAAATCATGTGCCGATATAGTACA

Nipah F Sequencing Primers

NiFFo	GCTAGCGCCGCCACCATG
NiFMi1	GAGCTGAGCCTGGATCTGGC
NiFMi2	GTGTCCACGGGAGCTGG
NiFRo	CGGACCCGCCGGTCCT

Nipah G Optimized Oligonucleotides

R0	CGAGCTCGTCACAGGTT
F0	AACCTGTGACGAGCTCGCCGCCACCATGCCTG
R17	CGCACTTTCTTGTTCTCTGCAGGCATGGTGGCGG
F32	CAGAGAACAAGAAAGTGCGGTTCGAGAATACTACATCCG
R51	GCTGGGAATCTTTCCCTTATCGGATGTAGTATTCTCGAAC
F71	ATAAGGGAAAGATTCCCAGCAAGGTGATTAAGAGCTACTATG
R91	TCTTCTTGATGTCCATTGTTCCATAGTAGCTCTTAATCACCTT
F113	GAACAATGGACATCAAGAAGATCAACGAAGGACTGCT
R134	CGCTCAGGATCTTGCTATCCAGCAGTCCTTCGTTGA
F150	GGATAGCAAGATCCTGAGCGCCTTCAACACTGTCATTGC
R170	GATGCTGCCCAGCAGTGCAATGACAGTGTTGAAGG
F189	ACTGCTGGGCAGCATCGTGATCATTGTGATGAACATTAT
R205	TCCGTGTATAATTCTGGATAATCATAATGTTCATCACAATGATCAC
F228	GATTATCCAGAATTATACACGGAGCACCGATAATCAAGCAG
R251	AGTGCGTCCTTGATGACTGCTTGATTATCGGTGC
F269	TCATCAAGGACGCACTGCAGGGCATCCAACA
R285	GCCAGTCCTTTGATCTGCTGTTGGATGCCCTGC
F300	GCAGATCAAAGGACTGGCCGACAAGATCGGAACAGA
R318	CTCACCTTAGGTCCGATCTCTGTTCCGATCTTGTCG
F336	GATCGGACCTAAGGTGAGCCTGATCGACACTTCCT
R354	TGGAATGGTGATGGTGGAGGAAGTGTCGATCAGG
F371	CCACCATCACCATTCCAGCAAACATTGGGCTGC
R388	GCTAATCTTGCTGCCCAGCAGCCCAATGTTTGC
F404	TGGGCAGCAAGATTAGCCAGAGCACTGCAAGCA
R421	CTCGTTCACATTCTCGTTAATGCTTGCAGTGCTCTG
F437	TTAACGAGAATGTGAACGAGAAGTGTAAGTTTACTCTGCC
R457	TTCATGGATCTTCAGAGGAGGCAGAGTAAACTTACACTT
F477	TCCTCTGAAGATCCATGAATGTAACATTTCCTGTCCCA
R496	CGGAAAGGCAGAGGATTGGGACAGGAAATGTTACA
F515	ATCCTCTGCCTTTCCGGGAGTACCGGCCTCA
R531	TGCTCACTCCTTCTGTTTGAGGCCGGTACTCC
F546	AACAGAAGGAGTGAGCAATCTGGTCGGACTGCC
R563	TCTTCTGCAGACAGATATTGTTAGGCAGTCCGACCAGAT
F579	TAACAATATCTGTCTGCAGAAGACATCCAACCAAATCCTGAA
R602	TATAGCTAATCAGCTTTGGCTTCAGGATTTGGTTGGATG
F621	GCCAAAGCTGATTAGCTATACCCTGCCAGTGGTGG
R641	AGGTGCCGGACTGGCCCACCACTGGCAGGG
F656	GCCAGTCCGGCACCTGCATCACCGACCCAC

R671	CGTCCATGGCCAGCAGTGGGTCGGTGATGC
F686	TGCTGGCCATGGACGAGGGGTACTTCGCCTA
R701	CGTTCCAGGTGGCTGTAGGCGAAGTACCCCT
F717	CAGCCACCTGGAACGGATCGGCAGCTGTAGC
R732	TTTGGACACGCCCCGGCTACAGCTGCCGATC
F748	CGGGGCGTGTCCAAACAACGGATCATTGGCG
R763	CCAGGACTTCGCCGACGCCAATGATCCGTTG
F779	TCGGCGAAGTCCTGGATCGGGGCGACGAGG
R794	CATGAACAGGCTAGGGACCTCGTCGCCCCGAT
F809	TCCCTAGCCTGTTCATGACCAACGTCTGGACAC
R826	TGTGTTGGGATTGGGAGGTGTCCAGACGTTGGT
F842	CTCCCAATCCCAACACAGTGTACCACTGCAGCG
R859	AACTCGTTGTTGTACACAGCGCTGCAGTGGTACAC
F875	CTGTGTACAACAACGAGTTCTACTATGTCCTGTGCGC
R894	CCCACAGTGCTCACTGCGCACAGGACATAGTAG
F912	AGTGAGCACTGTGGGAGACCCAATTCTGAACTCC
R927	TCCGCTCCAGTAGGTGGAGTTCAGAATTGGGTCT
F946	ACCTACTGGAGCGGATCCCTGATGATGACACG
R961	GGTTTCACGGCCAGCCGTGTCATCATCAGGGA
F978	GCTGGCCGTGAAACCTAAATCCAACGGCGGA
R993	GCTGATGTTGGTTATAGCCTCCGCCGTTGGATTTA
F1009	GGCTATAACCAACATCAGCTGGCACTGCGGAGCA
R1028	ACCGGCCTTTCTCAATGCTCCGCAGTGCCA
F1043	TTGAGAAAGGCCGGTACGACAAAGTGATGCCC
R1058	GCCGGAGGGTCCGTAGGGCATCACTTTGTCGT
F1075	TACGGACCCTCCGGCATTAAACAGGGAGACACACT
R1090	CCACGGCTGGAAAGTACAGTGTGTCTCCCTGTTTAAT
F1110	GTACTTTCCAGCCGTGGGCTTCCTGGTGCGGA
R1127	CGTTGTACTTGAACTCGGTCCGCACCAGGAAGC
F1142	CCGAGTTCAAGTACAACGACTCCAATTGTCCAATCAC
R1160	GCTTGGAGTACTGACACTTTGTGATTGGACAATTGGAGT
F1179	AAAGTGTCAGTACTCCAAGCCTGAAAACTGCCGGCT
R1199	GGCCGAATTCCCATGCTCAGCCGGCAGTTTTCAG
F1215	GAGCATGGGAATTCGGCCCAATAGCCACTACATCCTGC
R1233	TCAGCAGCCCGGACCGCAGGATGTAGTGGCTATTG
F1253	GGTCCGGGCTGCTGAAATACAACCTGAGCGACG
R1268	ACCTTAGGGTTCTCGCCGTCGCTCAGGTTGTATT
F1286	GCGAGAACCCTAAGGTGGTCTTCATCGAAATCAGC
R1302	GGACAGCCGCTGATCGCTGATTTCGATGAAGACC
F1321	GATCAGCGGCTGTCCATTGGAAGCCCTAGCA
R1336	CCAGGCTGTCATAGATCTTGCTAGGGCTTCCAAT
F1352	AGATCTATGACAGCCTGGGCCAGCCTGTGTTCTA
R1370	GGAGAAGGAGGCCTGATAGAACACAGGCTGGC
F1386	TCAGGCCTCCTTCTCCTGGGATACCATGATTAAGTTC
R1402	TGTCAGCACGTCGCCGAACTTAATCATGGTATCCCA
F1423	GGCGACGTGCTGACAGTGAACCCACTGGTGG
R1438	GTTGTTCCGCCAATTCACCACCAGTGGGTTCAC
F1454	TGAATTGGCGGAACAACACCGTGATCTCCCGG

R1471	CTGGCTTTGCCCAGGCCGGGAGATCACGGT
F1486	CCTGGGCAAAGCCAGTGTCCTCGGTTCAACA
R1501	ACAAATCTCGGGACAGGTGTTGAACCGAGGACA
F1517	CCTGTCCCGAGATTTGTTGGGAAGGAGTGTATAACG
R1534	CGATCAATCAGGAAAGCATCGTTATACACTCCTTCCCA
F1553	ATGCTTTCCTGATTGATCGGATTAATTGGATTTCCGCAG
R1572	GTTGGAATCCAGAAACACTCCTGCGGAAATCCAATTAATC
F1592	GAGTGTTTCTGGATTCCAACCAGACAGCCGAGAACC
R1612	CTTTAAACACGGTAAACACTGGGTTCTCGGCTGTCTG
F1628	CAGTGTTTACCGTGTTTAAAGATAACGAAATTCTGTACCGGG
R1649	CGGAGGCCAGCTGGGCCCGGTACAGAATTTCGTTAT
F1670	CCCAGCTGGCCTCCGAGGATACCAACGCTCAA
R1685	GGAAGCAATTAGTGATAGTCTTTTGAGCGTTGGTATCCT
F1702	AAGACTATCACTAATTGCTTCCTGCTGAAGAACAAGATTTGG
R1724	GACCAGGCTGATGCACCAAATCTTGTTCTTCAGCA
F1744	TGCATCAGCCTGGTCGAGATCTACGATACCGGC
R1759	GGCCGAATCACGTTATCGCCGGTATCGTAGATCTC
F1777	GATAACGTGATTCGGCCTAAGCTGTTCGCCGT
R1794	TACATTGCTCAGGAATCTTGACGGCGAACAGCTTA
F1809	CAAGATTCCTGAGCAATGTACATAAGCTAGCAACCTGTG
R1829	CACAGGTTGCTAGCTTATG

Nipah G Sequencing Primers

NiGNewSeqFo	AACCTGTGACGAGCTCGC
NiGNewSeqMi1	GCAATCTGGTCGGACTG
NiGNewSeqMi2	TGGCCGTGAAACCTAAATCC
NiGNewSeqMi3	CTGTCCATTGGAAGCCCTAG
NiGNewSeqMi4	CTCCGAGGATACCAACGCT

CCHF Optimized Oligonucleotides

R0	Tccgctttgcgccat
F0	atggcgcaaagcggATGCATATCTCCCTGATGTA
R15	AGGCACAGGATTGCGTACATCAGGGAGATATGCA
F34	CGCAATCCTGTGCCTGCAGCTGTGCGGCC
R49	CATGAGTCTCGCCCAGGCCGCACAGCTGC
F63	TGGGCGAGACTCATGGCTCCCATAACGAGACC
R78	TGTATCTGTCTTATTATGCCGGGTCTCGTTATGGGAGC
F95	CGGCATAATAAGACAGATACAATGACTACCCCCGGA
R116	CGCTGCTGGGATTGTCTCCGGGGGTAGTCAT
F131	GACAATCCCAGCAGCGAGCCCCCAGTGAGC
R147	GGTAATGCTCAGAGCTGTGCTCACTGGGGGCT
F161	ACAGCTCTGAGCATTACCCTGGACCCTAGCACC
R179	GAGTAGTGGGGGTCACGGTGCTAGGGTCCAG
F194	GTGACCCCCACTACTCCAGCATCCGGGCTG

R210 TCGCCGCTGCCCTCCAGCCCGGATGCTG
F224 GAGGGCAGCGGCGAAGTGTACACCAGCCC
R238 GGGATCCAGTTGTAATAGGTGGGCTGGTGTACACT
F253 ACCTATTACAACTGGATCCCTGCCACTGTCCGAGA
R273 CAGCTCGGGGGTTGTCTCGGACAGTGGCA
F288 CAACCCCCGAGCTGCCTGTCACCACAGGG
R302 GGACAGTGTGTCGGTCCCTGTGGTGACAGG
F317 ACCGACACACTGTCCGCTGGAGACGTGGAT
R332 GGTCTGGGTGGAGGGATCCACGTCTCCAGC
F347 CCCTCCACCCAGACCGCAGGCGGGACCAG
R362 GCACAGTGGGGGCGCTGGTCCCGCCTGC
F376 CGCCCCCACTGTGCGGACTAGCCTGCCCA
R390 GGTGTGGATGGGGAGTTGGGCAGGCTAGTCC
F405 ACTCCCCATCCACACCAAGCACTCCCCAGGA
R421 CGGGGTGATGGGTGTCCTGGGGAGTGCTT
F436 CACCCATCACCCCGTGCGGAATCTGCTGA
R450 GGGGCTTGTCACGCTCAGCAGATTCCGCA
F465 GCGTGACAAGCCCCGGGCCTGACGAGAC
R479 CCGGAAGGTGTGGATGTCTCGTCAGGCCC
F493 ATCCACACCTTCCGGCACTGGCAAAGAGTCCA
R508 GGGAGCTAGTTGCGCTGGACTCTTTGCCAGTG
F525 GCGCAACTAGCTCCCCTCATCCTGTGAGCAACC
R540 GAGGAGTTGGTGGCCGGTTGCTCACAGGATGAG
F558 GGCCACCAACTCCTCCAGCTACAGCCCAAGG
R573 GAATCGTTCTCAGTTGGGCCTTGGGCTGTAGCTG
F589 CCCAACTGAGAACGATTCCCACAACGCTACCGA
R607 GGCTCTCGGGGTGCTCGGTAGCGTTGTGG
F622 GCACCCCGAGAGCCTGACCCAAAGCGCA
R636 GTCATCAGTCCAGGAGTTGCGCTTTGGGTCA
F650 ACTCCTGGACTGATGACAAGCCCCACCCAGA
R667 CTGGGGGTGCACGATCTGGGTGGGGCTT
F681 TCGTGCACCCCCAGTCCGCAACTCCAATCA
R695 GGTGTCCTGCACGGTGATTGGAGTTGCGGA
F711 CCGTGCAGGACACCCACCCTTCCCCAACTA
R725 CCGCTTGCTCCGATTAGTTGGGGAAGGGTG
F741 ATCGGAGCAAGCGGAATCTGAAGATGGAGATTATCC
R755 CCTGGCTCAGGGTCAGGATAATCTCCATCTTCAGATT
F777 TGACCCTGAGCCAGGGCCTGAAGAAATACTATGGAA
R792 GCAGCCGCAGAATTTTTCCATAGTATTTCTTCAGGC
F813 AAATTCTGCGGCTGCTGCAGCTGACACTGG
R828 AGTCCTTCTGTGTCTTCTTCCAGTGTCAGCTGCA
F843 AAGAAGACACAGAAGGACTGCTGGAGTGGTGTAAGC
R862 CAGGCCCAGGTTCCGCTTACACCACTCCAGC
F879 GGAACCTGGGCCTGGATTGCGACGACACATT
R893 TCGATCCGTTTCTGGAAGAATGTGTCGTCGCAATC
F910 CTTCCAGAAACGGATCGAAGAATTCTTTATCACTGGGG
R928 CTCGTTGAAGTGTCCCTCCCCAGTGATAAAGAATTCT
F948 AGGGACACTTCAACGAGGTGCTGCAATTCCGGA
R965 TGGACAGAGTTCCAGGAGTCCGGAATTGCAGCAC
F981 CTCCTGGAACTCTGTCCACCACTGAGAGCACACCC

R999 GGTTGGCAGTCCGGCGGGTGTGCTCTCAGTGG
F1016 GCCGGACTGCCAACCGCCGAGCCCTTTAAATC
R1031 AGGAACCCTTTTGCAAAATAGGATTTAAAGGGCTCGGC
F1048 CTATTTTGCAAAAGGGTTCCTGTCCATTGACTCCGGGTA
R1069 GAATAACACTTAGCGGAGTAATACCCGGAGTCAATGGAC
F1087 TTACTCCGCTAAGTGTTATTCCGGAACCTCCAACAGCG
R1108 TGTTAATCAGCTGCAGGCCGCTGTTGGAGGTTCCG
F1125 GCCTGCAGCTGATTAACATTACCCGGCATAGCACC
R1143 GGGTGTCGACGATCCGGGTGCTATGCCGGGTAA
F1160 CGGATCGTCGACACCCCAGGCCCCAAGATTACTA
R1176 ATGCAATTAATGGTTTTCAGATTAGTAATCTTGGGGCCTG
F1194 ATCTGAAAACCATTAATTGCATCAACCTGAAGGCCAGC
R1216 CCCGGTGTTCCTTAAAAATGCTGGCCTTCAGGTTG
F1232 ATTTTTAAGGAACACCGGGAAGTGGAGATTAATGTGCTG
R1251 GCGACTTGGGGCAGCAGCACATTAATCTCCACTT
F1271 CTGCCCCAAGTCGCAGTGAACCTGTCCAACT
R1285 GCTTTTAATCACCACATGGCAGTTGGACAGGTTCACT
F1302 GCCATGTGGTGATTAAAAGCCACGTGTGTGACTATTCCC
R1322 GCCCCGTCGATGTCCAGGGAATAGTCACACACGTG
F1341 TGGACATCGACGGGGCCGTGCGGCTGCCTC
R1357 GCCCTCGTGATAGATGTGAGGCAGCCGCACG
F1371 ACATCTATCACGAGGGCGTGTTTATTCCCGGAACCTA
R1388 TTCTTGTCAATCACGATCTTATAGGTTCCGGGAATAAACAC
F1408 TAAGATCGTGATTGACAAGAAAAATAAGCTGAACGATCGG
R1429 TCGGTGAACAGTGTACACCGATCGTTCAGCTTATTT
F1448 TGTACACTGTTCACCGACTGTGTGATTAAAGGCCG
R1465 CCCTTTCCGGACTTCCCGGCCTTTAATCACACAG
F1483 GGAAGTCCGGAAAGGGCAGTCCGTCCTGCG
R1499 GAATCTCTGTCTTGTACTGCCGCAGGACGGACTG
F1513 GCAGTACAAGACAGAGATTCGGATCGGCAAGGCC
R1533 CCGGCTTCCTGTGGAGGCCTTGCCGATCC
F1547 TCCACAGGAAGCCGGCGGCTGCTGTCCGA
R1562 CGTCGCTTGGCTCCTCGGACAGCAGCCG
F1576 GGAGCCAAGCGACGACTGCATCAGCCGG
R1590 CCGCAGCAGTTGAGTCCGGCTGATGCAGT
F1604 ACTCAACTGCTGCGGACAGAGACCGCTGAAAT
R1619 CGTAGTTGTCCCCGTGGATTTCAGCGGTCTCTGT
F1636 CCACGGGGACAACTACGGAGGACCCGGCGACA
R1653 CTGCCATTACAAATTGTAATCTTGTCGCCGGGTCCTC
F1668 AGATTACAATTTGTAATGGCAGCACCATCGTCGACCA
R1690 CGCTTCCCAGCCGCTGGTCGACGATGGTG
F1705 GCGGCTGGGAAGCGAGCTGGGCTGTTATACC
R1719 CCGGACCCGATTGATGGTATAACAGCCCAGCT
F1736 ATCAATCGGGTCCGGTCCTTCAAACTGTGCGA
R1751 CCAGTGGCGCTGTTCTCGCACAGTTTGAAGGA
F1768 GAACAGCGCCACTGGCAAGAACTGTGAAATCGATAGC
R1783 CGACACTTCACAGGCACGCTATCGATTTCACAGTTCTTG
F1805 GTGCCTGTGAAGTGTCGGCAGGGATACTGTCTGC
R1822 CCCTCCTGGGTAATCCGCAGACAGTATCCCTGC
F1839 GGATTACCCAGGAGGGCCGGGGCCATGTGA

R1855 CCCCCGGCTCAGCTTCACATGGCCCCGG
F1869 AGCTGAGCCGGGGGTCCGAAGTGGTGCTG
R1883 TAGTGTCGCAAGCGTCCAGCACCACTTCGGA
F1898 GACGCTTGCGACACTAGCTGTGAGATCATGATCCC
R1914 TCGCCTGTTCCCTTGGGGATCATGATCTCACAGC
F1933 CAAGGGAACAGGCGATATCCTGGTGGACTGTTC
R1948 GTTGCTGCCCTCCGGAACAGTCCACCAGGATA
F1966 CGGAGGGCAGCAACACTTCCTGAAGGATAACCT
R1980 CAGCCCAGGTCGATCAGGTTATCCTTCAGGAAGT
F1999 GATCGACCTGGGCTGCCCCAAGATTCCTCTGC
R2014 GATAGCCATTTTGCCCAGCAGAGGAATCTTGGGG
F2031 TGGGCAAAATGGCTATCTACATTTGCCGGATGAGC
R2048 TGTGGTTTTTGGATGGTTGCTCATCCGGCAAATGTA
F2066 AACCATCCAAAAACCACAATGGCCTTTCTGTTTTGG
R2084 CACATACCCAAAGCTGAACCAAAACAGAAAGGCCAT
F2102 TTCAGCTTTGGGTATGTGATCACTTGCATCCTGTGTA
R2120 GCAGGTAGAAAATGGCCTTACACAGGATGCAAGTGAT
F2139 AGGCCATTTTCTACCTGCTGATCATCGTGGGAACT
R2157 AGCCGTTTGCCCAGAGTTCCCACGATGATCA
F2174 CTGGGCAAACGGCTGAAGCAGTACCGGGAA
R2188 CATGTTTGGGGCTTCAGTTCCCGGTACTGCTTC
F2204 CTGAAGCCCCAAACATGCACCATTTGTGAGACTACTC
R2221 CGTCAATGGCATTGACTGGAGTAGTCTCACAAATGGTG
F2241 CAGTCAATGCCATTGACGCCGAAATGCATGACCTG
R2259 ACAGATGTTGTAGGAACAATTCAGGTCATGCATTTCGG
F2276 AATTGTTCCTACAACATCTGTCCCTATTGTGCATCCCG
R2297 CCGTCGCTAGTCAGCCGGGATGCACAATAGGG
F2314 GCTGACTAGCGACGGACTGGCCCGGCACG
R2329 TTTGGGACACTGGATGACGTGCCGGGCCAGT
F2343 TCATCCAGTGTCCCAAACGGAAGGAAAAAGTCGAG
R2360 CAGGTACAGCTCTGTCTCCTCGACTTTTTCCTTCCG
F2378 GAGACAGAGCTGTACCTGAATCTGGAACGGATCCC
R2396 TCCGGACGACCCATGGGATCCGTTCCAGATT
F2413 ATGGGTCGTCCGGAAGCTGCTGCAGGTG
R2427 CTCCAGTGGATTCGCTCACCTGCAGCAGCT
F2441 AGCGAATCCACTGGAGTGGCTCTGAAACGGTC
R2457 ACGATCAGCCAGCTGGACCGTTTCAGAGCCA
F2473 CAGCTGGCTGATCGTCCTGCTGGTGCTGT
R2488 GACAGGGACACGGTAAACAGCACCAGCAGG
F2502 TTACCGTGTCCCTGTCCCCCGTGCAGTCCG
R2518 CCTGCCCGATAGGGGCGGACTGCACGGGG
F2532 CCCCTATCGGGCAGGGCAAGACTATCGAGGCC
R2547 TCCCGAGCCCGATAGGCCTCGATAGTCTTGC
F2564 TATCGGGCTCGGGAAGGGTACACCTCCATCT
R2578 CCCAGCACGAACAGACAGATGGAGGTGTACCCT
F2595 GTCTGTTCGTGCTGGGATCCATCCTGTTCATCGT
R2611 CCTTCATCAGGCAGCTGACGATGAACAGGATGGAT
F2629 CAGCTGCCTGATGAAGGGCCTGGTGGATAGCG
R2646 GGGAAGAAGGAATTTCCGACGCTATCCACCAGGC
F2661 TCGGAAATTCCTTCTTCCCTGGCCTGTCCATTTGC

R2680 TGGAGATGCTACAAGTCTTGCAAATGGACAGGCCA
F2696 AAGACTTGTAGCATCTCCAGCATTAATGGGTTCGAAATTG
R2715 GCAGTAGCATTTATGGGACTCAATTTCGAACCCATTAATGC
F2736 AGTCCCATAAATGCTACTGCTCCCTGTTTTGTTGTCCC
R2756 GGAACAATGCCGACAGTAGGGACAACAAAACAGGGA
F2774 TACTGTCGGCATTGTTCCACCGACAAGGAGATCC
R2792 GCTCAGGTGCAGTTTGTGGATCTCCTTGTCGGT
F2808 ACAAACTGCACCTGAGCATTTGTAAGAAGCGGAAAAAG
R2825 CATGACGTTGCTGCCCTTTTTCCGCTTCTTACAAAT
F2846 GGCAGCAACGTCATGCTGGCAGTCTGCAAGC
R2861 GCCCGAAAGCACATCAGCTTGCAGACTGCCAG
F2877 TGATGTGCTTTCGGGCAACCATGGAGGTGTCC
R2893 TGAACAGAGCCCGATTGGACACCTCCATGGTT
F2909 AATCGGGCTCTGTTCATTCGGAGCATCATCAACA
R2925 GCACAGCACGAAAGTAGTGTTGATGATGCTCCGAA
F2943 CTACTTTCGTGCTGTGCATCCTGATCCTGGCCG
R2960 GTGGAGACGACACAGACGGCCAGGATCAGGAT
F2976 TCTGTGTCGTCTCCACATCCGCCGTGGAGA
R2992 CGGGCAGGTTCTCCATCTCCACGGCGGAT
F3006 TGGAGAACCTGCCCGCTGGCACATGGGAACG
R3021 GGTCAGGTCTTCCTCCCGTTCCCATGTGCCAG
F3037 GGAGGAAGACCTGACCAACTTCTGTCACCAGGA
R3053 CGGTCACCTGGCATTCCTGGTGACAGAAGTT
F3070 ATGCCAGGTGACCGAGACTGAGTGTCTGTGC
R3084 CCAGGGCCTCATATGGGCACAGACACTCAGTCT
F3101 CCATATGAGGCCCTGGTGCTGCGGAAGCCT
R3117 TGCTGTCCAGGAACAGAGGCTTCCGCAGCA
F3131 CTGTTCCTGGACAGCACCGCTAAGGGAATGAAAA
R3147 GGTGGAATTCAGCAGGTTTTTCATTCCCTTAGCGG
F3165 ACCTGCTGAATTCCACCAGCCTGGAAACCTCC
R3182 GGTGCTTCGATGGACAGGGAGGTTTCCAGGCT
F3197 CTGTCCATCGAAGCACCATGGGGCGCTATTAACG
R3214 GCTTATAGGTGGACTGGACGTTAATAGCGCCCCAT
F3231 TCCAGTCCACCTATAAGCCTACTGTCAGCACCGC
R3249 CAGCTCAGTGCGATATTGGCGGTGCTGACAGTAG
F3265 CAATATCGCACTGAGCTGGTCCTCCGTGGAACAC
R3283 GGATCTTGTTGCCCCGGTGTTCCACGGAGGAC
F3299 CGGGGCAACAAGATCCTGGTCAGCGGGCGG
R3315 TTCATGATGGACTCGCTCCGCCCGCTGACCA
F3329 AGCGAGTCCATCATGAAACTGGAAGAACGGACAG
R3346 CCAGGTCCCAGGAAATTCCTGTCCGTTCTTCCAGT
F3363 GAATTTCCTGGGACCTGGGCGTCGAAGATGCCT
R3381 AGCAGTTTGCTCTCGGAGGCATCTTCGACGC
F3396 CCGAGAGCAAACTGCTGACAGTCTCCGTGATGG
R3412 GTACATCTGGCTCAGGTCCATCACGGAGACTGTC
F3429 ACCTGAGCCAGATGTACTCCCCTGTCTTTGAATATCT
R3446 GCCGATCGCCGCTCAGATATTCAAAGACAGGGGA
F3466 GAGCGGCGATCGGCAGGTGGGGGAGTGG
R3480 AGTGCAAGTTGCCTTAGGCCACTCCCCCACCT
F3494 CCTAAGGCAACTTGCACTGGGGACTGCCCCGAG

R3512 GTGCAGCCGCACCGCTCGGGGCAGTCCCC
F3527 CGGTGCGGCTGCACTAGCTCCACTTGCCTG
R3541 GGGGCCACTCCTTATGCAGGCAAGTGGAGCTA
F3557 CATAAGGAGTGGCCCCATTCCCGGAACTGGC
R3573 ACCAGGTAGGATTACACCGCCAGTTCCGGGAAT
F3588 GGTGTAATCCTACCTGGTGCTGGGGAGTGGGC
R3606 CATGTGCACCCGGTGCCCACTCCCCAGC
F3620 ACCGGGTGCACATGCTGCGGCCTGGATG
R3634 TCAGTGAACAGGTCTTTGACATCCAGGCCGCAG
F3648 TCAAAGACCTGTTCACTGACTATATGTTCGTCAAGTGGAA
R3667 CGGTTTTGATATACTCGACCTTCCACTTGACGAACATATAG
F3688 GGTCGAGTATATCAAAACCGAGGCCATTGTGTGTGT
R3708 TGGGAAGTCAGCTCGACACACACAATGGCCT
F3724 CGAGCTGACTTCCCAAGAACGGCAGTGCA
R3739 CGGCCTCAATCAGGCTGCACTGCCGTTCT
F3753 GCCTGATTGAGGCCGGAACCCGGTTCAATCTG
R3768 ATGGTCACGGGGCCCAGATTGAACCGGGTTC
F3785 GGCCCCGTGACCATCACTCTGTCCGAGCC
R3799 TGCTGGATGTTCCGGGGCTCGGACAGAGTG
F3814 CCGGAACATCCAGCAGAAACTGCCACCCGA
R3829 GGATGCAGTGTGATGATCTCGGGTGGCAGTTTC
F3844 GATCATCACACTGCATCCACGGATTGAGGAGGGG
R3862 CGTGCATCAGATCGAAGAACCCCTCCTCAATCCGT
F3878 TTCTTCGATCTGATGCACGTCCAGAAGGTCCTGAGCG
R3897 GCTTACACACTGTGGAGGCGCTCAGGACCTTCTGGA
F3915 CCTCCACAGTGTGTAAGCTGCAGAGCTGCACC
R3933 CCTGGCACGCCGTGGGTGCAGCTCTGCA
F3947 CACGGCGTGCCAGGCGACCTGCAGGTGTA
R3961 CAGCAGATTGCCGATATGATACACCTGCAGGTCG
F3976 TCATATCGGCAATCTGCTGAAGGGCGATAAGGTCAAC
R3995 ATCTTATGAATCAGATGCCCGTTGACCTTATCGCCCTT
F4013 GGGCATCTGATTCATAAGATCGAACCTCACTTCAATACATC
R4033 CGTCCCAGCTCATCCAGGATGTATTGAAGTGAGGTTCG
F4054 CTGGATGAGCTGGGACGGGTGCGATCTGGATTATTAC
R4071 GCCAATCTCCCATGTTACAGTAATAATCCAGATCGCACC
F4091 TGTAACATGGGAGATTGGCCAAGCTGCACTTACACCG
R4110 GGTTGTGTTGGGTGACTCCGGTGTAAGTGCAGCTTG
F4128 GAGTCACCCAACACAACCACGCTAGCTTTGTGAATC
R4146 TAGTCAGTTTCGATATTCAGCAGATTCACAAAGCTAGCGT
F4164 TGCTGAATATCGAAACTGACTATACAAAGAACTTTCACTTCCA
R4186 TGTCACCCGTTTGGAGTGGAAGTGAAAGTTCTTTGTA
F4207 CTCCAAACGGGTGACAGCACACGGAGACACC
R4223 TCAGATCCAGCTGTGGGGTGTCTCCGTGTGC
F4238 CCACAGCTGGATCTGAAGGCTCGGCCCACC
R4254 TCCCCAGCGCCATAGGTGGGCCGAGCCT
F4268 TATGGCGCTGGGGAAATTACCGTGCTGGTGG
R4282 CATGTCGGCGACCTCCACCAGCACGGTAATT
F4299 AGGTCGCCGACATGGAGCTGCATACTAAGAAGATC
R4313 TTCAGGCCGGAAATTTCGATCTTCTTAGTATGCAGCTC
F4334 GAAATTTCCGGCCTGAAATTCGCCAGCCTGG

R4351	ACACCCGGTGCAAGCCAGGCTGGCGAAT
F4365	CTTGCACCGGGTGTTACGCTTGTAGCAGCG
R4379	ACCTTGCAGGAGATGCCGCTGCTACAAGCGTA
F4395	GCATCTCCTGCAAGGTGCGGATCCACGTCG
R4411	AGTTCATCGGGCTCATCGACGTGGATCCGC
F4425	ATGAGCCCGATGAACTGACCGTCCACGTGAA
R4441	CGGGATCGTCGCTCTTCACGTGGACGGTC
F4456	GAGCGACGATCCCGATGTGGTGGCTGCC
R4470	CCATCAGGGAGGAGGAGGCAGCCACCACAT
F4484	TCCTCCTCCCTGATGGCCCGGAAACTGGAGT
R4500	TGGAGTCAGTCCCGAACTCCAGTTTCCGGG
F4515	TCGGGACTGACTCCACATTCAAGGCCTTCAGC
R4530	GGTCTTGGGCATGGCGCTGAAGGCCTTGAATG
F4547	GCCATGCCCAAGACCAGCCTGTGCTTCTACAT
R4562	GTTCCCGCTCGACAATGTAGAAGCACAGGCT
F4579	TGTCGAGCGGGAACATTGCAAGAGCTGCTCC
R4593	GCACTTTTTAGTATCCTCCTCGGAGCAGCTCTTGCAAT
F4610	GAGGAGGATACTAAAAAGTGCGTGAACACTAAACTGGAGC
R4631	GGATGCTCTGTGGCTGCTCCAGTTTAGTGTTCAC
F4650	AGCCACAGAGCATCCTGATTGAGCACAAAGGAAC
R4665	GGAATTCTGTTTGCCAATGATAGTTCCTTTGTGCTCAATCA
F4684	TATCATTGGCAAACAGAATTCCACCTGCACCGCCAA
R4706	GCCAGCAGCTGGCTTTGGCGGTGCAGGT
F4720	AGCCAGCTGCTGGCTGGAGTCCGTCAAGTC
R4734	TTCAGCCCGTAAAAGAAGGACTTGACGGACTCCA
F4750	CTTCTTTTACGGGCTGAAGAATATGCTGAGCGGC
R4768	CATGAAGACATTGCCAAAAATGCCGCTCAGCATATTC
F4784	ATTTTTGGCAATGTCTTCATGGGAATCTTCCTGTTTCTGG
R4805	AGCAGGATGAATGGGGCCAGAAACAGGAAGATTCC
F4824	CCCCATTCATCCTGCTGATCCTGTTCTTTATGTTCGG
R4840	AACAGGATCCGCCAGCCGAACATAAAGAACAGGATC
F4861	CTGGCGGATCCTGTTTTGTTTCAAATGCTGTCGG
R4876	AGGCCCCGTGTCCGCCGACAGCATTTGAAACAA
F4895	CGGACACGGGGCCTGTTCAAGTATCGGCACC
R4909	TCTCCTCGTCATCTTTCAGGTGCCGATACTTGAAC
F4926	TGAAAGATGACGAGGAGACAGGATATCGGCGGAT
R4944	TTTATTGTTCAGCTTCTCAATGATCCGCCGATATCCTG
F4960	CATTGAGAAGCTGAACAATAAAAAGGGCAAGAATAAACTGC
R4982	CCGCTCCCCATCCAGCAGTTTATTCTTGCCCTT
F5001	TGGATGGGGAGCGGCTGGCTGACCGGCG
R5015	AGAACAGCTCTGCGATCCGCCGGTCAGCCAG
F5029	GATCGCAGAGCTGTTCTCCACCAAGACTCATATTGG
F5046	CCAATATGAGTCTTGGTGG

CCHF Primers

CCHFF	AACCTGTGACGAGCTCGCCGCCACCATGCATATCTCCCTGATGTACGCT
CCHFR	GTCACAGGTTGCATGCTCACCCAATATGAGTC

CCHF Sequencing Primers

CCHFF0	ACGGTCCTAAGGTAGCGAAA
CCHFMi1	CATAGCCCATATATGGAGTT
CCHFMi2	TACGTATTAGTCATCGCTAT
CCHFMi3	CGGGAGTCGCTGCGTTGCCT
CCHFMi4	GCCTGGGCGAGACTCATGGC
CCHFMi5	CCGCAGGCGGGACCAGCGCC
CCHFMi6	TGATGACAAGCCCCACCCAG
CCHFMi7	ACGAGGTGCTGCAATTCCGG
CCHFMi8	TTAATGTGCTGCTGCCCCAA
CCHFMi9	GGCGGCTGCTGTCCGAGGAG
CCHFMi10	GCCATGTGAAGCTGAGCCG
CCHFMi11	TCATCGTGGGAACTCTGGGCAA
CCHFMi12	CTCTGAAACGGTCCAGCTGG
CCHFMi13	TGTTTTGTTGTCCCTACTGT
CCHFMi14	GTCACCAGGAATGCCAGGTGA
CCHFMi15	CAGGAATTTCCTGGGACCTG
CCHFMi16	TCACTGACTATATGTTCGTC
CCHFMi17	GCGACCTGCAGGTGTATCAT
CCHFMi18	CCACCTATGGCGCTGGGGAAA
CCHFMi19	CCAGCCTGTGCTTCTACATT
CCHFMi20	GCTGGCGGATCCTGTTTTGT
CCHFMi21	ATTGGGTGAGCATGCAACCT
CCHF1Fo	GCCGCCACCATGCATATCTCC
CCHF1Re	CGATAGTCTTGCCCTGCCCGATAGG
CCHF2Fo	CCTATCGGGCAGGGCAAGACTATCG
CCHF2Re	GCTCACCCAATATGAGTCTTGGTGGAGAA
CCHFMi2Re	ACAGTGTGTCGGTCCCTGTG
CCHFAdXFo	GGACACTTCAACGAGGTGCTGCAA
CCHFAdXRe	CTTGTCGCCGGGTCCTCCGT

RVFV M Segment Optimized Oligonucleotides

R0	GCGAGCTCGTCACAGGTT
F0	AACCTGTGACGAGCTCGCCGCCACCATGGCTGGA
R18	GCAGGACAGTCATAGCGATTCCAGCCATGGTGGCG
F34	ATCGCTATGACTGTCCTGCCTGCACTGGCTGTCTTCG
R53	CACGACTGGTGCCAGAGCGAAGACAGCCAGTGCAG
F71	CTCTGGCACCAGTCGTGTTCGCTGAAGATCCACACC
R88	CCAGGCCGATTCCGCAGGTGTGGATCTTCAGCGAA
F107	TGCGGAATCGGCCTGGCAAGGGACACAATTACATCGAC
R123	CGTCCTCTTGTGTCATGCCGTCGATGTAATTGTGTCCCTTG
F145	GGCATGACACAAGAGGACGCCACCTGTAAGCCTGTGA
R164	CATGCGCCAGCGTAGGTCACAGGCTTACAGGTGG
F182	CCTACGCTGGCGCATGCAGCTCCTTCGACGTCC

R198	CTTGCCCTTCTCCAGCAGGACGTCGAAGGAGCTG
F215	TGCTGGAGAAGGGCAAGTTCCCTCTGTTTCAGAGCTAC
R232	AGTGTCCGATGGTGTGCGTAGCTCTGAAACAGAGGGAA
F253	GCACACCATCGGACACTGCTGGAGGCCGTGCAC
R270	GCCTTGGCGATAATGGTGTCGTGCACGGCCTCCAGC
F286	GACACCATTATCGCCAAGGCAGATCCACCTTCCTGTGATC
R306	CCGTGGGCGCTCAGCAGATCACAGGAAGGTGGATCT
F326	TGCTGAGCGCCCACGGCAACCCATGCATGAAAGAGAA
R342	AGTGTGTCTTCATCACCAGCTTCTCTTTCATGCATGGGTTG
F363	GCTGGTGATGAAGACACACTGTCCTAATGACTATCAGTCCGC
R383	CGTCGTTGTTCAGATGGTGAGCGGACTGATAGTCATTAGGAC
F405	TCACCATCTGAACAACGACGGCAAGATGGCTTCCGTGA
R425	CTCGTACTTTGGAGGACACTTCACGGAAGCCATCTTGC
F443	AGTGTCCTCCAAAGTACGAGCTGACCGAAGACTGTAACTTCT
R463	CCGGTCATCTGCCGACAGAAGTTACAGTCTTCGGTCAG
F485	GTCGGCAGATGACCGGCGCATCCCTGAAGAAGGG
R501	TCCTGCAGAGGGTAGCTTCCCTTCTTCAGGGATGCG
F519	AAGCTACCCTCTGCAGGACCTGTTCTGCCAAAGCAG
R537	TGGAGCCATCGTCTTCGCTGCTTTGGCAGAACAGG
F555	CGAAGACGATGGCTCCAAGCTGAAGACTAAGATGAAAGGC
R572	CGCCGACCTCGCACACGCCTTTCATCTTAGTCTTCAGCT
F595	GTGTGCGAGGTCGGCGTCCAGGCTCTGAAGAAATGC
R611	TGCTCAGCTGCCCATCGCATTTCTTCAGAGCCTGGA
F631	GATGGGCAGCTGAGCACAGCTCACGAGGTGGTC
R647	GTTCTTAAAGACGGCGAAAGGGACCACCTCGTGAGCTG
F664	CCTTTCGCCGTCTTTAAGAACTCCAAGAAGGTGTATCTGGATAA
R685	CGGTCTTCAGGTCCAGCTTATCCAGATACACCTTCTTGGA
F708	GCTGGACCTGAAGACCGAAGAGAACCTGCTGCCA
R725	GTTCGAAACACACGAAGCTATCTGGCAGCAGGTTCTCTT
F742	GATAGCTTCGTGTGTTTCGAACATAAAGGCCAGTACAAAGGCA
R764	TGGCCGCTATCCATGGTGCCTTTGTACTGGCCTTTAT
F785	CCATGGATAGCGGCCAGACTAAGCGGGAACTGAAGA
R801	GCACTGGGAGATATCGAAGCTCTTCAGTTCCCGCTTAGTC
F821	GCTTCGATATCTCCCAGTGCCCTAAGATCGGCGGACAT
R841	CGGTACACTTCTTGCTGCCATGTCCGCCGATCTTAGG
F859	GGCAGCAAGAAGTGTACCGGCGACGCCGCCTTCTG
R878	CGGTACATTCGTATGCGGAGCAGAAGGCGGCGTCGC
F894	CTCCGCATACGAATGTACCGCACAATACGCAAACGCTTATTG
R914	ACCCATTGGCATGGGAGCAATAAGCGTTTGCGTATTGTG
F936	CTCCCATGCCAATGGGTCCGGCATTGTCCAGATTCA
R953	CCAGACGCCGGACACCTGAATCTGGACAATGCCGG
F972	GGTGTCCGGCGTCTGGAAGAAGCCACTGTGTGTGG
R988	ACCACCCGCTCATAGCCCACACACAGTGGCTTCTT
F1007	GCTATGAGCGGGTGGTCGTGAAACGGGAGCTGA
R1023	GCTGGATGGGTTTAGCGCTCAGCTCCCGTTTCACG
F1040	GCGCTAAACCCATCCAGCGGGTGGAACCTTGTACTACC
R1058	GGTTCGCATTTAGTGATGCAGGTAGTACAAGGTTCCACCC
F1078	TGCATCACTAAATGCGAACCTCACGGCCTGGTGGTG
R1098	TTGAACCCTGTGGACCGCACCACCAGGCCGTGA
F1114	CGGTCCACAGGGTTCAAGATCTCCAGCGCCGTC

R1131	CTCCGCTTGCGCAGGCGACGGCGCTGGAGATC
F1147	GCCTGCGCAAGCGGAGTGTGTGTGACTGGAAGCC
R1163	TCTCTGTGGAAGGGCTCTGGCTTCCAGTCACACACA
F1181	AGAGCCCTTCCACAGAGATCACCCTGAAATATCCCGG
R1199	GCCGCTGGATTGGGAAATCCCGGGATATTTCAGGGTGA
F1218	GATTTCCCAATCCAGCGGCGGAGACATCGGCGTGC
R1237	GTCATCGTGGGCCATGTGCACGCCGATGTCTCC
F1253	ACATGGCCCACGATGACCAGTCCGTCTCCTCCAAG
R1270	GGCAGTGAGCGACGATCTTGGAGGAGACGGACTG
F1288	ATCGTCGCTCACTGCCCTCCTCAGGACCCATGTC
R1304	ACACAATACAATCGTGGACCAGACATGGGTCCTGAGGAG
F1322	TGGTCCACGATTGTATTGTGTGCGCCCATGGCCTGAT
R1343	CGGTGTGGCACTGATAGTTGATCAGGCCATGGGCGC
F1359	CAACTATCAGTGCCACACCGCTCTGAGCGCCTTTGTGG
R1379	GCTGCTAAACACGAAGACCACCACAAAGGCGCTCAGAG
F1397	TGGTCTTCGTGTTTAGCAGCATTGCCATTATTTGTCTGGCC
R1417	CAGCACCCGGTACAGAATGGCCAGACAAATAATGGCAAT
F1438	ATTCTGTACCGGGTGCTGAAGTGTCTGAAGATTGCTCCA
R1456	GGGTTCAGCACCTTCCGTGGAGCAATCTTCAGACACTT
F1477	CGGAAGGTGCTGAACCCACTGATGTGGATTACAGCTTTCA
R1494	CATCTTCTTATAGATCCACCGGATGAAAGCTGTAATCCACATCAGT
F1517	TCCGGTGGATCTATAAGAAGATGGTGGCACGGGTGGCAG
R1540	CCGATTCACCTGGTTGATATTGTCTGCCACCCGTGCCAC
F1556	ACAATATCAACCAGGTGAATCGGGAGATTGGATGGATGGAGGG
R1579	CAGGACCAGTTGGCCTCCCTCCATCCATCCAATCTC
F1599	AGGCCAACTGGTCCTGGGCAACCCTGCTCCTAT
R1615	GGGTGCATGCCGAGGGATAGGAGCAGGGTTGCC
F1632	CCCTCGGCATGCACCCATTCCACGGTACTCCACC
R1648	CAGCAGCAGCATCAGGTAGGTGGAGTACCGTGGAAT
F1666	TACCTGATGCTGCTGCTGATTGTGTCCTACGCCAGC
R1684	TTGAATCAGTTCGCTACAAGCGCTGGCGTAGGACACAAT
F1702	GCTTGTAGCGAACTGATTCAAGCATCCTCCCGGATTACT
R1723	TCCTTCGGTGGAACATGTAGTAATCCGGGAGGATGC
F1741	ACATGTTCCACCGAAGGAGTGAACACTAAGTGTCGGCT
R1759	ATCAGAGCTGTTCCGGACAGCCGACACTTAGTGTTCAC
F1779	GTCCGGAACAGCTCTGATTCGGGCTGGAAGCGTGG
R1797	AGGCATGCCTCGGCGCCCACGCTTCCAGCCCGA
F1814	GCGCCGAGGCATGCCTGATGCTGAAAGGAGTGAAAGAAG
R1830	TCAGTTTCAGAAACTTGGTTTGATCTTCTTTCACTCCTTTCAGCATC
F1853	ATCAAACCAAGTTTCTGAAACTGAAGACTGTGTCCTCCGAGC
R1877	CCCTCCCGACAGCTCAGCTCGGAGGACACAGTCT
F1895	TGAGCTGTCGGGAGGGCCAGAGCTACTGGACCG
R1911	ACTTAGGGCTGAAGCTGCCGGTCCAGTAGCTCTGG
F1928	GCAGCTTCAGCCCTAAGTGTCTGTCCAGCCGGCG
R1946	CGCCGACCAGATGACACCGCCGGCTGGACAGAC
F1962	GTGTCATCTGGTCGGCGAGTGCCACGTGAACCG
R1979AgeI	CCGCCAGGACAGGCACCGGTTCACGTGGCACT
F1995	GTGCCTGTCCTGGCGGGACAACGAGACAAGCGCC
R2011	CTCCCACGAAGGAGAACTCGGCGCTTGTCTCGTTGTC
F2029	GAGTTCTCCTTCGTGGGAGAGAGCACAACAATGCGGGA

R2048	CCACATTGTTCGAAGCACTTATTCTCCCGCATTGTTGTGCTCT
F2067	GAATAAGTGCTTCGAACAATGTGGCGGCTGGGGCTGTGGA
R2091	GCTAGGGTTCACATTGAAACATCCACAGCCCCAGCCG
F2107	TGTTTCAATGTGAACCCTAGCTGTCTGTTCGTGCATACATACC
R2128	TTCCGGACGCTCTGCAGGTATGTATGCACGAACAGACA
F2150	TGCAGAGCGTCCGGAAAGAGGCTCTGCGGGTC
R2166	GGACCCAGTCGATACAATTGAAGACCCGCAGAGCCTCT
F2182	TTCAATTGTATCGACTGGGTCCATAAACTGACCCTGGAGATCAC
R2204	CGGAGCCGTCAAAGTCAGTGATCTCCAGGGTCAGTTTAT
F2226	TGACTTTGACGGCTCCGTGTCCACTATTGACCTGGG
R2243	CCGGCTGGAGCTTGCGCCCAGGTCAATAGTGGACA
F2262	CGCAAGCTCCAGCCGGTTTACAAATTGGGGATCCGTCT
R2278	GCATCCAGGCTCAGGGAGACGGATCCCCAATTTGTAAA
F2300	CCCTGAGCCTGGATGCCGAGGGCATCTCCGGC
R2316	GATAAAGCTGAAGCTGTTGCTGCCGGAGATGCCCTCG
F2332	AGCAACAGCTTCAGCTTTATCGAATCCCCAGGGAAAGGG
R2353	GGGTTCATCCACGATTGCATACCCTTTCCCTGGGGATTC
F2371	TATGCAATCGTGGATGAACCCTTTAGCGAAATCCCTCGGC
R2392	CGAATTTCTCCCAGAAATCCCTGCCGAGGGATTTCGCTAAA
F2411	AGGGATTTCTGGGAGAAATTCGGTGTAACAGCGAGTCCAGC
R2433	CGTGGGCGGACAGGACGCTGGACTCGCTGTTACAC
F2452	GTCCTGTCCGCCCACGAAAGCTGCCTGCGGGC
R2468	GCTTATAGGAGATCAGGTTTGGAGCCCGCAGGCAGCTTT
F2484	TCCAAACCTGATCTCCTATAAGCCTATGATTGACCAACTGGAATGT
R2507	TGGATCAATCAGGTTAGTGGTACATTCCAGTTGGTCAATCATAG
F2530	ACCACTAACCTGATTGATCCATTTGTGGTGTTCGAACGGG
R2551	GTCTGTGGCAGGGACCCCCGTTCGAACACCACAAA
F2570	GGTCCCTGCCACAGACTCGGAACGACAAGACCTTTG
R2586	GGTTGCCTTTGGAAGCGGCAAAGGTCTTGTCGTTCCGA
F2606	CCGCTTCCAAAGGCAACCGGGGCGTGCAGGCCTT
R2624	CACGGAGCCCTTGGAGAAGGCCTGCACGCCCC
F2640	CTCCAAGGGCTCCGTGCAGGCAGACCTGACTCTG
R2656	TCGACTTCAAAGTTGTCAAACATCAGAGTCAGGTCTGCCTG
F2674	ATGTTTGACAACTTTGAAGTCGATTTCGTCGGCGCTGCT
R2697	GCAGCGTCACAGGACACAGCAGCGCCGACGAAA
F2713	GTGTCCTGTGACGCTGCCTTCCTGAATCTGACAGGGT
R2730	GCCTGCGTTACAGGAGTAACACCCTGTCAGATTCAGGAAG
F2750	GTTACTCCTGTAACGCAGGCGCTCGGGTGTGTCTGAG
R2770	GGTGCCGGTGCTAGTAATGCTCAGACACACCCGAGC
F2787	CATTACTAGCACCGGCACCGGATCCCTGAGCGCAC
R2806	GCAGGCTTCCGTCCTTATTATGTGCGCTCAGGGATCC
F2822	ATAATAAGGACGGAAGCCTGCATATTGTGCTGCCATCCGA
R2843	TGATCCTTGGTCCCGTTCTCGGATGGCAGCACAATAT
F2862	GAACGGGACCAAGGATCAGTGCCAGATTCTGCACTTTAC
R2880	CTTCGACCTCAGGCACAGTAAAGTGCAGAATCTGGCAC
F2901	TGTGCCTGAGGTCGAAGAGGAATTTATGTACAGCTGTGACG
R2918	AGAGGCCGTTCGTCGCCGTCACAGCTGTACATAAATTCCT
F2942	GCGACGAACGGCCTCTGCTGGTCAAAGGAACTCTGA
R2958	CGAAAGGATCGATGGCGATCAGAGTTCCTTTGACCAGC
F2978	TCGCCATCGATCCTTTCGACGATCGGCGGGAAGC

R2996	GGTGGATTCGCCGCCAGCTTCCCGCCGATCGT
F3012	TGGCGGCGAATCCACCGTCGTGAATCCTAAGAGCGG
R3028	CCAATCAAAGAAGTTCCAGCTTCCGCTCTTAGGATTCACGAC
F3048	AAGCTGGAACTTCTTTGATTGGTTCTCCGGACTGATGAGC
R3070	GTGGGCCGCCAAACCAGCTCATCAGTCCGGAGAA
F3088	TGGTTTGGCGGCCCACTGAAGACCATCCTGCTGATC
R3104	GTGCCACGTACAGGCAGATCAGCAGGATGGTCTTCA
F3124	TGCCTGTACGTGGCACTGAGCATCGGACTGTTCTT
R3140	GCCCAGGTAGATCAGCAGAAAGAACAGTCCGATGCTCA
F3159	TCTGCTGATCTACCTGGGCGGAACAGGGCTGAGCA
R3178	GCGGCGAGCCACATCTTGCTCAGCCCTGTTCC
F3194	AGATGTGGCTCGCCGCTACCAAGAAGGCCAGCTAAG
R3210	ccatgGTCACAGGTTGCTAGCTTAGCTGGCCTTCTTGGTA
F3230	CTAGCAACCTGTGACcatggaacacacccacaagtggc
R3250	gccacttgtgggtgtgtt

RVFV M Segment Primers

RVFVcFoSacI	AACCTGTGACGAGCTCGCCGCCACCATGGCTGGAATCGCTATG
RVFVcReNheI	GTCACAGGTTGCTAGCTTAGCTGGCCTTCTTGGT

RVFV M Segment Sequencing Primers

RVFVcseqF1	ATGGCTGGAATCGCT
RVFVcseqF2	GTCCAGGCTCTGAAG
RVFVcseqF3	ACCTTGTACTACCTG
RVFVcseqF4	ACCAGGTGAATCGGG
RVFVcseqF5	GATGTTTCAATGTGA
RVFVcseqF6	GCGCTGCTGTGTCCT
RVFVcseqR1	TTAGCTGGCCTTCTT

CCHF ELISA Raw Data

	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400	1:204800
PB	0.105	0.104	0.091	0.094	0.096	0.104	0.1	0.095	0.107	0.1	0.107	0.106
TB1	0.1	0.098	0.092	0.097	0.102	0.104	0.107	0.104	0.092	0.089	0.113	0.1
TB1	0.11	0.106	0.098	0.099	0.12	0.116	0.134	0.099	0.092	0.104	0.112	0.129
TB2	0.145	0.103	0.1	0.107	0.117	0.128	0.158	0.111	0.155	0.122	0.124	0.134
TB2	0.194	0.108	0.102	0.108	0.14	0.15	0.134	0.119	0.108	0.108	0.115	0.087
MI3	1.374	0.474	0.276	0.226	0.171	0.14	0.132	0.126	0.117	0.208	0.2	0.119
HuPos	1.81	1.56	1.261	0.766	0.431	0.325	0.257	0.191	0.193	0.196	0.13	0.139
HuCtrl	1.394	1.121	0.635	0.418	0.295	0.172	0.158	0.107	0.106	0.11	0.137	0.095

CCHF Mouse ELISPOT Raw Data

Plate Data - Spots Number Mouse 1, 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	51	300	80	54	55	1	0	79	257	135	76	43
B	45	85	101	66	49	0	0	86	115	148	94	68
C	101	110	95	55	101	0	0	102	111	138	83	127
D	91	98	109	87	116	0	0	235	154	155	94	150
E	109	170	179	95	98	0	0	245	241	197	122	88
F	43	332	128	328	71	0	0	134	312	267	272	79
G	33	307	67	102	45	0	0	96	249	111	92	49
H	56	84	133	273	647	0	0	110	108	160	274	1

Plate Data - Spots Number Mouse 3, 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	48	343	83	46	85	0	0	182	395	212	166	123
B	42	140	106	65	55	0	0	219	250	274	199	169
C	39	122	102	82	174	0	1	230	285	304	209	211
D	139	137	71	93	128	0	0	295	268	236	200	166
E	90	145	112	56	101	0	0	223	286	208	184	167
F	46	308	96	281	60	0	0	234	465	285	589	127
G	35	235	51	61	49	0	0	187	599	197	201	115
H	77	56	107	348	578	0	0	196	204	223	377	1

Plate Number	Data - Spots Mouse 5 and 6											
A	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
B	129	320	107	83	74	1	0	45	228	135	113	45
C	127	117	177	80	81	0	0	97	80	90	142	64
D	109	161	162	87	206	0	0	142	199	161	139	137
E	207	121	94	96	208	0	0	255	169	155	169	112
F	140	173	194	86	96	0	0	281	166	140	181	113
G	98	245	177	243	50	1	0	121	399	148	475	54
H	122	218	114	100	93	0	0	126	381	99	111	65
	81	105	219	355	1	1	0	86	113	127	327	1

Supplementary Data

This study attempted the creation of full M-segment Rift Valley Fever Virus in an adenoviral particle. Due to difficulties with rescue procedures, the project was put on hold to concentrate on CCHF AdX, Nipah G AdX and Nipah F AdX. The procedures involved are outlined below.

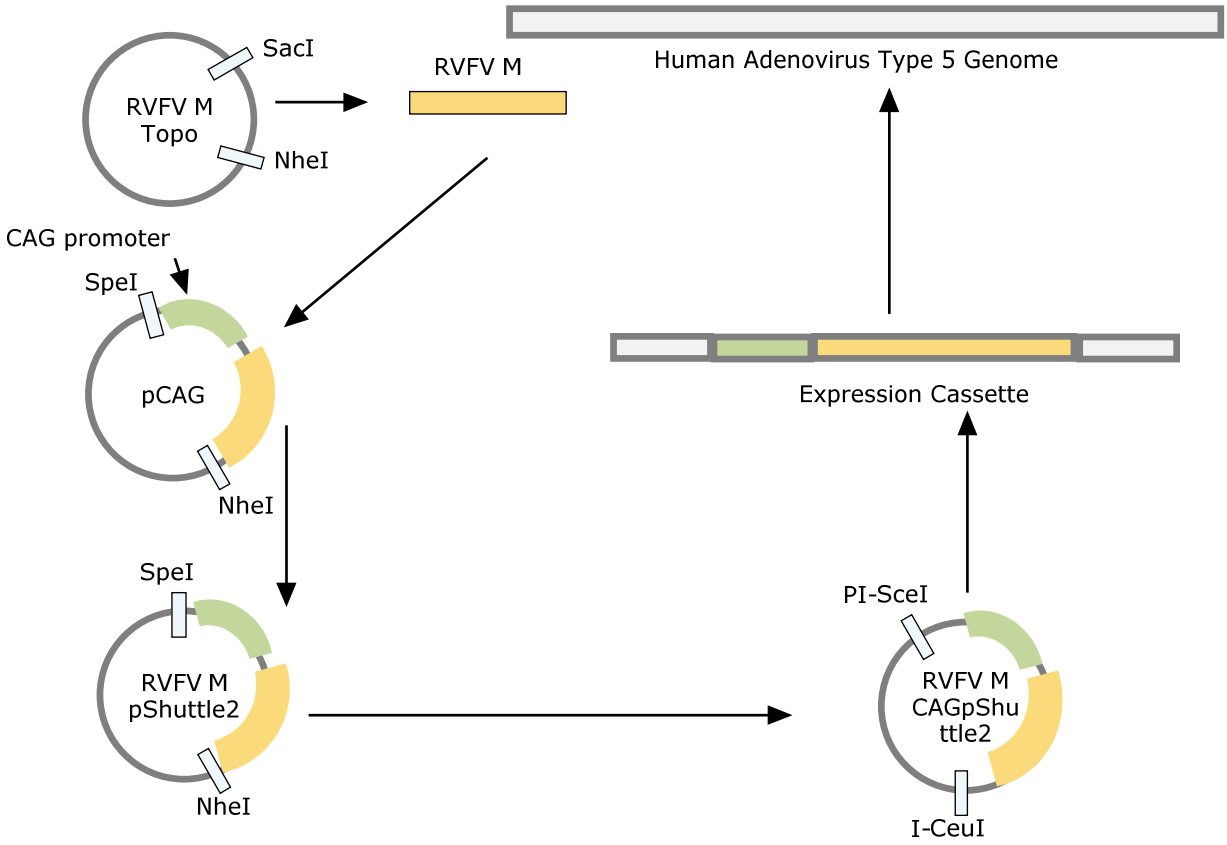

Figure S.1. RVFV Cloning Strategy. Proposed cloning strategy for RVFV M-Segment. The entire M segment was synthesized and cloned into pCAGα. The insert with the promoter was cloned into pShuttle2, replacing the CMV promoter with the truncated CAGα promoter. The entire expression cassette was then cloned into pAdenoX® (AdHu5).

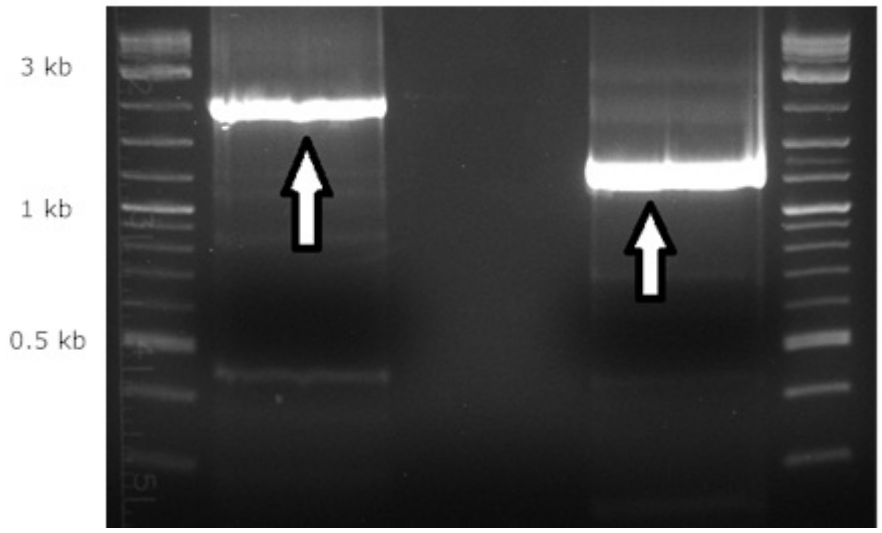

Figure S.2 Gene Synthesis of Rift Valley Fever Virus M Segment.

The M segment was divided into two portions for ease of synthesis. The two fragments are approximately 1.8 and 1.3 kb in length. Arrows indicate bands that were excised.

The Rift Valley Fever Virus M segment was synthesized using the modified 4-step PCR protocol and generated very distinct bands (Figure 3.2). The two resulting fragments were successfully assembled into one fragment through PCR. DNA sequencing of the RVFV M segment indicated that there was a deletion at position 1327, which generated a frame-shift mutation; this mutation was corrected using overlapping PCR.